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PROSTAGLANDIN REGULATION OF MACROPHAGE FUNCTION

DISSERTATION

Presented in Partial Fulfillment of the Requirements for

the Degree Doctor of Philosophy in the Graduate

School of The Ohio State University

By

Mary Ellen McCarthy, B.S., M.S.

* * * * *

The Ohio State University

1982

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STATEMENT OF THE PROBLEM

Macrophages are required for the initiation and expression of immunity. This requirement represents several functional capacities including such effector functions as phagocytosis, resistance to infection by intracellular microorganisms, secretion of regulatory and enzymatic molecules and resistance to neoplasm. Activation results in biochemical and metabolic alterations which are manifested as changes in morphology and function. The results of macrophage activation are increases in macrophage size, membrane ruffling, spreading, adherence, chemotaxis, phagocytosis and secretions(1). There are also the acquisition of the capacity for glucose oxidation by the hexose monophosphate shunt, increased numbers of cytoplasmic granules, an increase in the amount of adenyl cyclase and a greater capacity for tumor growth inhibition and tumoricidal activity. The macromolecular secretion products or monokines that are increased include lysozyme, lysosomal enzymes, plasminogen activator, collagenase, elastase, complement components, interferon and prostaglandins(2,3,4). Hydroxyl radical, hydrogen peroxide and superoxide are highly reactive molecules also secreted by activated macrophages(5,6).

The macrophage is activated in a step-wise fashion through a² cascade of poorly defined events. The generation of three distinct populations of mouse peritoneal macrophages can be accomplished in vivo and in vitro. Resident normal macrophages are thought to be resting cells and are collected from untreated animals. Elicited macrophages are primed in vivo by the injection of sterile irritants or treated in vitro with agents such as lipopolysaccharide(LPS). This subpopulation of cells exhibits elevated functional capacity but no tumoricidal activity. Activated macrophages are obtained from animals chronically infected with intracellular organisms or treated with lymphocyte products like macrophage activating factor(MAF). The activated cell is an operationally defined population that acquires the capacity to lyse tumor cells.

Regulation of the activation sequence is not well understood. It seemed reasonable to assume that cyclic nucleotides might be involved since they control differentiation in several cells examined(7). Additionally, there was growing evidence that prostaglandins (PGs) produced by macrophages were acting as immunoregulators(8). These hormone-like compounds are intercellular regulators which trigger changes in the intracellular cyclic nucleotide and calcium levels.

The effector functions of the macrophage that are affected by treatment with PGs include proliferation of myeloid progenitor cells(9), chemotaxis(10), lymphokine-induced inhibition of migration(11), collagenase production(12), plasminogen activator production(13), phagocytosis and anti-tumor activity(14). Schultz, et al(15) hypothesized that macrophage derived PGE may cause feedback inhibition of activated macrophage tumoricidal activity. My results,

as well as others(16), support the theory whereby the expression of macrophage anti-tumor activity was limited by the accumulation of PGs.

Agents which affect macrophage activation have been shown to alter PG metabolism(17). LPS activation is thought to stimulate PGE production by increasing arachidonic acid release through the action of phospholipase A2. Evidence is accumulating that other arachidonic acid metabolites are involved in immunoregulation (8). Macrophages produce PGE, PGF_{2α}, PGI₂, thromboxane, leukotrienes and hydroxyfatty acids at various levels depending on the method of collecting the cells and the method of detection utilized. It has been suggested that arachidonic acid metabolism varies with the state of activation; however, most reports only examined one population of macrophages at an arbitrary incubation time.

The relationship between endogeneous arachidonic acid metabolites and macrophage activation is the subject of this investigation. My studies involved several independent approaches. First, the pattern of arachidonic acid and cyclic nucleotide metabolism was determined for resident, elicited and activated macrophages. Second, the effect of alterations in metabolism and exogeneously added PGs on macrophage tumoricidal activity was examined. Lastly, we evaluated the role of endogeneous PGs in controlling macrophage activation, in vitro. The results of my investigation indicate that arachidonic acid metabolites have a pivotal role in controlling macrophage functional differentiation. Resident and elicited macrophages are activated by PGs while already activated cells appear to dedifferentiate.

CHAPTER I

Production of Arachidonic Acid Metabolites by Operationally Defined Macrophage Subsets

INTRODUCTION

Macrophages metabolize arachidonic acid into prostaglandins(PGs), thromboxane(Tx), leukotrienes and hydroxyfatty acids. These metabolites are thought to be intercellular hormone-like compounds which can regulate the immune response. Alteration in synthesis of and/or immune cell responsiveness to these products have been found to be associated with various disease states(8). Macrophages have a central role in controlling the immune response by regulating several lymphocyte and macrophage functions. The role of macrophage derived arachidonic acid metabolites in affecting immunity is mostly speculative, since definitive studies on macrophage intermediate metabolism have not been reported. One of the problems in evaluating macrophage metabolism is that they are a functionally and biochemically heterogeneous population of cells. This heterogeneity reflects varying stages of macrophage activation. The work reported here represents a systematic approach to evaluate several macrophage populations which differ in their activation state. I found that the state of macrophage functional differentiation influenced the pattern of PGs production.

METHODS

Animals

Male C57Bl/6 mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. The mice were used between 6-12 weeks of age. They were housed 25 per cage and given food and water ad libitum.

Reagents

All tissue culture media were prepared from a powdered mix (Gibco, Grand Island, NY). Hanks balanced salt solution (HBSS) was used for obtaining peritoneal exudate cells and Selegmans balanced salt solution (SBSS) was used for washing macrophage monolayers, and aided in the removal of polymorphonuclear leucocytes. McCoy's 5A medium was supplemented with 100µg/ml streptomycin, 100 units/ml penicillin, 2mM glutamine, 25µg/ml gentamycin, 110µg/ml sodium pyruvate and 10% fetal calf serum (Gibco). The serum contained approximately 100 ng/ml lipopolysaccharide (LPS). Macrophages were adhered to a 35mm petri plate (Falcon, Oxnard, Ca.) for prostaglandin and cyclic nucleotide determinations.

Mice were injected intraperitoneally with either peptone (Difco ⁷ Laboratory, Detroit, MI), thioglycollate (Difco), pyran co-polymer (Hercules Chemical, Wilmington, Delaware) or Mycobacterium bovis, (strain BCG)(TMC 1029, Trudeau Institute, Saranac Lake, NY) in order to obtain operationally defined macrophage subpopulations. Differential cell counts were done routinely on total peritoneal exudate cells and adherant cells from each treatment group after staining with Camco Quik Stain (Scientific Products, McGraw Park, Ill.). All reagents were screened for endotoxin with a limulus amoebocyte lysate assay (Mallinckrodt Inc., St. Louis, Mo) at a sensitivity of 0.03ng/ml to the established reference E. coli lipopolysaccharide (LPS) lot EC-2 kindly provided by Dr. Richard Prior(Ohio State Univ., Division of Infectious Diseases). All tissue culture reagents, peptone, thioglycollate, pyran and BCG contained no detectable LPS. However, fetal calf serum and the 1% gelatin solution used for BCG injection were positive until diluted in LPS free water at least 1:3000 and 1:100, respectively.

Radioactive prostaglandins (3HPG) and Iodine-125 were obtained from New England Nuclear, Boston, Ma.(NEN). Unlabelled prostaglandins (PG) were kindly provided by Dr. John Pike, The Upjohn Co., Kalamazoo, MI. and unlabelled cyclic nucleotides were purchased from Sigma Chemical Co., St. Louis, MO. Thrift-solv (Kew Scientific, Columbus, Oh) was used as the scintillation cocktail.

Isolation of Macrophages

Peritoneal cells were washed from five groups of mice. Resident macrophages were obtained from untreated mice. Elicited macrophages were taken from mice injected i.p. 3 days prior with either 1 ml of 10% peptone or 10% thioglycollate. Activated macrophages were obtained from mice injected i.p. 7 days prior with either 25mg/kg of pyran co-polymer or 0.5ml 1% gelatin containing 6 million colony forming units of BCG. Peritoneal cells were removed by repeatedly lavaging with a total of 10mls of HBSS. The cells were centrifuged at $1100 \times g$, viability was determined by trypan blue exclusion and cell number was determined using a hemocytometer. Five million cells were purified by adherence in McCoy's 5A (no serum) for 1 hour at $37^{\circ}C$ in a humidified atmosphere containing 5% CO_2 in air. The monolayer was washed with SBSS, then allowed to incubate for 1-3 minutes in SBSS and finally rinsed again to remove non-adherent cells. The resultant monolayer ranged from 90-99% macrophages. Peritoneal cells obtained from mice treated with activating agents contained more polymorphonuclear leucocytes than the elicited or normal groups.

Culture of Macrophages

Peritoneal cells were cultured for up to 48 hours with 2 mls of McCoy's medium and 10% serum. At designated time intervals, the medium was removed and saved from 3-5 replicate plates, rinsed with HBSS and then the monolayers were immediately treated with 0.5ml of 5%

trichloroacetic acid (TCA). The cells were scraped, rinsed with an additional 0.5ml TCA and collected in a 12 X 75mm glass tube. The cellular protein precipitate was pelleted at 1500 x g. The supernatant (cell extract) was saved in another 12 X 75mm tube for cyclic nucleotide determination. The protein pellet was dissolved in 1ml of 0.1N sodium hydroxide and used for protein determination by the method of Lowry, et al(18). The 2ml medium sample was assayed directly for PG content. All samples were stored at 4°C.

Assay for Prostaglandins

The concentration of PGE₂, PGF_{2α}, 6-Keto PGF_{1α} (a stable metabolite of prostacyclin) and Tx B₂ (a stable metabolite of TxA₂) was assessed by radioimmunoassay. Antibodies were obtained from chicken egg yolks after injections of PG coupled to keyhole limpet hemocyanin(19). The concentration of antibody used for each metabolite was sufficient to bind 30-40% of its specific PG ligand. Ten to twelve concentrations of unlabelled PG standard ranging from 1 pg/0.1ml to 10,000 pg/0.1ml were utilized. All reagents were adjusted to the appropriate concentration with 50mM Tris buffer (0.1% BSA, 0.05% sodium azide, pH 7.5). Each 10 x 75mm glass tube contained 0.05ml of antibody, 0.05ml of 3HPG (17pg or 15,000dpm) and either 0.01ml of PG standard or medium sample. The final volume of 0.2ml was mixed by vortexing and incubated at 4°C for 18 hours. The unbound PG was removed by mixing with a 0.5ml suspension of dextran-coated charcoal (250mg Norit A, 25mg Dextran T-70, 100ml Tris buffer) and

centrifuging at 1500 x g for 20 minutes. The supernatant containing the antibody bound PG was counted on a Beckman 7000 LSC after decanting into a 5ml scintillation vial containing 3.5ml Thrift-Solv scintillation cocktail. The results were expressed as the percent of labelled PG bound versus the concentration of unlabelled PG present. The sample PG content was extrapolated from the standard displacement curve with a sensitivity of 1 pg/0.1ml. Macrophage PG levels were expressed as pg per million cells.

Assay for Cyclic Nucleotides

Macrophage cell extracts were obtained by treatment with 5% TCA, as described above. TCA was extracted 3 times with 3ml of water-saturated ethyl ether (5ml water/liter ethyl ether). Residual ether was allowed to evaporate. Samples were adjusted to pH 6.2 with 1M acetate buffer (pH 6.5). Adenosine 3',5'-monophosphate (cAMP) and guanosine 3',5'-monophosphate (cGMP) concentrations were determined by radioimmunoassay(20). Samples and unlabelled standards were treated with 0.01ml of a mixture of triethylamine and acetic anhydride in a ratio of 1.0:0.4 to increase the assay sensitivity. Antibodies were produced in rabbits by a conventional method (21) coupling cyclic nucleotides to keyhole limpet hemocyanin. The concentration of antibody was adjusted in buffer containing 0.25% bovine gamma globulin and 0.1% bovine serum albumin and was sufficient to provide approximately 30% binding. The standards were adjusted in 0.05M sodium acetate buffer. Each 12 x 75mm glass tube contained 0.1ml

sample or standard, 0.05ml I-125 cAMP or cGMP (12,000 dpm) and 0.05ml antibody. The final volume of 0.2ml was vortexed and incubated at 4°C for 18 hours. The antibody bound cyclic nucleotides were precipitated with 2.5ml 60% ammonium sulfate and pelleted after centrifugation at 1500 x g for 20 minutes. The unbound fraction was discarded. Each tube was carefully wiped dry and the antibody-antigen complex in the pellet was counted on the Beckman 7000 gamma counter. The sample nucleotide levels were extrapolated from a 10-12 point standard curve ranging from 1 fmol to 10,000 fmol. The macrophage cyclic nucleotide concentrations were expressed as pm/mg protein or as a cAMP/cGMP ratio.

Analysis of Data

PG and cyclic nucleotide experiments contained 3-5 plates per data point and were assayed in duplicate. Dunnett's t test was done with mean cpm and standard error of the mean. The data are expressed as mean $\pm$ SEM of a representative experiment and each experiment was repeated a minimum of 4 times.

RESULTS

Prostaglandin Production by Macrophage Populations

Macrophage PG metabolism was assessed using resident, elicited and activated macrophage populations. The results in Figure 1a show the concentration of PGE₂ produced by the macrophage populations during 48 hours of culture in McCoy's 5A medium with 10% serum containing LPS. Pyran and BCG activated macrophages produced 1,146 pg and 723 pg PGE₂, respectively, after 12 hours in culture; these levels increased to 1,706 pg and 1,114 pg after 48 hours. PGE₂ was the major arachidonic acid metabolite released by activated macrophages among the four metabolites measured. Resident macrophages released 602 pg within 12 hours. Thioglycollate and peptone elicited macrophages produced the least amount of PGE₂. There was no detectable level of PGE₂ released by peptone elicited macrophages until after 24 hours in culture. Thioglycollate elicited macrophages also produced low levels of PGE₂, reaching only 367 pg after 24 hours.

Resident macrophages produced the most prostacyclin (Figure 1b). There was a rapid increase in release of prostacyclin during the first 20 hours of culture reaching a final concentration of 721 pg. Both activated macrophage populations (BCG and pyran) produced 200-250 pg within 48 hours of culture. The elicited macrophage populations, in

Figure 1. Accumulation of arachidonic acid metabolites in culture supernatants of operationally defined macrophage subsets. The concentrations of PGE_2 (A), 6-Keto $\text{PGF}_{1\alpha}$ (B), TxB_2 (C) and $\text{PGF}_{2\alpha}$ (D) were determined using radioimmunoassay. Resident (●), peptone elicited (Δ), thioglycollate elicited (□), pyran activated (◇) and BCG activated (×) macrophages were incubated in McCoys 5A medium with 10% fetal calf serum.

Fig. 1

Fig. 1 (continued)

Fig. 1 (continued)

Fig. 1 (continued)

contrast, produced very low levels of prostacyclin. Thioglycollate¹⁸ and peptone elicited macrophages secreted 145 pg and 61 pg, respectively, after 48 hours.

Thromboxane metabolism for the five representative macrophage populations can be seen in Figure 1c. The overall concentration of TxB₂ does not change significantly during 48 hours of incubation, except for the activated macrophages. Production of TxB₂ increased steadily to 542 pg during the first 20 hours of culture for BCG activated macrophages. Thromboxane production by pyran activated macrophages also increased during the 48 hour incubation to 413 pg. The other macrophage populations released Tx levels which ranged between 90 pg and 250 pg.

The production of PGF₂ α by the macrophages are shown in Figure 1d. Activated macrophages produce the most PGF₂ α in culture. The first 20 hours resulted in a rapid increase to 218 pg and 208 pg for BCG and pyran activated macrophages, respectively. Peptone elicited cells produced detectable levels after 24 hours in culture and the concentration reached 95 pg at 48 hours. Thioglycollate elicited macrophages released PGF₂ levels ranging from 24 pg to 48 pg. Resident cells produced the least PGF₂ ranging between 6 pg and 17 pg.

Cyclic Nucleotide Concentration in Macrophages at Different States of 19 Activation

Cyclic nucleotide levels were determined immediately following adherence of macrophages (Table 1). Resident macrophages contained 2.09 pm cAMP/mg protein and 1.75 pm cGMP/mg protein which resulted in a cAMP:cGMP ratio of 1.19. BCG activated macrophages had a 32 fold higher cyclic nucleotide ratio. The cAMP content was elevated to 15.9 pm and the lower cGMP content of 0.41 pm resulted in a ratio of 38.78. The elicited macrophages had an intermediate cyclic nucleotide ratio of 6.67. Figure 2 shows the changes in cAMP levels for the different macrophage populations during a 24 hour incubation period in McCoy's 5A medium with 10% serum containing LPS. Resident macrophage cAMP levels increased from 2.09 pm to 8.47 pm. Peptone elicited macrophage cAMP levels were initially at an intermediate level of 5.54 pm and rose to 14.0 pm, a level similar to that initially observed for activated macrophages. The cAMP concentration of the BCG activated cells decreased from 15.9 pm to 3.67 pm.

Table 1. Cyclic Nucleotide Concentrations of Mouse Peritoneal Macrophages.^a

Macrophage	cAMP (pm/mg protein)	cGMP (pm/mg protein)	cAMP:cGMP Ratio
Resident	2.09 $\pm$ 0.6	1.75 $\pm$ 0.4	1.19
Peptone- Elicited	5.54 $\pm$ 0.5	0.83 $\pm$ 0.1	6.67
BCG- Activated	15.90 $\pm$ 0.3	0.41 $\pm$ 0.1	38.78

^aResults are mean values $\pm$ SEM determined following macrophage purification.

Figure 2. Alteration in cAMP content of cultured macrophages. Resident($\bullet$), peptone elicited(Δ) and BCG activated($\times$) macrophage monolayers were extracted with TCA and the concentration of cAMP was determined by radioimmunoassay.

Fig. 2

DISCUSSION

Arachidonic acid metabolites are one of the major secretory products of the macrophage. Macrophages have been shown to produce PGE₂(22), 6KF1 α (23), TxB₂(24), PGF₂ α (25), HETEs(26) and leukotrienes(27). Resident, elicited and activated macrophages are defined as being in different states of activation with distinct functional and biochemical properties. Generalities concerning macrophage metabolism, therefore, must be approached cautiously when only one macrophage population is studied. Culture conditions can also alter macrophage metabolism and it is difficult to compare results of different laboratories. There has been no systematic investigation of the pattern of arachidonic acid metabolism in operationally defined macrophage populations. I, therefore, have investigated the pattern of arachidonic acid metabolism by various macrophage subsets under the same culture conditions.

My results indicate that the PGs produced by the macrophages are influenced by their state of activation. The finding that PGE₂ was the major metabolite produced by the BCG and pyran activated macrophage populations is similar to that reported by Farzad, et al (25) and others (3) using C. parvum or LPS activated macrophages. Resident macrophages secreted less PGE₂ than the activated macrophages but more than the elicited cells. Humes also reported that elicited

cells produce little PGE₂(28). Several investigators have found that²⁴ resident cells also produce PGE₂ and have reported that production of PGE₂ can be stimulated by phagocytosis(29,30).

The macrophage populations used in our study exhibited a rapid linear increase in the PGE₂ concentration in the culture medium during the first 24 hours with the exception of the peptone elicited cells. The increase in PGE₂ production by these elicited cells was delayed for 24 hours. While the thioglycollate elicited macrophages did not exhibit a delay in PGE₂ production, the concentrations released during the second day in culture paralleled those released by peptone elicited macrophages. Both elicited populations were functionally similar in culture but their arachidonic acid metabolism differed slightly. This apparent difference between elicited macrophages emphasizes the need for careful evaluation of macrophage subsets.

The rapid increase in PGE₂ synthesis by activated and resident macrophages in our experiments was probably due to changes in culture conditions during the incubation period. Washing the monolayers after 24 hours and providing fresh medium resulted in the same linear increase (data not shown). This indicated that the plateau reached was in part due to the exhaustion of substrates and/or increased degradation of metabolites. Several investigators have shown that agents which affect macrophage activity can stimulate PG production(3,17). At least one of these agents, LPS, was present in the serum we used in our tissue culture medium.

My finding that resident macrophages produce the most prostacyclin is similar to that reported by Stringfellow, et al(23).

They suggested that an elevated PGI₂ synthetase activity may correlate with decreased macrophage function since the most active cells from C. parvum treated mice had a much lower synthetase activity. At present, the role of PGI₂ in controlling macrophage function is not known. PGI₂ may influence intracellular cyclic nucleotides or calcium levels. Prostacyclin is a potent agonist in stimulation of intracellular cAMP levels in cells that have been shown to contain specific receptors. Very little work has been done to elucidate the biological effects of PGI₂ on immune cells since its half-life is less than 5 minutes in neutral media. The rapid rate of PGI₂ production by resident cells may influence the functional capacity of those cells, in vitro. Reports in the literature suggest that PGE₂ is the major metabolite produced by resident macrophages, however, no attempt to measure PGI₂ was made(31). Humes, et al(28) found that basal levels of PGE₂ were slightly higher than PGI₂ levels after 30 hours of culturing. I found that the PGI₂ levels were not different than the PGE₂ concentration after a 30 hour incubation. The differences in absolute concentrations of PGs may be due to the differences in tissue culture medium and serum source. Bockman(24) has shown that in vitro synthesis of PGs is in part a function of the time of culture. The effect of varying culture conditions on PG production and macrophage anti-tumor activity is the subject of chapter 2.

Thromboxane was the major product of elicited macrophages; they produced little PGE₂, 6KF1 α and PGF₂. Morley, et al(32) also found that guinea pig elicited macrophages produced more TxB₂ than PGE₂. Bockman (24) suggested that stimulation of the Tx pathway was related

to cell adherence rather than a response to inflammatory stimulus.²⁶

The concentration of PGF_{2α} was the lowest concentration among the arachidonic acid metabolites measured. Activated macrophages produced the most while elicited and resident cells released very low levels of PGF_{2α}. I have not seen any comparable information in the literature.

Activated macrophages produced the most arachidonic acid metabolites overall. The diminished products produced by elicited macrophages may result from the decreased release and deacylation of arachidonic acid from phospholipids as observed by Humes, et al(28). They found that resident and elicited cells incorporated a similar percentage of arachidonic acid into phospholipids. However, zymosan treatment resulted in a greater PGE release by resident cells than the elicited cells. Bonney, et al(33) found that PGE₂ synthetase is more easily stimulated in resident cells than elicited cells. The biological significance of the differing patterns of PG production by macrophage subpopulations is unclear at this time. However, it is clear that macrophages have a critical role in control of inflammation and immunity. PGs mediate symptoms of inflammation and have been shown to inhibit numerous lymphocyte, as well as, macrophage functions. Several reports suggest that PGE₂ negatively regulates the expression of macrophage anti-tumor activity(15,16). The accumulation of PGE₂ by activated macrophages may be responsible for the eventual loss of activity after prolonged culturing. It has been proposed that PGE₂ production provides a negative feedback signal to control excessive tissue damage(12). Since resident cells have no anti-tumor activity, the role of elevated PGE₂ and PGI₂ concentrations are

unclear. It is possible that arachidonic acid metabolites have a positive feedback effect on non-activated macrophages. The accumulation of these products in culture may be responsible for changing the intracellular concentrations of second messengers (cyclic nucleotides and calcium) which regulate cell function and could result in increased activity of these cell populations. Elicited cells have been shown to incorporate PG precursors but they initially fail to synthesize or secrete comparable levels of PGs. This delay in activation of the arachidonic acid pathway may allow these primed cells to express certain functions at an elevated level, in vitro. The thromboxane levels observed in elicited macrophages may influence the amount of cGMP or calcium, as reported in platelets(34). Elevated cGMP and calcium are associated with enhanced phagocytosis, enzyme secretion, monokine secretion and motility(35). Studies have shown that certain metabolites stimulate phagocytosis and secretion by macrophages(12,36). Changing patterns of PG metabolism may reflect a homeostatic regulatory mechanism which first stimulates cell function in non-activated macrophages and then inhibits activated macrophage function following the accumulation of certain arachidonic acid metabolites to avoid pathological consequences. The mechanism(s) for controlling the PG pathway in macrophages have not been studied. Regulation can be achieved at various steps including uptake of precursors, release and deacylation of arachidonic acid, PG synthetase activity and release. Cyclic nucleotides and calcium levels may influence any of these steps by activating cyclic nucleotide dependent protein kinases. Therefore, knowledge of these controlling influences

may aid in understanding changes in macrophage metabolism.

Elevated cAMP:cGMP ratios caused by high cAMP and low cGMP concentrations are associated with non-dividing, differentiating cell types. In contrast, low cAMP and high cGMP levels are observed in less differentiated and less metabolically active cells. My findings support this hypothesis with regard to macrophage differentiation. The generally accepted theory proposes that activated macrophages are a more differentiated cell type than resident macrophages while elicited cells are intermediate. My data show that activated cells have a 32 fold higher nucleotide ratio than the resting resident cells and elicited macrophages are intermediate. Resident macrophage cAMP levels increased to the initially observed elicited macrophage level after a 24 hour incubation period. The level in elicited cells rose to the concentration observed for activated macrophages. The cAMP concentration of the activated macrophages decreased over time in culture. One could speculate from this data that the activated macrophage was dedifferentiating while the resident cells were maturing during the incubation period. This possibility is the subject of further investigation.

CHAPTER II

Effect of Endogeneous and Exogeneous Prostaglandin

INTRODUCTION

Macrophages synthesize arachidonic acid metabolites. Arachidonic acid metabolites also affect macrophage function(8). Schultz, et al(15) showed that exogenously added PGE inhibited macrophage anti-tumor activity. They proposed that endogenous PGE may serve as a negative feedback signal preventing activated macrophage function. Taffet, et al(16), in support of this hypothesis, found that activated macrophages lose their capacity to lyse tumor cells because of an accumulation of endogenously produced PGs. I have previously shown that PGs had a differential effect on macrophage anti-tumor activity based on their prior state of activation(14). Some investigators reported that stimulation of the macrophage to an activated state alters the sensitivity of the cell to an exogenous source of PGs(37). Treatment with agents resulting in macrophage activation have been associated with an increased release of certain PGs(12,16,22,29,38). My eariler findings indicated that the pattern of arachidonic acid metabolism was influenced by the state of macrophage activation. All of this information taken together indicated that PGs may exert a differential role in controlling macrophage function based on their prior state of activation. This investigation was designed to examine the relationship of endogeneous arachidonic acid metabolism with

macrophage function. The results indicate that culture conditions³¹ significantly affect the synthesis of arachidonic acid products and macrophage anti-tumor activity. I found that conditions which favor elevated PGE metabolism as well as the direct addition of PGs resulted in activation of resident and elicited macrophages while activated macrophage tumoricidal activity was inhibited.

METHODS

Animals

Male C57Bl/6 mice were purchased from The Jackson Laboratory, Bar Harbor, Maine. The mice were used between 6-12 weeks of age. They were housed 25 per cage and given food and water ad libitum.

Reagents

All tissue culture media were prepared from a powdered mix (Gibco, Grand Island, NY). Hanks balanced salt solution (HBSS) was used for obtaining peritoneal exudate cells and Selegmans balanced salt solution (SBSS) was used for washing macrophage monolayers, and aided in the removal of polymorphonuclear leucocytes. McCoy's 5A medium and Dulbeccos modified Eagles medium (DMEM) were supplemented with 100µg/ml streptomycin, 100 units/ml penicillin, 2mM glutamine, 25µg/ml gentamycin, 110µg/ml sodium pyruvate and 10% fetal calf serum. Serum was obtained from 2 sources: Gibco serum containing approximately 100 ng/ml lipopolysaccharide (LPS) and Sterile Systems serum (Sterile Systems, Inc., Logan, Utah) with no detectable LPS, will be referred to as LPS-free serum. Macrophages were adhered in 96 well microtest plates (Costar, Cambridge, Ma) for the assessment of

anti-tumor activity and 35mm petri plates (Falcon, Oxnard, Ca.) for prostaglandin determination. L-929 tumor cell line derived from C3H connective tissue was maintained in 75cm² culture flasks (Falcon) with DMEM containing 10% Gibco serum. The cells were passaged at confluency every 4-6 days following a brief exposure to 0.25% trypsin(Gibco).

Mice were injected intraperitoneally with either peptone (Difco Laboratory, Detroit,MI) or Mycobacterium bovis, (strain BCG)(TMC 1029, Trudeau Institute, Saranac Lake, NY) in order to obtain operationally defined macrophage subpopulations. Differential cell counts were done routinely on total peritoneal exudate cells and adherant cells from each treatment group after staining with Camco Quik Stain (Scientific Products, McGraw Park, Ill.). All reagents were screened for endotoxin with a limulus amebocyte lysate assay (Mallinckrodt Inc., St. Louis, Mo) at a sensitivity of 0.03ng/ml to the established reference E. coli LPS lot EC-2 kindly provided by Dr. Richard Prior(Ohio State Univ., Division of Infectious Diseases). All tissue culture reagents, peptone and BCG contained no detectable LPS. However, Gibco serum and the 1% gelatin solution used for BCG injection were positive until diluted in LPS free water at least 1:3000 and 1:100, respectively. Sterile Systems serum had no detectable LPS. Prostaglandins E₂, F_{2α} and indomethacin (Sigma, St. Louis, MO) were dissolved in 95% ethanol and diluted with tissue culture medium. The LPS, E. coli serotype #0128:B12,(Sigma) used in macrophage experiments was diluted in tissue culture medium.

Radioactive thymidine (3HTdR) and prostaglandins (3HPG) were³⁴ obtained from New England Nuclear, Boston, Ma.(NEN). Unlabelled prostaglandins (PG) were kindly provided by Dr. John Pike, The Upjohn Co., Kalamazoo, MI. Toluene-liquiflour (NEN) with 10% Biosolv BBS-3 (Beckman Inst., Cincinnati, Oh) and Thrift-solv (Kew Scientific, Columbus, Oh) were used as scintillation cocktails.

Isolation of Macrophages

Peritoneal cells were washed from three groups of mice. Resident macrophages were obtained from untreated mice. Elicited macrophages were taken from mice injected i.p. 3 days prior with 1 ml of 10% peptone. Activated macrophages were obtained from mice injected i.p. 7 days prior with 0.5ml 1% gelatin containing 6 million colony forming units of BCG. Peritoneal cells were removed by repeatedly lavaging with a total of 10mls of HBSS. The cells were centrifuged at 1100 x g, viability was determined by trypan blue exclusion and cell number was determined using a hemocytometer. The appropriate cell number was purified by adherence in DMEM (no serum) for 1 hour at 37°C in a humidified atmosphere containing 5% CO₂ in air. The monolayer was washed with SBSS, then allowed to incubate for 1-3 minutes in SBSS and finally rinsed again to remove non-adherent cells. The resultant monolayer ranged from 90-99% macrophages. Peritoneal cells obtained from mice treated with BCG contained more polymorphonuclear leucocytes than the elicited or normal groups.

Macrophage destruction of tumor cells was assessed as previously described(39). Cytolysis was measured by the release of ³HThdR from prelabelled L-cells. Three million L-cells were seeded into a 75cm² flask with 10uCi of ³HThdR (specific activity 2.0 Ci/mmol) in 10mls of DMEM with serum 24 hours prior to assay. Four hundred thousand peritoneal cells per well were adhered to a 96 well microtest plate in DMEM with no serum and washed as already described. Twenty thousand L-cells in 0.2mls of DMEM with 10% LPS-free serum were added to each well (unless otherwise specified) resulting in a ratio of approximately 20:1 at the beginning of the experiment. After 48 hours of co-cultivation, 0.1ml of the culture medium was placed into a scintillation cocktail containing toluene-liquiflour and 10% Biosolv BBS-3. The total radioactivity in the target cells was determined by digestion of the cells in the beginning of the experiment with 0.5% sodium dodecylsulfate (SDS). Cytolysis was calculated by the formula:

$$\frac{\text{cpm with macrophages} - \text{cpm without macrophages}}{\text{total cpm}} \times 100$$

Culture of Macrophages

Peritoneal cells were adhered to 35mm petri plates and cultured for up to 24 hours with 2 mls of DMEM medium and 10% serum. At designated time intervals, the medium was removed from 3-5 replicate plates, rinsed with HBSS and the monolayers were immediately treated with 0.5ml of 5% trichloroacetic acid (TCA). The cells were scraped,

rinsed with an additional 0.5ml TCA and collected in a 12 X 75mm glass tube. The cellular protein precipitate was pelleted at 1500 x g. The protein pellet was dissolved in 1ml of 0.1N sodium hydroxide and used for protein determination by the method of Lowry, et al(18). The 2ml medium sample was assayed directly for PG content. All samples were stored at 4°C.

Assay for Prostaglandins

The concentration of PGE₂, PGF₂ α , 6-Keto PGF₁ α (a stable metabolite of prostacyclin) and TxB₂ (a stable metabolite of TxA₂) was assessed by radioimmunoassay. Antibodies were obtained from chicken egg yolks after injections of PG coupled to keyhole limpet hemocyanin(19). The concentration of antibody used for each metabolite was sufficient to bind 30-40% of its specific PG ligand. Ten to twelve concentrations of unlabelled PG standard ranging from 1 pg/0.1ml to 10,000 pg/0.1ml were utilized. All reagents were adjusted to the appropriate concentration with 50mM Tris buffer (0.1% BSA, 0.05% sodium azide, pH 7.5). Each 10 x 75mm glass tube contained 0.05ml of antibody, 0.05ml of 3HPG (17pg or 15,000dpm) and either 0.01ml of PG standard or medium sample. The final volume of 0.2ml was mixed by vortexing and incubated at 4°C for 18 hours. The unbound PG was removed by mixing with a 0.5ml suspension of dextran-coated charcoal (250mg Norit A, 25mg Dextran T-70, 100ml Tris buffer) and centrifuged at 1500 x g for 20 minutes. The supernatant containing the antibody bound PG was counted on a Beckman 7000 LSC after

decanting into a 5ml scintillation vial containing 3.5ml Thrift-Solv scintillation cocktail. The results were expressed as the percent of labelled PG bound versus the concentration of unlabelled PG present. The sample PG content was extrapolated from the standard displacement curve with a sensitivity of 1pg/0.1ml. Macrophage PG levels were expressed as pg per million cells.

Analysis of Data

Assays for the assessment of antitumor activity were performed with 6 replicates. PG experiments contained 3-5 plates per data point and were assayed in duplicate. Dunnett's t test was done with mean cpm and standard error of the mean. The data are expressed as mean $\pm$ SEM of a representative experiment and each experiment was repeated a minimum of 4 times.

RESULTS

Effect of Culture Conditions on Tumoricidal Activity

Increased PG production has been associated with decreased macrophage tumoricidal activity. I, therefore, determined the levels of PGs produced by operationally defined macrophage populations in the different media. Different culture media resulted in an alteration in the expression of tumoricidal activity of operationally defined populations of macrophages (Table 2). Resident and peptone elicited macrophages did not kill tumor cells in LPS free medium. McCoys 5A medium with serum containing LPS or LPS spiked medium resulted in 20.5% and 14.2% destruction. Resident macrophages became highly tumoricidal in DMEM in the presence of LPS resulting in 52.2% and 44.1% tumor cell killing. Peptone elicited macrophages also destroyed up to 69.6% of the tumor cells in DMEM with LPS present while only 14.5% destruction was detected in McCoys 5A with LPS. In contrast, BCG activated macrophages lysed 74.7% of the tumor cells in DMEM in the absence of LPS. The presence of either LPS or McCoys 5A medium resulted in lower tumoricidal activity by BCG activated macrophages.

Table 2. Effect of Culture Conditions on the Tumoricidal Activity of Operationally Defined Macrophages.

Medium	LPS Containing Serum	LPS Added ^b	% Tumor Cell Killing ^a		
			Resident	Elicited	Activated
McCoy's 5A	Yes	No	20.5 ± 1.7	14.5 ± 1.0	29.1 ± 1.1
	No	No	9.8 ± 0.8	6.1 ± 0.2	22.4 ± 0.7
	No	Yes	14.2 ± 0.6	9.4 ± 1.0	27.9 ± 1.3
DMEM	Yes	No	52.5 ± 3.8	69.6 ± 2.8	54.5 ± 2.3
	No	No	4.7 ± 0.8	8.5 ± 0.9	74.7 ± 4.5
	No	Yes	44.1 ± 1.9	44.1 ± 3.2	59.3 ± 1.3

^aData are expressed as mean values ± SEM of 6 replicates.

^bOne serum source contained approximately 10 ng LPS and the other was spiked with an equivalent amount.

Macrophage Prostaglandin Metabolism in Different Culture Conditions

BCG activated macrophages produced higher levels of PGE₂ than resident or elicited macrophages in McCoy's 5A with LPS containing serum (Table 3). However, resident and elicited macrophages produced more PGE₂ when cultured in DMEM with LPS present. No PGE₂ was produced in LPS free serum with McCoy's 5A and very low levels were released when the cells were cultured in DMEM with no LPS. Resident macrophage PGE₂ levels increased 3.7 fold from 243.5 pg in McCoy's 5A medium with LPS to 902.2 pg in LPS containing DMEM. Elicited macrophages released 6.3 fold more PGE₂ in DMEM with LPS (780.5 pg) than in McCoy's 5A medium (123.5 pg). PGE₂ levels decreased 7.8 fold from 510.6 pg to 65.4 pg for BCG activated macrophages when cultured in DMEM.

Resident macrophages produced the most prostacyclin (PGI₂) when compared to the elicited and activated cells (Table 4). The concentration of 6-Keto PGF_{1 α} reached 30 pg for resident cells, 20.2 pg for elicited cells and 14.1 pg for activated cells. All three populations released approximately 3 fold higher levels of the metabolite when incubated in DMEM than they did in McCoy's 5A medium. The absence of LPS resulted in a significantly lower level of PGI₂ produced, except for the BCG activated macrophages.

The concentrations of thromboxane produced by the macrophage populations are shown in Table 5. The cells released more Tx when cultured in DMEM than in McCoy's 5A medium. Peptone elicited macrophage Tx levels increased 3.7 fold from 345.9 pg to 1,292.6 pg in

Table 3 . Effect of Culture Conditions on PGE₂ Production by Operationally Defined Macrophages.^a

Medium	LPS Containing Serum	LPS Added ^b	PGE ₂ (pg/10 ⁶ cells)		
			Resident	Elicited	Activated
McCoy's 5A	Yes	No	243.5 ± 16	123.2 ± 43	510.6 ± 60
	No	No	0	0	0
	No	Yes	0	123.2 ± 44	75.0 ± 22
DMEM	Yes	No	902.2 ± 21	780.5 ± 24	65.4 ± 14
	No	No	70.6 ± 23	153.6 ± 32	23.7 ± 3
	No	Yes	619.5 ± 32	890.2 ± 77	52.3 ± 3

^aPGE₂ was measured in media collected from macrophages cultured for 24 hours and the data was expressed as mean values ± SEM.

^b10 ng LPS was added, as described in Table 2.

Table 4. Effect of Culture Conditions on PGI₂ Production by Operationally Defined Macrophages.^a

Medium	LPS Containing Serum	LPS Added	PGI ₂ (pg/10 ⁶ cells)		
			Resident	Elicited	Activated
McCoy's 5A	Yes	No	30.0 ± 4.0	20.2 ± 0.7	14.1 ± 4.0
	No	No	14.4 ± 1.8	2.4 ± 0.8	9.1 ± 0.5
	No	Yes	18.5 ± 1.5	15.8 ± 0.2	7.7 ± 1.8
DNEM	Yes	No	89.1 ± 2.2	68.3 ± 2.4	41.2 ± 0.3
	No	No	60.2 ± 1.5	39.7 ± 3.4	40.4 ± 2.1
	No	Yes	64.5 ± 7.0	62.2 ± 3.7	42.0 ± 0.5

^a6-Keto PGF_{1α}, a stable metabolite of PGI₂, was measured as described in Table 3.

Table 5. Effect of Culture Conditions on Thromboxane Production by Operationally Defined Macrophages.^a

Medium	LPS Containing Serum	LPS Added	TxB ₂ (pg/10 ⁶ cells)		
			Resident	Elicited	Activated
McCoy's 5A	Yes	No	269.5 ± 12	345.9 ± 24	321.8 ± 14
	No	No	182.6 ± 18	234.2 ± 10	263.3 ± 8
	No	Yes	195.6 ± 1	429.3 ± 30	279.2 ± 14
DMEM	Yes	No	717.4 ± 22	1292.6 ± 25	824.5 ± 80
	No	No	597.8 ± 32	829.2 ± 25	664.9 ± 38
	No	Yes	663.1 ± 11	1207.3 ± 12	784.6 ± 14

^aTxB₂ was measured as described in Table 3.

the presence of LPS containing serum. Resident and BCG activated macrophages released approximately 2.5 fold more Tx in the minimal medium resulting in 717.4 pg for the resident cells and 824.5 pg for the activated cells. Medium with no detectable LPS resulted in lower Tx levels. LPS free serum spiked with 10 ng LPS restored the Tx concentration to the level observed with serum containing LPS.

BCG activated macrophages produced the highest level of PGF2_α in either McCoy's 5A medium or DMEM (Table 6). Culturing the cells in DMEM resulted in a 2.3 fold increase from 199.0 pg to 465.4 pg for the BCG macrophages, 1.8 fold increase from 134.8 pg to 243.5 pg for resident cells and 1.9 fold increase from 139.0 pg to 273.2 pg for the peptone elicited macrophages. The absence of LPS reduced the level of PGF2_α detected which could be restored after spiking the serum with LPS.

Effect of Prostaglandin Pretreatment on Macrophage Tumoricidal Activity

Culturing BCG activated macrophages for 48 hours resulted in a 42.7% decrease in tumoricidal activity (Table 7). Macrophages obtained from mice treated with BCG destroyed 78.6% of the tumor cells while cells incubated for 48 hours lysed 33.6% of the tumor cells. This loss of activity has been attributed to the production of PG by the macrophage(16). The addition of 10^{-6}M indomethacin inhibited the decrease in tumor cell destruction. Exogenous addition of PGE_2 (10^{-7}M) further reduced the tumoricidal activity to 8.4%.

Table 6. Effect of Culture Conditions on $\text{PGF}_{2\alpha}$ Production by Operationally Defined Macrophages.^a

Medium	LPS Containing Serum	LPS Added	$\text{PGF}_{2\alpha}$ (pg/ 10^6 cells)		
			Resident	Elicited	Activated
McCoy's 5A	Yes	No	134.8 ± 6.0	139.0 ± 7.3	199.5 ± 8.0
	No	No	46.7 ± 1.1	63.4 ± 0.9	66.5 ± 5.0
	No	Yes	65.1 ± 4.3	128.1 ± 1.2	121.0 ± 1.3
DMEM	Yes	No	243.5 ± 17	273.2 ± 9.8	465.4 ± 12
	No	No	115.2 ± 6.5	178.1 ± 9.5	202.2 ± 16
	No	Yes	186.9 ± 4.3	263.4 ± 4.9	345.7 ± 48

^a $\text{PGF}_{2\alpha}$ was measured as described in Table 3.

Table 7. Effect of Pretreatment with PGE₂ on Tumoricidal Activity of BCG Activated Macrophages.^a

Pretreatment	Concentration	% Tumor Cell Killing
None		78.6 ± 3.3
Medium		33.6 ± 1.2 ^b
Indomethacin	10 ⁻⁶ M	59.6 ± 2.0
PGE ₂	10 ⁻⁷ M	8.4 ± 0.8 ^b
	10 ⁻⁶ M	9.2 ± 5.6 ^b
	10 ⁻⁵ M	24.4 ± 4.8 ^b

^aTumor cell destruction was measured after 48 hours preincubation in DMEM with LPS free serum.

^bp<0.01.

Resident macrophages did not lyse tumor cells, in vitro, unless cultured in the presence of activating agents, ie LPS or MAF. However, resident macrophages pretreated with PGE₂ in LPS free medium became tumoricidal (Figure 3). Tumor cell killing measured after preincubation in medium alone increased from the control value of 1.6% (with no preincubation) to 9.3% at 24 hours and 15% at 48 hours. Pretreatment of resident macrophages with 10⁻⁵M PGE₂ resulted in the lysis of 40% of the tumor cells. The increase in tumoricidal activity did not differ following 24 or 48 hour pretreatment with PG. The development of anti-tumor activity caused by culturing for a prolonged period or the presence of LPS was prevented by indomethacin (Table 8). The presence of LPS induced resident macrophages to destroy 19.5% of the tumor cells with no preincubation. Indomethacin treated cells lysed only 2.5% of the targets in LPS containing medium. Preincubation for 24 hours with medium containing LPS resulted in a tumoricidal activity of 30.6%. The addition of indomethacin prevented the development of cytotoxic activity; only 6.0% of the tumor cells were destroyed.

Inflammatory macrophages elicited by peptone did not lyse tumor cells in vitro; only 4.8% of the tumor cells were destroyed (Figure 4). Exogeneously added PGE₂ and PGF₂ α activated peptone elicited macrophages to destroy tumor cells. A concentration of 10⁻⁹M PGE₂ and PGF₂ α resulted in 79.3% and 75.1% lysis of tumor cells. Tumor cell killing was elevated to 44.5% after preincubation in LPS free medium for 24 hours. This increased killing can be attributed to the accumulation of endogeneous PGs since indomethacin treatment prevented the rise (Table 8). Twenty four hour preincubation with medium

containing LPS caused 69.1% tumor cell lysis while indomethacin treated cells only destroyed 6.2% of the targets.

Figure 3. Effect of pretreatment with PGE_2 on the tumoricidal activity of resident macrophages. Tumor cell destruction was assessed following either no pretreatment (Δ), pretreatment for 24 hours ($\square$) or 48 hours ($\times$).

Fig. 3

Figure 4. Effect of pretreatment with PGE_2 and $\text{PGF}_{2\alpha}$ on the tumoricidal activity of peptone² elicited^{2a} macrophages. Tumor cell destruction was assessed following no pretreatment (Δ) and pretreatment with either PGE_2 ($\square$) or $\text{PGF}_{2\alpha}$ ($\times$) for 24 hours.

Fig. 4

Table 8. Effect of Indomethacin on Macrophage Activation in Serum Containing LPS.

LPS Containing Serum	Indomethacin	24 Hour Preincubation	% Tumor Cell Killing ^a	
			Resident	Elicited
No	No	No	1.6 $\pm$ 0.6	4.8 $\pm$ 1.0
Yes	No	No	19.5 $\pm$ 2.3 ^b	34.2 $\pm$ 2.9 ^b
Yes	Yes	No	2.5 $\pm$ 0.2	6.3 $\pm$ 1.1
No	No	Yes	9.3 $\pm$ 1.0	44.5 $\pm$ 2.7
Yes	No	Yes	30.6 $\pm$ 1.5 ^b	69.1 $\pm$ 3.2 ^b
Yes	Yes	Yes	6.0 $\pm$ 1.1	6.2 $\pm$ 0.8 ^b

^aTumor cell destruction was determined using DMEM and the data are expressed as mean values $\pm$ SEM of 6 replicates.

^bSignificantly different than LPS free control values (p<0.01).

DISCUSSION

I have shown in chapter 1 that the pattern of PG metabolism is influenced by the state of macrophage activation. The effect of arachidonic acid metabolites produced by the macrophage on macrophage function is unclear. This investigation was designed to elucidate the role of endogeneously produced PGE₂ in controlling macrophage function.

Operationally defined subsets of macrophages were routinely screened for their tumoricidal activity. I found that activated macrophages destroyed tumor cells best when assayed in a minimal medium, DMEM, with no detectable LPS. Resident and elicited cells had no tumoricidal activity when tested under the same conditions. In contrast, the anti-tumor activity of activated macrophages was lower in McCoy's 5A medium. Resident and peptone elicited macrophages were activated by lower LPS concentrations in DMEM than in McCoy's 5A medium.

I hypothesized that the apparent differences in tumoricidal activity were due, in part, to the varying constituents of the tissue culture media which could result in changes in cellular metabolism. Several arachidonic acid metabolites produced by the 3 macrophage subpopulations were measured using a variety of culture conditions. McCoy's 5A medium or DMEM were supplemented with fetal bovine serum

from two sources, one of which was LPS free. Each medium with LPS free serum was also spiked with the approximate concentration of LPS found in the other serum. Our results indicate that the pattern of PGE₂ production by the macrophages changed in the different media. Activated macrophages produced the highest PGE₂ levels in McCoy's 5A with LPS present while resident and elicited cells produced more in DMEM. The pattern of PGE₂ released correlated with changes in the level of tumoricidal activity. The diminished activity of the activated cells in McCoy's 5A medium may be due to the elevated PGE₂ accumulating in culture. The change in PGE₂ metabolism in the non-tumoricidal macrophages may be responsible for the alteration in LPS-induced activation. The other products, PGF₂ α , TxB₂ and 6-keto PGF₁ α , were found in higher concentrations in DMEM with LPS containing serum. It is not clear, however, why macrophages cultured in DMEM produced elevated levels of these arachidonic acid metabolites. A comparison of the media indicates that DMEM contains twice as much calcium as McCoy's 5A medium. Calcium has been shown to activate phospholipase A₂ which cleaves arachidonic acid from the membrane phospholipids(40).

I examined the effect of exogenously added PGE₂ on anti-tumor activity to further establish the significance of macrophage derived PGE₂. I had previously found that PGE₂ or PGF₂ α had no effect on activated macrophage tumor cell killing when the PG were added at the onset of the experiment(14). However, tumoricidal activity was completely inhibited by PGs when the activated macrophages were pretreated for 48 hours prior to assay. The decrease in anti-tumor

activity following 48 hours incubation in DMEM was blocked by the addition of indomethacin. This observation supports those of Taffet, et al(16) who found that PGE2 accumulation in culture was responsible for loss of anti-tumor activity. My findings further demonstrate that macrophages destroyed the tumor cells better in DMEM because they produced less PGE2.

I have previously reported that resident macrophages acquire the capacity to inhibit tumor cell proliferation when treated with PGs(14). I have now provided evidence that resident and elicited cells become tumoricidal when pretreated with PGE. This is based on several observations. First, exogeneously added PGE2 activated the macrophages in the absence of LPS or MAF. Secondly, macrophages were activated optimally under culture conditions which resulted in increased arachidonic acid metabolism. Finally, indomethacin treatment prevented the activation of the macrophage to tumoricidal activity induced by preincubation for 24 hours or by LPS treatment. The macrophage subpopulations were more easily activated because they produced higher levels of PGE2. The enhanced tumoricidal activity of resident and elicited cells after preincubation in medium alone can also be attributed to the accumulation of PGs.

The observation that PGs can stimulate macrophages is not new. The addition of PGE2 enhanced the phagocytic capacity of elicited macrophages and restored the phagocytic defect in C3H/HeJ mice(41). This report represents the first with regard to anti-tumor activity of macrophage populations. The differing pattern of PG metabolism by macrophages in different states of activation may reflect a self

regulatory mechanism of controlling activation. Resident macrophages are stimulated to produce PGs which in turn activated these macrophages to become tumoricidal. Further accumulation of the PGs can then prevent longterm, uncontrolled expression of anti-tumor cell activity by the activated macrophages.

CHAPTER III

Role of Prostaglandin in Macrophage Activation

INTRODUCTION

The ability of a population of macrophages to perform an effector function is governed by its state of activation. Acquisition of tumoricidal activity is one of the functional phenotypes used to define an activated macrophage. These operationally defined activated macrophages have several distinct biochemical markers(42). Macrophage populations representing different states of activation can be obtained following either in vivo or in vitro treatment with various agents. Resident peritoneal mouse macrophages from untreated animals demonstrate lower levels of functional activity while elicited cells from mice treated with sterile irritants have elevated functional activity. Neither of these operationally defined populations have the capacity to destroy tumor cells, in vitro. However, elicited cells are activated, in vitro, with either macrophage activating factor(MAF) or bacterial lipopolysaccharide(LPS) treatment and resident cells require the presence of both preparations. This in vitro model has proven useful in studying the regulation of macrophage functional differentiation.

Several investigators have shown that macrophages produce high levels of arachidonic acid metabolites. Previous work in my laboratory demonstrated that the pattern of arachidonic acid

metabolism was influenced by the state of macrophage activation. Varying culture conditions resulted in changes in the metabolism of arachidonic acid(chapter 2). The alteration in the endogeneous production of certain arachidonic acid metabolites or the addition of PGE also induced activation of resident and elicited cells. Taken together, these data suggested that arachidonic acid products regulated the functional differentiation of the macrophage. I now report that PGE provides a priming signal for resident macrophages and an activating signal for elicited macrophages, both independent of LPS. Additionally, I have shown that PGE may be acting synergistically with MAF resulting in the induction of anti-tumor activity.

METHODS

Animals, reagents, collection of macrophages, anti-tumor assay and prostaglandin assay are all described in chapter 2.

Assay for Cyclic Nucleotides

Macrophage cell extracts were obtained by treatment of purified monolayers with 5% trichloroacetic acid(TCA). TCA was extracted 3 times with 3ml of water-saturated ethyl ether (5ml water/liter ethyl ether). Residual ether was allowed to evaporate. Samples were adjusted to pH 6.2 with 1M acetate buffer(pH 6.5). Adenosine 3',5'-monophosphate (cAMP) concentration was determined by radioimmunoassay(20). Samples and unlabelled standards were treated with 0.01ml of a mixture of triethylamine and acetic anhydride in a ratio of 1.0:0.4 to increase the assay sensitivity(21). Antibodies were produced in rabbits by a conventional method coupling cyclic nucleotides to keyhole limpet hemocyanin. The concentration of antibody was adjusted in buffer containing 0.25% bovine gamma globin and 0.1% bovine serum albumin and was sufficient to provide approximately 30% binding. The standards were adjusted in 0.05M sodium acetate buffer. Each 12 x 75mm glass tube contained 0.1ml sample or standard, 0.05ml I-125 cAMP (12,000 dpm) and 0.05ml

antibody. The final volume of 0.2ml was mixed by vortexing and incubated at 4°C for 18 hours. The antibody bound cyclic nucleotides were precipitated with 2.5ml 60% ammonium sulfate and pelleted after centrifugation at 1500 x g for 20 minutes. The unbound fraction was discarded. Each tube was carefully wiped dry and the antibody-antigen complex in the pellet was counted on a Beckman 7000 gamma counter. The sample concentrations were extrapolated from a 10-12 point standard curve ranging from 1 fmol to 10,000 fmol. The macrophage cyclic nucleotide levels were expressed as pm/mg protein.

Lymphokine Preparation

Lymphokine-containing supernatants were prepared as previously described by Pace, et al(43). Briefly, spleens were removed from normal mice, cells were dispersed by teasing with a rubber policeman through a wire cell sieve and passage through sequentially smaller syringe needles. The cells were centrifuged at 1100 x g for 10 minutes and the cell number was adjusted to 5×10^6 /ml in DMEM containing 5×10^{-5} M 2-mercaptoethanol and 2 ug/ml concanavalin A(Con A, Sigma Chemical Co., St. Louis, MO). Fifty ml of this cell suspension was placed into a 75 cm² tissue culture flask. Control cultures were prepared similarly, except they did not contain Con A. The flasks were incubated for 72 hours at 37°C in humidified air containing 5% CO₂. Culture supernatants were obtained by centrifugation at 1100 x g for 20 minutes, filtered and stored at 4°C. Con A (2 ug/ml) was added to cell free control supernatants. These

control lymphokine preparations had no effect on macrophage⁶³ tumoricidal activity. Endotoxin could not be detected in the lymphokine preparations. PG concentrations were determined as described in chapter 2. The Con A stimulated spleen cell supernatant will be referred to as macrophage activating factor(MAF).

Macrophage Activation

The activated state of macrophages was operationally defined as the ability to destroy tumor cells. Resident or elicited macrophages had no detectable tumoricidal activity when tested in an LPS free media. Macrophage monolayers were exposed to activating agents (MAF and/or LPS) for a minimum of 2 hours prior to the addition of prelabelled L-cells. The assay was done in DMEM with 10% LPS free serum. The tumoricidal assay contained activating agents during the 48 hour co-cultivation. The tumor cells alone were also treated with the activating agents. Indomethacin (10⁻⁶M) was added to certain monolayers for 1 hour prior to the addition of L-cells. Macrophages secreted no detectable PGs following indomethacin treatment. All monolayers pretreated with drugs were washed thoroughly at the beginning of the assay.

Analysis of Data

Assays for the assessment of antitumor activity were performed with 6 replicates. PG and cyclic nucleotide experiments contained 3-5

plates per data point and were assayed in duplicate. Dunnett's t test⁶⁴ was done with mean cpm and standard error of the mean. The data are expressed as mean $\pm$ SEM of a representative experiment and each experiment was repeated a minimum of 4 times.

RESULTS

Activation In Vitro

The experiments in chapter 2 demonstrated that PGs had a differential effect on macrophages at different levels of activation, inhibiting activated macrophage tumoricidal activity and activating normal or elicited macrophages. The critical relationship between the state of macrophage activation and PGs produced by the cells was examined by inducing tumoricidal activity in vitro with activating agents.

Resident macrophages destroyed 52.2% of the tumor cells when treated with a 1/120 dilution of MAF and 50 ng LPS (Figure 5). MAF alone had no effect on the tumoricidal activity which ranged from 0.6% to 4.0%. Concentrations of LPS exceeding 500 ng induced resident macrophages to destroy up to 20% of the tumor cells while lower concentrations had no effect (data not shown).

Elicited macrophages were activated from control levels of 6.9% to 42.2% with a 1/80 dilution of MAF alone (Figure 6). Treatment with 10 ng LPS alone resulted in 35.7% lysis of tumor cells. The addition of 10 ng of LPS with a 1/400 dilution of MAF caused 50.5% tumor cell lysis.

Figure 5. Activation of tumoricidal activity in resident macrophages. Adherent monolayers were treated with varying dilutions of MAF alone (□) or MAF with 50 ng LPS (X). The release of ³HTdR from prelabelled L cells was determined after a 48 hour incubation.

Fig. 5

Figure 6. Activation of tumoricidal activity of elicited macrophages. Adherent monolayers were treated with varying dilutions of MAF alone (□) or MAF with 10 ng LPS (x). The release of ³HTdR from prelabelled L cells was determined after a 48 hour incubation.

Fig. 6

I have shown in chapter 1 that activated macrophages have higher⁷⁰ cAMP levels than resident or elicited macrophages. The results in Figure 7 demonstrates that the intracellular cAMP concentration in peptone elicited macrophages increased after in vitro activation. The elicited macrophages contained 3.26 pg cAMP/mg protein which increased in a dose dependent fashion to 5.34 pg/mg protein with 50 ng LPS. MAF did not by itself cause a rise in cAMP. A 1/120 dilution of MAF in the presence of 50 ng LPS caused the level of cAMP to rise to 7.59pg/mg protein.

Correlation of Prostaglandin Metabolism and Macrophage Activation

LPS caused a dose dependent increase in tumoricidal activity of elicited macrophages which went from 3.1% with no LPS to 57.9% with 500 ng LPS (Figure 8). A parallel increase in PGE₂ production with increasing LPS concentration was observed. Elicited macrophages produce 438 pg PGE₂/10⁶ cells in LPS free medium after 48 hours while 500 ng LPS caused the release of 2,557 pg PGE₂/10⁶ cells. MAF also activated elicited macrophages in a dose dependent fashion resulting in 48.8% lysis of tumor cells with a 1/20 dilution (Figure 9). Treatment of macrophage cultures with MAF alone did not result in an increase in arachidonic acid metabolites(data not shown). However, MAF containing supernatant fluids had detectable levels of PGE₂. The LPS free medium used in macrophage activation experiments had no detectable PGE₂. A 1/8 dilution of MAF contained 90 pg of PGE₂. The increasing concentration of PGE₂ in the MAF preparation paralleled the capacity of MAF to activate peptone macrophages.

Figure 7. Change in cAMP concentration in elicited macrophages following in vitro activation. Adherent monolayers were treated with varying doses of LPS alone (□) or with LPS and a 1/120 dilution of MAF(X). Cyclic AMP content of TCA extracts were determined by radioimmunoassay.

Fig. 7

Figure 8. Correlation of PGE₂ synthesis and tumoricidal activity. Tumor cell destruction (□) was evaluated following activation with LPS and PGE₂ content (x) was determined using radio-immunoassay in parallel cultures.

Fig. 8

Fig. 9 Correlation of MAF PGE₂ concentration with macrophage anti-tumor activity. PGE₂(□), tumor cell destruction(X)

To explore the possibility that PGs were increasing the sensitivity of resident macrophages to MAF mediated activation, the cells were pretreated with PGE2 for 24 hours prior to the addition of MAF. Untreated resident macrophages are unresponsive to MAF, however pretreatment with PGE2 resulted in activation (Figure 10). Resident macrophages incubated in LPS free medium for 24 hours lysed 8.4% of the tumor cells which increased to 24.1% with a 1/8 dilution of MAF. Pretreatment with 10^{-7} M PGE2 alone activated resident macrophages to destroy 21.0% of the tumor cells. Resident macrophages cultured previously with PGE2 exhibited an increased sensitivity to MAF activation. Tumoricidal activity increased to 69.8% with 10^{-7} M PGE2 pretreatment followed by a 1/8 MAF dilution.

Figure 10. Priming of resident macrophages with PGE_2 for MAF activation. Adherent monolayers were pretreated with 10^{-7} M PGE_2 (x) or LPS free media alone ($\square$) for 24 hours prior to the addition of varying dilutions of MAF. Tumoricidal activity, as determined by $^3\text{HTdR}$ release, was also evaluated with no pretreatment (Δ).

Fig. 10

DISCUSSION

Prostaglandins exert positive and negative effects on macrophage function. The addition of exogenous PGs inhibited activated macrophage tumoricidal activity(chapter 1) and phagocytic activity(14). In contrast, I found that resident and elicited macrophages were activated to become tumoricidal by the addition of PGs. This differential effect of PGs on macrophage anti-tumor activity lead to an investigation of the role of PGs in controlling macrophage activation.

Macrophage activation, in vitro, is believed to represent a sequence of events which results in cellular differentiation. The activated or differentiated macrophage is operationally defined by the aquisition of a new function, tumor cell killing. The resident and elicited macrophage are resting and stimulated cells which represent earlier stages in the activation cascade. Kuo, et al(44) provided evidence that two signals were required to activate resident cells, in vitro. The first signal primes or stimulates the cell to express higher functional potential. This step is thought to occur in vivo following the induction of an inflammatory response with sterile irritants or in vitro with LPS. The work of Lang(45) and Adams (46) suggests that the second signal induces the appearance of receptors responsible for target cell binding or secretion of a cytolytic

factor. Products of responding T lymphocytes(MAF) are thought to provide the second signal. Several investigators have proposed that the physiological role of LPS may be to initiate an endogeneous activation signal(47). I report that PGs are responsible for the priming or potentiation of macrophage functional differentiation.

My results showed that LPS activation of elicited macrophages paralleled an increase in PGE2 production. MAF alone did not stimulate the release of PGE2. This observation has also been reported by Meltzer, et al(48) and by Taffet, et al(49). The activation capacity of the MAF, however, was associated with the concentration of PGE2 in the lymphokine preparation. I suggest that the variability of potency between each MAF preparation may reflect the level of arachidonic acid products produced by the stimulated spleen cells used for the lymphokine preparation. This hypothesis is supported by the observation that LPS free lymphokine supernatants are less active then those contaminated with LPS(43,49). Presumably the LPS stimulates arachidonic acid metabolism thus priming the macrophages for MAF activation.

Other investigators have also observed that an increase in PG synthesis correlates with the state of macrophage activation (22,23,50). They concluded that stimulation of the PG pathway and the acquisition of anti-tumor activity were regulated by a common mechanism but that there was no direct relationship. I beleive that PGs have a direct role in regulation of macrophage activation.

To further substantiate the effect of PGs on macrophage activation, I examined the two signals required for resting resident

macrophages to become tumoricidal. Resident cells were unresponsive⁸¹ to MAF unless LPS was provided. My results show that the addition of PGE could replace the requirement for LPS, rendering resident cells tumoricidal with MAF alone. The signal mediated by PGE appears to prime the cells and acts synergistically with MAF to induce tumoricidal activity. Taffet, et al(49) suggested that MAF was involved in priming macrophages for a second stimulus, however, my data indicates that the PGE content of the lymphokine preparation may be, in part, responsible. The limited work done with chromatographically enriched MAF suggests that this may be the case since expression of tumoricidal activity either required the addition of LPS or contaminating LPS was present in the reagents.

Elicited macrophages can be activated by either LPS or MAF in a dose dependent fashion. The combined treatment of LPS and MAF resulted in an additive effect, in contrast to the synergistic response by resident macrophages. The work reported here demonstrates that the development of tumoricidal activity correlates with the presence of PGE₂. Work presented in chapter 2 showed that the addition of exogenous PG resulted in activation. Additionally, LPS and culture induced activation was blocked by indomethacin treatment. Drysdale, et al(50) described PG dependent and independent phases of macrophage activation. They found that activation of elicited macrophages by LPS or MAF could be blocked by indomethacin and restored by the addition of PGE. However, PGs alone were unable to activate elicited macrophages with a 4 hour pulse. They concluded that PGs were involved in regulating the effector phase and not the

activation phase. Unfortunately, the level of LPS contamination in⁸² their reagents was not assessed and a different tissue culture medium was utilized. Previous work by others(24) and the results in chapter 1 have shown that culture conditions significantly influenced arachidonic acid metabolism. I suggest that PGs may be one of the endogenous stimuli responsible for direct activation of primed macrophages.

Culture induced activation also resulted in changes in the cAMP and cGMP content of the cell(chapter 1). Functionally activated macrophages have high cAMP and low cGMP levels. One might speculate that PGs may prime macrophages by elevating intracellular cAMP. Bonney, et al(37) found that macrophages exhibited a differential sensitivity to PGE₂. Elicited macrophages produced more cAMP than resident cells in response to PGE treatment. This hypothesis may explain the different sensitivities of the macrophage populations to activation agents.

The ability of PG to regulate macrophage activation is not a new concept. However, direct evidence in support of this theory has not been provided. I hypothesize that endogenous PGs may be responsible for influencing intracellular regulators in control of macrophage functional differentiation. I have shown that the state of macrophage activation influences the endogenous production of arachidonic acid metabolites. The acquisition of tumoricidal activity correlates with the macrophages synthesis and release of PGs. Based on these observations, I propose that macrophages, while responding to inflammatory stimuli, increase the production of PGE. The PGE primes

the macrophages to receive a second signal mediated by MAF. Several⁸³ stimuli have been shown to result in an increase in the release of PGE by resident cells including zymosan, LPS, antigen-antibody complexes and antibody coated red blood cells(8). The biological significance of PGE may be to potentiate the diverse functional activities of macrophages during an inflammatory response.

SUMMARY

Arachidonic acid metabolites modulate immune responses at various levels. Lymphocyte and macrophage function can be directly affected by these metabolites. Additionally, products from arachidonic acid metabolism can affect the release and/or action of specific factors which regulate the outcome of an immune response. Macrophages have a central role in controlling immunity. They are also the major source of arachidonic acid products among immunocompetent cells. Control of arachidonic acid metabolism in macrophages may provide a useful tool for regulating immune cell function.

Macrophage effector functions are affected by treatment with arachidonic acid metabolites, mostly the prostaglandins. We have previously reported that PGs have a differential effect on macrophages based on their prior state of activation; while BCG activated macrophage tumoricidal activity was inhibited by the addition of exogenous PGs, resident macrophages acquired the capacity to effect tumor growth. These opposing effects led me to explore the role of PGs on macrophage activation.

I assessed the interrelationships between macrophage activation, cyclic nucleotides and arachidonic acid products by several approaches. First, three macrophage populations, representative of different stages of activation, were utilized to define the baseline

concentrations of four major arachidonic acid metabolites and of the⁸⁵ cyclic nucleotides. The profile of these intermediate metabolites was followed during a two day incubation period. Secondly, the association of functional changes with arachidonic acid metabolism was assessed. The concentration of arachidonic acid products in the culture supernatants were altered by changes in metabolism induced by differing culture conditons and also by the addition of exogeneous PGs. Macrophage tumoricidal activity was assessed using these varying culture conditions. Lastly, the in vitro macrophage activation model was utilized to examine cyclic nucleotide and arachidonic acid metabolism during the acquistion of anti-tumor activity. I tested the hypothesis that arachidonic acid metabolites regulate macrophage functional differentiation. The results in this investigation supported this hypothesis.

The pattern of arachidonic acid metabolites produced by the macrophage varies with the state of activation. The predominant metabolite of activated macrophages was PGE₂. They also produced substantial quantities of the other arachidonic acid metabolites. Elicited cells produced lower levels of PGs while the thromboxane levels were comparable to the other macrophage subsets. Resident macrophages produced the most prostacyclin.

The mechanism by which PGs exert their effects is thought to be by influencing intracellular cyclic nucleotides. Changes in cyclic nucleotides could influence macrophage function and, therefore, activation. Cyclic nucleotide metabolism, therefore, was also examined. The profile of cAMP and cGMP levels revealed that activated

macrophages had the highest cAMP and the lowest cGMP. In vitro ⁸⁶ culture induced a rapid decline in cAMP levels for the activated macrophage while these levels in elicited and resident cells increased. These alterations in the cAMP/cGMP ratio indicated that the macrophage subsets were progressing to the next stage of activation, in vitro. The functional changes that occurred during this time also support this concept.

Manipulation of cell function and metabolism provided an additional tool for examining the role of PGs in macrophage function. The use of different tissue culture conditions resulted in significant changes in arachidonic acid metabolism and functional activity. The medium in which activated macrophages destroyed the most tumor cells, DMEM, was the medium in which they produced the least PGE₂, as well as the other metabolites. The diminished activity of the activated cells in McCoy's 5A medium was probably due to the accumulation of arachidonic acid products since indomethacin blocked the effect. LPS in the medium increased metabolism of arachidonic acid in all of the macrophages and induced anti-tumor activity of resident and elicited macrophages. These results suggested that endogenous PGs and/or thromboxane regulated the expression of tumoricidal activity. Exogenously added PGs inhibited the capacity of activated macrophages to lyse tumor cells and induced anti-tumor activity in resident and elicited macrophages. This finding further established the significance of PGs in controlling macrophage function.

The relationship of metabolism and function was also assessed by controlling macrophage activation, in vitro. The results of studies

using in vivo derived macrophage subsets were compatible with the in ⁸⁷
vitro model. Acquisition of anti-tumor activity which indicates functional differentiation was paralleled by an increase in PG and cAMP concentrations. Pulsing elicited macrophages with LPS or MAF induced anti-tumor activity. This activity was closely associated with PGE₂ production or the PGE₂ content in the MAF preparation. Resident macrophages required both activating agents to destroy tumor cells. However, pulsing with PGE₂ replaced the requirement for LPS in the medium. LPS may serve as a signal to induce PGE₂ production in vivo. Our results indicate that PGE₂ could be an important endogeneous stimulus to promote macrophage anti-tumor activity.

Figure 11 shows a schematic representation of macrophage functional differentiation. Resident macrophages are resting cells with a low basal ratio of cAMP to cGMP. They are capable of several effector functions including phagocytosis, monokine secretion and motility. PGE₂ and possibly PGI₂ produced by resident cells may potentiate their response to inflammatory insult rendering them primed for further stimulus. Thromboxane production by primed macrophages may be associated with the expression of elevated functional activity. Lymphokines released during a specific immune response induce the activation of primed macrophages. Activated macrophages predominately release PGE₂ which can inhibit the expression of anti-tumor activity following its accumulation in culture supernatants. The exact mechanism and specificity of action of the derivatives of arachidonic acid in controlling macrophage activation are unclear at this time. However, this study has shown that components of the arachidonic acid

Scheme for Macrophage Functional Differentiation

Fig. 11

cascade affect macrophage function differentially and the state of 89
macrophage activation determined the pattern of their production.

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