THE MACROPHAGE ADHERENCE PHENOMENON: ITS RELATIONSHIP TO PROSTAGLANDIN E_2 AND SUPEROXIDE ANION PRODUCTION AND CHANGES IN TRANSMEMBRANE POTENTIAL

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Abstract

Mononuclear phagocytes are undoubtedly the sine qua non of chronic inflammatory reactions. This is demonstrated by their unique ability to function as phagocytic, secretory, or effector cells during the course of an immune event. Although macrophages can perform a variety of immune tasks, their ability to function appropriately is dependent upon the mode of elicitation, the stimulus under investigation, the source of the macrophages (peritoneal, alveolar, etc.), and whether the macrophages are monolayers or in suspension. We have examined the relationship between adherent and non-adherent elicited peritoneal macrophages in terms of prostaglandin E_2 (PGE_2) and superoxide anion (O_2^-) production; in addition, we have studied these elicited macrophages in suspension for their ability to undergo transmembrane potential changes in response to several stimuli. Non-adherent, elicited peritoneal macrophages demonstrated an increase in basal PGE_2 production, and were refractory to particulate stimulus. After monolayer formation, basal PGE_2 levels dropped and the cells could respond to both soluble and particulate stimuli. Only adherent macrophages could respond to a specific challenge and synthesize O_2^-. Both O_2^- production and depolarization of the transmembrane potential were suppressed in cells in suspension. Furthermore, both exogenous PGE_2 and supernatant from macrophages in suspension could modulate O_2^- production by PMA challenged macrophages monolayers. These studies indicate that PGE_2 may modulate macrophage function and dictate activity as macrophages go from the non-adherent to adherent state.

Introduction

Mononuclear phagocytes are known to play a key role in immune/inflammatory reactions by serving as an effector cells for the induction and regulation of immune responses and acting as secretory cells for the synthesis and release of specific phlogistic mediators. The ability of macrophages to function in any one of the above capacities is not only dependent upon the nature of the inflammatory stimulus (1), but also on the source of the mononuclear phagocytes (2,3,4) and whether these cell are adherent or not (5,6). With regard to specific macrophage populations, the function of peritoneal resident macrophages differs dramatically from that of thioglycollate elicited macrophages (1,4,7,8). Resident peritoneal macrophage demonstrate an increase in lipid deacylation, resulting in an increase in arachidonic acid metabolites (7), contain higher levels of 5'-nucleotidase (9), and participate more efficiently in antibody-dependent cell-mediated cytolytic reactions (4) compared to thioglycollate elicited populations. In contrast, elicited macrophages contain high levels of lysosomal enzymes (10), are more activity in pinocytosis and IgG-dependent phagocytosis (4), and have an increased capacity to express I region associated (Ia) antigen in the presence of lymphokine (6) than resident macrophages. In addition to specific population differences, the *in vitro* adherence phenomenon appears to play an extremely important role with respect to macrophage effector (4,6) or secretory (5,11) cell function. In the present study we have examined the relationship between adherent and non-adherent elicited peritoneal macrophages in terms of prostaglandin E_2 (PGE_2) and superoxide anion (O_2^-) production. Also, we have examined the ability of elicited non-adherent macrophages to undergo transmembrane potential changes in response to various inflammatory stimuli, as assessed by the potentiometric cyanine dye,

3-3'dipropyl thiodicarbocyanine iodide [diS-C_3-(5)]. Since the precise relationship between the adherence phenomenon and macrophage function is not entirely understood, their examination is important in view of in vitro discrepancies concerning the activity of various macrophage populations.

Our results indicate that elicited peritoneal macrophages in suspension displayed a dramatic increase in the production of PGE_2 and, the production of this prostaglandin was refractory to specific stimulus. Only after the macrophages were adherent for 2 hrs or more did the basal level drop and the macrophages could respond to a specific challenge and produce PGE_2 above control levels. While macrophages in suspension produced large amounts of PGE_2, superoxide anion (O_2^-) generation was suppressed. Upon being adherent for 2 hours or more, the macrophages could respond to a stimulus and produce O_2^- well above basal levels. In addition, cell-free supernatant from elicited macrophages in suspension, which was devoid of superoxide dismutase activity, could suppress the production of O_2^- from adherent cells. Furthermore, depolarization of the transmembrane potential in elicited macrophages in suspension was not responsive to a number of stimuli. These studies suggest that the endogenous production of PGE_2 may act to immuno-modulate macrophage function while in a non-adherent state and thereby constitute a natural means to regulate macrophage activity.

Methods

Animals. Female, C57B/6 mice (Jackson Laboratories, Bar Harbor, ME) were used in all experiments. Mice were maintained under standard care and given food and water ad libitum.

Macrophage preparation. Peritoneal macrophages from C57B/6 mice were elicited by the intraperitoneal injection of 2 ml thioglycollate medium (Becton Dickinson, Cockeysville, MD). Four days after injection, peritoneal macrophages were harvested, washed in RPMI-1640 medium (Gibco, Grand Island Biological Co., Grand Island, NY) and resuspended at 5×10^5/ml in RPMI-10% fetal calf serum containing 100 units/ml penicillin and 100 µg/ml of streptomycin. One milliter of cells (5×10^5) were added to plastic dishes (35mm diameter; Costar, Cambridge, MA) and were allowed to adhere for either 2, 8, or 16 hours at 37°C in 5% CO_2/95% air prior to assay. The number of macrophages adherent to the surface of the culture dishes were determined by direct counting of adherent cells, as described elsewhere (12). This methodology has been confirmed by the use of biochemical parameters (13). Typically greater than 90% of the cells were macrophages and 3×10^5 macrophages were found to adhere to individual plates. Non-adherent cells were harvested as above, washed 4x in Hanks balanced salt solution (HBSS), and resuspended to 3×10^5 cells/ml. The following protocol was employed for the examination of macrophage monolayers: cells cultured for 2,8 and 16 hours were challenged with either PMA or zymosan, incubated 2 additional hours, and assayed for either PGE_2 or O_2^-, as described below. Non-adherent cells were also incubated for 2 hours in the presence of the appropriate stimulus and assayed for PGE_2 or O_2^-.

Assay for superoxide anion. A modification of the procedure of Johnston et al. (12) was used to quantitate the production of O_2^-. Briefly, adherent monolayers or non-adherent macrophages, prepared as described above, were incubated in 1 ml of 80 µM ferricytochrome C in the presence or absence of opsonized zymosan (500 µg) or phorbol myristate acetate (1 µg, PMA). An

additional set of cells were incubated with the above reagents in the presence of 50 µg/ml of superoxide dismutase. The cell cultures were allowed to incubate for 120 minutes at 37C in 5% CO_2 95% air. The reaction mixtures were then removed by decantation, the fluids cleared of cells by centrifugation, and the optical density of the supernatants determined spectrophotometrically at 550 nm. Superoxide anion concentration was calculated as the superoxide dismutase inhibitable reduction of ferricytochrome C and expressed as nMoles $O_2^-/10^6$ cells/2 hr. (12). Each sample was examined in triplicate.

Assay for prostaglandin E_2. Radioimmunoassay (RIA) using antibody and the method developed by Fitzpatrick et al. (14) was used to quantitate prostaglandin E_2 (PGE_2) in the macrophage culture fluids. Prior to assay, samples were extracted to remove protein and free fatty acids. Bound ligand was separated from free ligand by the use of dextran-coated charcoal. The limit of sensitivity for PGE_2 was 8 picograms/ml. Levels of PGE_2 are expressed as $ng/10^6$ cells/2 hr. All samples were examined in triplicate.

Effect of exogenous PGE_2 on O_2^- production by elicited adherent macrophages. The ability of in vitro PGE_2 treatment to alter O_2^- production by adherent macrophages was examined by incubating 2×10^{-5}M PGE_2 with monolayer macrophages for 30 min prior to and during PMA or zymosan challenge. In addition, 2×10^{-5}M PGE_2 was incubated with adherent macrophages for 30 min prior to, but not during, stimulation. The monolayer macrophages were examined for O_2^- production as described above.

Changes in transmembrane potential. The changes in transmembrane potential (ΔF) were determined according to the procedure of Duque et al. (15). Briefly, 1.5×10^6 elicited peritoneal macrophages were allowed to equilibrate with the fluorescent dye diS-C_3-(5) (2×10^{-6}M). Upon reaching a steady level of fluorescence, the following stimuli were examined for their ability to alter membrane fluorescent intensity: PMA (1 µg/ml), f Met-Leu-Phe (FMLP) (10^{-6}M), C5a (10^{-8}M), LTB_4 (10^{-6}M), LTC_4 (10^{-6}M), LTD_4 (10^{-6}M) arachidonic acid (10^{-5}M), and A23187 (2×10^{-5}M). The difference (F_1-Fo) between the initial and final fluorescence intensities of each reagent was recorded and expressed as ΔF.

Superoxide dismutase and glucose determinations. Superoxide dismutase levels were determined in macrophage culture supernatants employing the autooxidation of epinephrine as described by Misra and Fridovich (16). Glucose levels in cultured supernatant was assessed by the use of dextrostix reagent strips (Miles Laboratory,Elkhart, Indiana).

Reagents. Ferricytochrome C (Horse heart, type II), PMA, zymosan, superoxide dismutase (bovine blood, type I), DMSO, and FMLP were all obtained from Sigma Chemical Co. (St. Louis, MO). C5a was isolated and purified according to the procedure of Manderino et al. (17). The fluorescent dye diS-C_3-(5) was obtained from Molecular Probes (Junction City, OR). Arachidonic acid was purchased from Nu-Chec Prep (Elysian, MN). Prostaglandin E_2 was a gift of Dr. John Pike, the Upjohn Co. (Kalamazoo, MI). LTB_4, LTC_4, and LTD_4 were all a gift from Dr. J. Rokach, Merck Frost Canada Inc. (Dorval, Quebec).

Results

Superoxide anion and prostaglandin E_2 production by adherent and monolayer macrophages. Numerous studies have demonstrated that vast differences exist between the functional activities of adherent and non-adherent macrophages (5,6). As shown in Figure 1, thioglycollate elicited macrophages

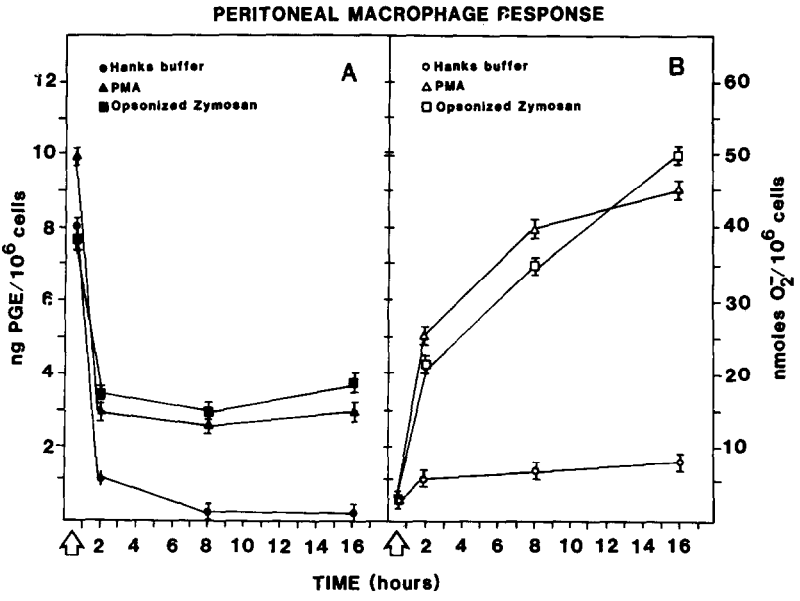

Figure 1. A comparative study of PGE_2 synthesis (A) and O_2^- production (B) between elicited macrophages in suspension (↑) and those in monolayers for 2, 8, and 16 hours. Points represent mean ± S.E.M. The concentration of PMA zymosan used throughout this study was 1 µg/ml and 500 µg/ml, respectively.

adherent for 2,8, or 16 hours could respond to opsonized zymosan (500 μg/ml) or PMA (1 μg/ml) challenge with an increase above basal levels in both PGE_2 and O_2^- production. In contrast, the same macrophage population in suspension (non-adherent) did not produce O_2^- above basal levels with either a particulate or soluble stimulus. Interestingly, PGE_2 levels synthesized by nonstimulated macrophages in suspension were elevated, as compared to their adherent counterpart, yet remained refractory to particulate stimulus and responded with only a moderate increase when treated with PMA (Figure 1A). Only after the macrophages were adherent for 2 hours or more did they respond to either a zymosan or PMA challenge and release O_2^- and PGE above basal levels. Macrophage monolayers cultured for either 8 or 16 hours and then stimulated with either zymosan or PMA exhibited a 6 to 8 fold increase in PGE_2 production above basal levels. Although less dramatic, PGE_2 synthesis from monolayers in culture for 2 hours demonstrated an approximate 3 fold increase above basal levels. Macrophages adherent for 2,8 and 16 hours responded to either zymosan or PMA challenge with an increase in O_2^- production above control values of approximately 5, 6, and 7 fold, respectively.

Modulation of macrophage O_2^- production by exogenous PGE_2: Since non-adherent macrophages in their basal state produced significant amounts of PGE_2, concommitant with little O_2^- production, it was important to determine whether exogenous PGE_2 could decrease the production of O_2^- by stimulated adherent cells. Macrophage monolayers cultured for 8 hours were incubated with 20 μM PGE_2 30 min prior to and during either PMA or zymosan stimulation. As shown in Figure 2, PGE_2 reduced O_2^- production in response to zymosan and PMA by 43% and 17%, respectively. No suppression was observed if monolayers were washed and incubated in the absence of PGE_2 during the zymosan or PMA challenge period. Basal levels of O_2^- were not altered by the PGE_2 treatment.

Modulation of macrophage O_2^- production by supernatant of non-adherent cells. Since macrophages in suspension produced significant amounts of PGE_2, while O_2^- production was reduced, it was of interest to determine the effect of supernatant from non-adherent cells on O_2^- production by macrophage monolayers. Supernatant from non-adherent macrophages were examined for its effect on monolayer O_2^- production in the following manner: monolayers were incubated with supernatant during the 8 hour adherence period, washed with HBSS, and stimulated 1 μg/ml PMA for 2 hours in the presence or absence of supernatant. In this study only macrophage monolayers incubated continually in the presence of supernatant was able to suppress O_2^- production in response to PMA (Table 1). The supernatant from nonadherent macrophage resulted in an approximate 60% reduction in PMA-induced O_2^- production. Those macrophage monolayers incubated with supernatant prior to but not during PMA stimulation could respond normally to PMA challenge. The suppression of O_2^- appeared to be due to mechanism(s) independent of superoxide dismutase, since the supernatant of macrophages in suspension contained no detectable levels of this particular enzyme. In addition, glucose levels remained adequate in the supernatant for normal metabolic function.

Transmembrane potential changes in non-adherent elicited macrophages. The binding of a ligand to the plasma mem-brane receptor is intimately associated with depolarization of the transmem-brane potential (18). Since depolarization of the transmembrane potential precedes many functional activities of inflammatory cells (19), we examined the ability of non-adherent thioglycollate-elicited macrophages to undergo membrane depolarization to a variety of sti-

Incubation Conditions	O_2^- (nmoles/10^6 cells/2 hrs) ± SEM
Macrophages in suspension	5.2 ± 3.2
Macrophage monolayer	38.5 ± 6.2
Macrophage monolayer + continuous incubation with non-adherent macrophage supernatant	15.3 ± 5.4
Macrophage monolayer + non-adherent macrophage supernatant incubated prior to but not during PMA challenge	34.7 ± 7.5

Table 1. Suppression of elicited macrophage O_2^- production by supernatant of non-adherent macrophages. Macrophage monolayers were stimulated with 1 µg/ml PMA in the presence or absence of non-adherent macrophage supernatant.

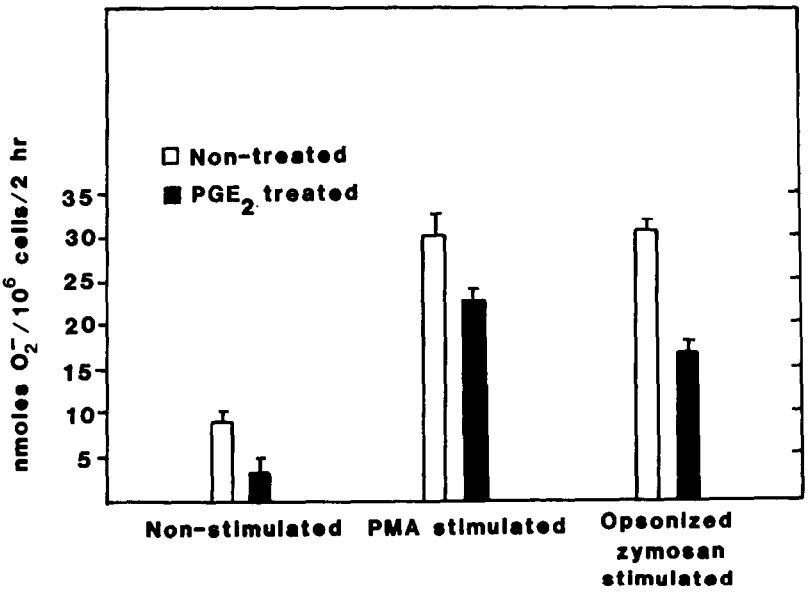

Figure 2. Modulation of O_2^- production by zymosan (500 µg/ml) and PMA (1 µg/ml) challenged macrophages monolayers (adherent for 8 hours) by exogenous PGE_2 (20 µM).

muli. As shown in Table 2, macrophages in suspension were refractory to a number of soluble stimuli; including PMA, FMLP, C5a, LTB_4, LTC_4, LTD_4 and arachidonic acid. Only the calcium ionophore, A23187, induced a change in fluorescence intensity of considerable magnitude. A typical tracing of fluorescent changes in response to A23187 is shown in Figure 3. The calcium ionophore induced a monophasic sustained rise in diS-C_3-(5) fluorescence, indicative of prolonged depolarization, while PMA, C5a, FMLP, LTB_4 and LTD_4 induce no change in fluorescent intensity.

Discussion

The ability of mononuclear phagocytes to function appropriately as effector or secretory cells is dependent upon a number of variables including: species (2), anatomical location of the cells (5), mode of elicitation (3,4), stimulus (1), and whether the macrophages are examined in suspension or as monolayers (5,6). Numerous investigations have demonstrated dramatic functional differences between adherent macrophages and macrophages in suspension (5,6,10,11). In addition to functional changes, metabolic alterations have also been identified between adherent and non-adherent macrophage populations. Caseinate-elicited mouse peritoneal macrophages in suspension were found to be more active in the oxidation of glucose than when examined in the adherent state (20). This phenomonen was explained by differences which exist in the intracellular pool of glycogen and glucose between non-adherent and monolayer macrophages. Cohen et al. (5) have examined the oxidative metabolism of thioglycollate-elicited macrophages in suspension and in the adherent state. When the elicited macrophages were allowed to adhere to glass, H_2O_2 and O_2^- production in response to PMA challenge were found to increase dramatically compared to the same cell in suspension. In addition, Badweg et al (21) have demonstrated that freshly isolated thioglycollate induced peritoneal macrophages produce negligible amounts of O_2^- upon stimulation with PMA, while Johnston et al. (12) have shown that thioglycolate macrophages adherent for 24 hrs can synthesize significant amounts of O_2^-. This suggests that thioglycollate-elicited macrophages possess a very active oxidative metabolic capacity, but the recovery of oxygen reduction products was dependent upon the conditions of incubation. Other investigators have reported alterations in the membrane transport of amino acids and nucleotides between non-adherent and monolayer macrophages (22). Specifically, the membrane transport of both lysine and adenosine was shown to be reduced by rabbit alveolar macrophage in suspension, but not the adherent state.

The underlying mechanism(s) which is responsible for controlling the metabolic activity between macrophages in suspension or in monolayers may also dictate the functional activities of these cells. An interesting concept developed a decade ago by Nelson and Boyden (23) suggested that adherence is a required step in the activation process of macrophages. Their study demonstrated that an antigenic challenge of immunized animals could induced peritoneal macrophages to undergo a reversible adherence reaction, which was associated with the initiation of macrophage activation. More recent studies have shown that the adherence phenomenon is closely associated with lymphokine-induced macrophage Ia antigen expression (6) and interleukin 1 production (24). In the former study adherent macrophages and macrophages in suspension were examined for their ability to express Ia and serve as antigen-presenting

Stimulus (final concentration)	ΔF (Arbitrary units)
PMA (1 μg/ml)	1
f Met-Leu-Phe (10^{-6}M)	2
Human C5a (10^{-8}M)	1
Arachidonic acid (1 x 10^{-5}M)	0
LTB_4, LTC_4, LTD_4 (1 x 10^{-7}M)	2
A23187 (2 x 10^{-5}M)	35

Table 2. Effect of various reagents on the transmembrane potential changes of elicited macrophages in suspension. 1.5 x 10^6 thioglycollate elicited peritoneal macrophages were allowed to equilibrate with diS-C_3-(5) 2 x 10^{-6}M, at 37°C with constant stirring, upon reaching a stable fluorescence intensity the stimulus was added.

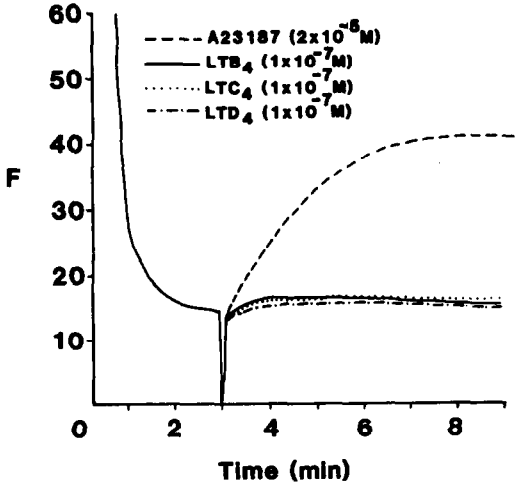

Figure 3. Effect of calcium ionophore A23187 and various leukotrienes on changes in membrane fluorescence of non-adherent macrophage.

cells. Only macrophage monolayers incubated in the presence of lymphokine could function as effector cells and express Ia antigen. Those cells maintained in a non-adherent state neither expressed Ia antigen in response to lymphokine challenge nor functioned as an antigen presenting cell. In an extension of these studies Snyder et al. (25) have demonstrated that PGE_1 and PGE_2 could both regulate the expression of Ia antigen on adherent macrophages induced by lymphokine and modulate T-cell independent development of the basal levels of Ia-positive macrophages in tissue. This loss in Ia antigen expression after exposure to PGE is associated with impaired antigen presention to immune T-cells. In a corroboration of these studies, we have recently demonstrated that PGE_1 can dramatically suppress Ia expression by lymphokine treated granuloma macrophages *in vitro* (26), as well as modulate granuloma development and the expression of Ia antigen by macrophages *in vivo* (26,27). Either macrophages in suspension or adherent cells treated with PGE possessed an impaired ability to express Ia antigen and function as effector cells.

In the present study we have examined the relationship between adherent and non-adherent elicited peritoneal macrophages with regard to PGE_2 and O_2^- production. In addition, we have examined changes in transmembrane potential in macrophages in suspension. Our results indicate that non-stimulated cells in suspension demonstrated a dramatic elevation in PGE_2 production as compared to macrophage monolayers. This elevation in PGE_2 production was concomitant with the inability of macrophages in suspension to produce O_2^- or undergo transmembrane potential changes in response to PMA. Only after the macrophages were adherent, did the elevated basal level of PGE_2 decrease and the cells able to respond to either a soluble or particulate stimulus. The ability of macrophage monolayers to produce O_2^- *in vitro* was shown to be suppressed by exogenous PGE_2. At 2×10^{-5} M PGE_2, O_2^- production by monolayer macrophages in response to zymosan or PMA was dramatically suppressed. This finding is similar to that of Metzger et al. (28), whereby PGE_2 could suppress oxygen radical production by PMA treated, LPS-activated macrophage monolayers. This suppressive effect of PGE_2 on the LPS-induced activation of macrophages could also be induced by cAMP agonists, suggesting a cAMP-mediated mechanism for the inhibitory effect of PGE_2 on macrophage activation. In an earlier study, Lehmeyer and Johnston (29) have examined agents that elevate intracellular levels of cAMP in macrophages and have shown a decrease in chemiluminescence and O_2^- production by these cells. In order to further evaluate the possible effects of endogenous PGE_2 on macrophage monolayer activity we examined the ability of supernatant from macrophages in suspension, shown to contain elevated levels of PGE_2, to modulate O_2^- production by macrophage monolayers. Supernatant obtained from macrophages in suspension could dramatically suppress PMA-induced O_2^- production when continuously incubated with macrophage monolayers. This effect appeared to be reversible, since those monolayers incubated with supernatant prior to, but not during, PMA stimulation showed no suppressive effect. A similar observation was demonstrated when exogenous PGE_2 was incubated with macrophage monolayers. Only when exogenous PGE_2 was continually incubated with macrophage monolayers was O_2^- production suppressed. The observation that macrophages in suspension did not undergo depolarization of the transmembrane potential to a number of soluble stimuli is important in light of the intimate association between ligand receptor interactions and depolarization. Although recent studies have suggested that O_2^- may be destroying the activity of fluorescent probes (18), this occurs only after the change in fluorescence intensity is recorded (19).

The calcium ionophore, A23187, was the only compound out of many examined shown to induce depolarization of the transmembrane potential. We postulate that high levels of endogenously produced PGE_2 by macrophages in suspension may suppress depolarization, since exogenous PGE compounds can inhibit neutrophil membrane depolarization to soluble stimuli (30). Measurement of membrane depolarization of adherent cells is presently restricted because of methodological constraints.

A number of investigations have demonstrated the ability of various arachidonic acid metabolites to alter inflammatory cell activity and dictate immune responsiveness (31,32). Although these studies demonstrate the potent modulating activity of both exogenous and endogenous PGE, they all lend common support to the theory that specific prostaglandins play a profound role in regulating inflammatory cell function. The present investigation also supports this hypothesis, since it appears that elevated PGE_2 production by non-adherent macrophages may provide a means to modulate cell activity and the production of phylogistic mediators until the cells are fully adherent and can respond to a specific challenge. The failure of macrophages in suspension to undergo depolarization or synthesize O_2^- may be due in part to an environment with elevated PGE_2 levels; thus, providing the macrophage with a natural control mechanism to modulate cellular activity.

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