

# Thromboxane A<sub>2</sub> Mediates Augmented Polymorphonuclear Leukocyte Adhesiveness

PHILIP J. SPAGNUOLO, JERROLD J. ELLNER, AVIV HASSID, and MICHAEL J. DUNN,  
*Departments of Medicine, Veterans Administration Hospital and University  
Hospitals of Cleveland, and Department of Biochemistry, School of Medicine,  
Case Western Reserve University, Cleveland, Ohio 44106*

**ABSTRACT** We examined the role of prostaglandins and thromboxanes as mediators of plasma-dependent increased polymorphonuclear leukocyte adhesiveness induced by *Escherichia coli* lipopolysaccharide. The cyclo-oxygenase inhibitors—indomethacin and *d,l*-6-chloro- $\alpha$ -methyl-carbozole-2-acetic acid (R020-5720)—reduced lipopolysaccharide-induced adherence of polymorphonuclear leukocytes by 74 and 62%, respectively. In addition, inhibitors of thromboxane synthetase—imidazole, 9,11-azoprosta-5,13-dienoic acid, and 1-benzylimidazole—suppressed the stimulation of adherence by 31, 66, and 83%, respectively. Exogenous prostaglandins E<sub>1</sub>, E<sub>2</sub>, and F<sub>2</sub> $\alpha$  did not increase polymorphonuclear leukocyte adherence, nor were they detected in significant quantities in supernates of polymorphonuclear leukocytes exposed to lipopolysaccharide. However, inhibitors of both cyclo-oxygenase and thromboxane synthetase reduced increases in adherence induced by arachidonic acid (10  $\mu$ g/ml), suggesting that lipopolysaccharide-mediated increases in adherence were due to an arachidonic acid product other than prostaglandin E<sub>2</sub> or F<sub>2</sub> $\alpha$ . 8,11,14-Eicosatrienoic acid, a precursor of monoenoic prostaglandins, did not enhance polymorphonuclear leukocyte adhesiveness.

We next demonstrated lipopolysaccharide-stimulated generation, by polymorphonuclear leukocytes, of a labile, low molecular weight, dialyzable substance capable of enhancing the adherence of unstimulated leukocytes. In parallel experiments, a 10-fold increase

in immunoreactive thromboxane B<sub>2</sub> over basal levels was detected after exposure of leukocytes to lipopolysaccharide. The inhibition of lipopolysaccharide enhancement of adherence by specific rabbit antibodies to thromboxane B<sub>2</sub> strongly supported a primary role for thromboxane A<sub>2</sub> as the mediator of the observed increases in adherence. Lipopolysaccharide-stimulated purified platelets did not increase leukocyte adherence, whereas thrombin-stimulated platelets did increase adherence.

These studies suggest that lipopolysaccharide stimulates polymorphonuclear leukocytes to produce thromboxane A<sub>2</sub>, which enhances their adhesiveness to nylon.

## INTRODUCTION

Polymorphonuclear leukocytes (PMN)<sup>1</sup> release biologically active lipids, the prostaglandins (PG) and thromboxanes (Tx), during the course of inflammation (1–8). PG play a crucial role in modulating inflammation by enhancing vascular permeability (9); in addition, PGE<sub>1</sub> and PGE<sub>2</sub> may recruit PMN to local inflammatory sites by acting as chemokinetic (10) and chemotactic factors (11, 12). Although TxA<sub>2</sub> is a vasoconstrictor (13), the only recognized role for Tx in directly modulating PMN inflammation is the chemotactic activity of TxB<sub>2</sub>, which occurs at pharmacologic concentrations (14).

PMN adhesiveness and margination are important components of the early neutrophilic response to inflammatory stimuli (15). It has been shown previously that in vitro PMN adhesiveness to nylon correlates well with in vitro adhesiveness to vascular endothelium (16). *Escherichia coli* lipopolysaccharide (LPS) enhances PMN adhesiveness to nylon. Moreover, this effect of LPS on PMN adhesiveness can be blocked

Portions of this work have appeared in abstract form: Program abstracts of the 18th International Conference on Antimicrobial Agents and Chemotherapy, 1978; and (1979.) *Clin. Res.* 27: 465.

Dr. Spagnuolo is the recipient of a Career Development Award from the Veterans Administration. Dr. Ellner is the recipient of a Young Investigator's Award from the National Institute of Allergy and Infectious Diseases.

Received for publication 27 September 1979 and in revised form 28 April 1980.

<sup>1</sup> Abbreviations used in this paper: HBSS, Hanks' balanced salt solution; LPS, *Escherichia coli* lipopolysaccharide; PG, prostaglandin; PMN, polymorphonuclear leukocyte; Tx, thromboxane.

by acetylsalicylic acid, an inhibitor of fatty acid cyclo-oxygenase (17). Our current studies indicate that LPS stimulates PMN to produce  $\text{TxA}_2$ , which increases PMN adhesiveness.

## METHODS

**Reagents.**  $\text{PGE}_1$ ,  $\text{PGE}_2$ ,  $\text{PGF}_2\alpha$ , and  $\text{TxB}_2$  (generously provided by Dr. John Pike, Upjohn Co., Kalamazoo, Mich.) were dissolved in Hanks' balanced salt solution (HBSS, KC Biological, Lenexa, Kans.) to appropriate concentrations. Prostacyclin ( $\text{PGI}_2$ , Upjohn Co.) was dissolved in a 9:1 solution of ethanol:Tris buffer, pH 9, stored at  $-70^\circ\text{C}$ , and diluted in HBSS immediately before use. Indomethacin (Sigma Chemical Co., St. Louis, Mo.), R020-5720 (*d,l*-6-chloro- $\alpha$ -methyl-carbazole-2-acetic acid, kindly supplied by Hoffman-LaRoche Co., Nutley, N. J.), 9,11-azoprostano-5,13-dienoic acid (Azo analogue 1, Upjohn Co.), and 8,11,14-eicosatrienoic acid (Nu-Chek Prep. Elysian, Minn.) were dissolved in 95% ethanol and diluted in HBSS. Ethanol at 0.1%, equivalent to its concentration in the reagents used in these studies, had no effect on LPS-induced increases in PMN adherence. Imidazole (Aldrich Chemical Co. Inc., Milwaukee, Wis.) and 1-benzylimidazole (Aldrich Chemical Co.) were dissolved in HBSS. Arachidonic acid (Sigma Chemical Co.) was purified by chromatography on silicic acid columns as described by Flower et al. (18) and stored in hexane at  $-35^\circ\text{C}$ . Before use the hexane was evaporated, and the arachidonic acid was resuspended in 0.2%  $\text{Na}_2\text{CO}_3$  and diluted to final concentration with HBSS. LPS (*E. coli* 026:B6, Difco Laboratories, Detroit, Mich.) was dissolved in HBSS and stored at  $-70^\circ\text{C}$  until use. Thrombin (Sigma Chemical Co.) was dissolved in HBSS at appropriate concentrations and stored at  $-70^\circ\text{C}$ .

One of the rabbit anti- $\text{TxB}_2$  antibodies used in our experiments was prepared in our laboratory by immunization of a rabbit with  $\text{TxB}_2$  coupled to keyhole limpet hemocyanin. Antisera to  $\text{TxB}_2$  were also generously provided by Dr. W. Campbell, Dallas, Tex. and Dr. Carlo Patrono, Rome, Italy. These three antibodies show <0.3% crossreactivity with all tested prostanoids.

**PMN and platelet preparation.** Venous blood from healthy volunteers was obtained using sterile heparinized syringes, and PMN were separated by successive Ficoll-Hypaque (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) and dextran (Sigma Chemical Co.) gradient sedimentations (7). Erythrocytes were removed by hypotonic lysis, and the PMN ( $98 \pm 2\%$  purity) were resuspended in HBSS at concentrations of  $10^7/\text{ml}$  until use. Platelet concentrations in PMN preparations were generally three to five platelets per PMN and were reduced, in selected experiments, by low-speed (200 g) centrifugation of the PMN during all washing procedures, to a ratio of one platelet per PMN.

Platelet-rich plasma was prepared by collecting blood into acid-citrate-dextrose anticoagulant. Blood was centrifuged at 1,200 g for 3 min and the supernate, containing platelet-rich plasma, was removed and centrifuged at 3,000 g for 6 min to concentrate the platelets. The pellet was resuspended in a small volume of platelet-poor plasma and recentrifuged at 180 g for 3 min to sediment remaining erythrocytes and leukocytes. The platelets were then diluted to  $2 \times 10^7/\text{ml}$  in HBSS before use. The leukocyte contamination of platelet preparations was  $<5 \times 10^4/\text{ml}$ .

**PMN nylon adherence assay.** Nylon adherence was modified from the method of MacGregor et al. (19). PMN at  $3.0 \times 10^6/\text{ml}$  in HBSS were preincubated for 10 min at  $37^\circ\text{C}$  with or without cyclo-oxygenase inhibitors. Appropriate agents plus autologous plasma (to a final concentration of 10%)

were added to the cell suspension, which was rotated at  $37^\circ\text{C}$  for 1 h. 0.9-ml aliquots of the cell suspension were allowed to filter by gravity through Pasteur pipettes containing 30 mg of nylon fibers (Fenwal Inc., Walter Kidde & Co. Inc., Ashland, Mass.). The quantity of PMN in the original sample immediately before column passage and in the eluate was determined with a Coulter counter (Coulter Electronics Inc., Hialeah, Fla.). The percent PMN adherence in triplicate nylon columns was calculated according to the following formula:

% PMN adherence to nylon = 100

$$\times \left( 1 - \frac{\text{PMN in eluate}}{\text{PMN in original sample}} \right)$$

In additional experiments, PMN or platelets were incubated in parabolic chambers (Bellco Glass Inc., Vineland, N. J.), which were separated by dialysis membranes excluding substances with molecular mass  $>3,500$  daltons (Fisher Scientific Co., Pittsburgh, Pa.).

PMN adherence to tissue culture plastic was quantitated by the method of Gallin et al. (20). Briefly,  $10^6$  PMN in autologous plasma (final concentration of 5%) were added to 16-mm tissue culture wells (Linbro Scientific, Hamden, Conn.), with or without cyclo-oxygenase inhibitors and LPS, for 60 min at  $37^\circ\text{C}$ . After this incubation, nonadherent PMN were removed by vigorous washing with HBSS. 30 mM xylocaine was added to each well, and adherent cells were scraped from monolayers and enumerated with a Coulter counter. Adherence was expressed as the number of PMN adhering per well in triplicate wells.

**Prostaglandin assay.**  $\text{PGE}_2$ ,  $\text{PGF}_2\alpha$ , and  $\text{TxB}_2$  concentrations were determined by radioimmunoassay. Antisera to  $\text{PGE}_2$  and  $\text{TxB}_2$  were obtained from Pasteur Institute, Paris, France. Their characteristics have been described elsewhere (21, 22). Anti- $\text{PGE}_2$  cross-reacts with  $\text{PGE}_1$  by 2.7%. All other PG and their metabolites cross-react by <0.1% (21). Anti- $\text{TxB}_2$  cross-reacts by <0.1% with all PG tested (22). Antiserum to  $\text{PGF}_2\alpha$  was produced in our laboratory and had the following characteristics: 50% inhibition of [ $^3\text{H}$ ] $\text{PGF}_2\alpha$  binding occurred with 20–40 pg  $\text{PGF}_2\alpha$ .  $\text{PGF}_1\alpha$ ,  $\text{PGE}_1$ ,  $\text{PGE}_2$ , 6-oxo- $\text{PGF}_1\alpha$ , 15-keto- $\text{PGF}_2\alpha$ , and 13,14-dihydro-15-keto- $\text{PGF}_2\alpha$  cross-reacted by 63, 0.22, 0.13, 0.17, 0.27, and 0.33%, respectively. Thus, this antiserum did not distinguish  $\text{PGF}_2\alpha$  from  $\text{PGF}_1\alpha$ .  $\text{PGB}_2$ , 15-keto- $\text{PGE}_2$ , and 13,14-dihydro-15-keto- $\text{PGE}_2$  cross-reacted by <0.01%.

## RESULTS

First, we examined the effect of the cyclo-oxygenase inhibitors on the increase in PMN adherence to nylon induced by LPS in the presence of plasma (Table I). The concentrations of inhibitors were selected on the basis of relative efficacy of inhibition in studies of bovine seminal vesicles (18) and human platelets (23). Indomethacin, 1.0  $\mu\text{g}/\text{ml}$  (3.5  $\mu\text{M}$ ), inhibited LPS-augmented PMN adherence by 74%. R020-5720 at concentrations up to 10.0  $\mu\text{g}/\text{ml}$  (36  $\mu\text{M}$ ) produced significantly less inhibition than 1  $\mu\text{g}/\text{ml}$  indomethacin,  $P < 0.05$ . Next we evaluated the effect of delayed addition of indomethacin on PMN adherence. The addition of indomethacin 10 min after LPS, rather than 10 min before, produced significantly less inhibition of adherence (mean of 44% as compared to 60%,  $P < 0.01$ ,

TABLE I  
Effect of Cyclo-oxygenase Inhibitors on LPS-induced  
Increases in PMN Adherence to Nylon

Addition to PMN	Concentration of inhibitor	Granulocyte adherence
	$\mu\text{g/ml}$	%
Basal adherence		$5 \pm 2$
LPS (5 $\mu\text{g/ml}$ )		$43.0 \pm 3$
LPS + indomethacin	0.1	$36.5 \pm 6$
	1.0	$11.4 \pm 0.5^*$
	10.0	$11.3 \pm 1.4^*$
LPS + R020-5720	0.5	$29.1 \pm 2.5^*$
	5.0	$17.9 \pm 6^*$
	10.0	$16.5 \pm 1^*$

$3 \times 10^6$  PMN were preincubated with each inhibitor for 10 min at 37°C before addition of LPS, 5  $\mu\text{g/ml}$ , plus 10% autologous plasma. Results are expressed as mean  $\pm$  SEM of three to six experiments.

\* Differs from LPS,  $P < 0.001$ ,  $t$  test.

data not shown). Thus, indomethacin interfered predominantly with the initial effects of LPS on PMN, rather than reversing already established increases in adherence.

The suppression of LPS-stimulated nylon adherence by preincubation of PMN with cyclo-oxygenase inhibitors suggested that arachidonic acid metabolites resultant from fatty acid cyclo-oxygenase activity might mediate changes in PMN adherence. Accordingly, we studied the effect of arachidonic acid on PMN adherence (Fig. 1). Arachidonic acid, at concentrations of 1.0–10  $\mu\text{g/ml}$ , enhanced PMN adhesiveness to nylon severalfold. The enhancement of adhesiveness was diminished by 10 min preincubation of PMN with 1 or 10  $\mu\text{g/ml}$  indomethacin before addition of 10  $\mu\text{g/ml}$  arachidonic acid.

PGE<sub>1</sub>, PGE<sub>2</sub>, and PGF<sub>2</sub> $\alpha$  at concentrations of 0.1–3.0  $\mu\text{g/ml}$  did not increase PMN adherence (Table II). Moreover, PGE<sub>1</sub>, PGE<sub>2</sub>, and PGF<sub>2</sub> $\alpha$  failed to enhance significantly the effects of 5  $\mu\text{g/ml}$  LPS. Neither PGE<sub>2</sub> nor PGF<sub>2</sub> $\alpha$  at 0.3  $\mu\text{g/ml}$  was effective in reversing the inhibition of adherence observed when PMN were preincubated with 10  $\mu\text{g/ml}$  indomethacin and then exposed to LPS (data not shown). Finally, in five of six experiments, we were unable to detect PGE<sub>2</sub> or PGF<sub>2</sub> $\alpha$  in supernates of PMN ( $3 \times 10^6/\text{ml}$ ) incubated with 5–20  $\mu\text{g/ml}$  LPS and 10% autologous plasma for 60 min. The radioimmunoassays of PGE<sub>2</sub> and PGF<sub>2</sub> $\alpha$  are capable of detecting these PG at minimal concentrations of  $\sim 30$  pg/ml. In the remaining experiment, however, PGE<sub>2</sub>, 100 and 300 pg/ml, was detected in supernates 60 and 120 min after incubation of PMN with LPS, 5  $\mu\text{g/ml}$ .

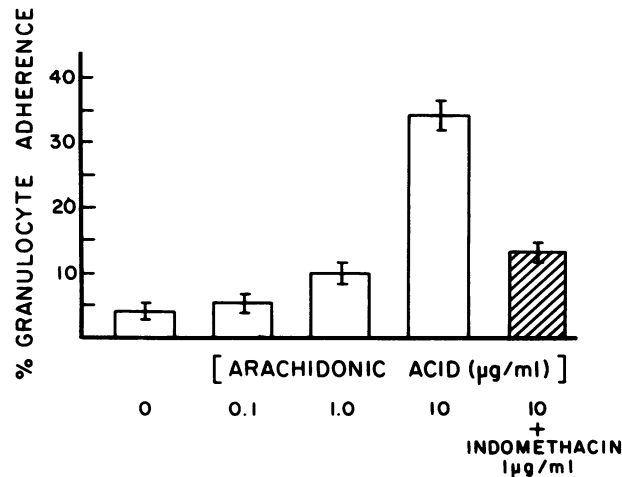


FIGURE 1 Arachidonic acid stimulation of PMN (granulocyte) adherence to nylon.  $3.0 \times 10^6$  PMN were incubated with various concentrations of arachidonic acid for 120 min at 37°C before column passage. Indomethacin, 1.0  $\mu\text{g/ml}$ , was added 10 min before addition of arachidonic acid. Adherence is expressed as mean  $\pm$  SEM of four separate experiments. Augmentation by 1 and 10  $\mu\text{g/ml}$  arachidonic acid differed from basal adherence,  $P < 0.01$ ,  $t$  test.

These data suggested that LPS-mediated increases in adherence were due to an arachidonic acid product different from PGE<sub>2</sub> or PGF<sub>2</sub> $\alpha$ . We therefore examined the role of an alternative pathway of arachidonic acid metabolism, the Tx synthetic pathway. In five separate experiments with each, the two Tx synthetase inhibitors imidazole and 1-benzylimidazole, at concentrations known to inhibit Tx synthetase in platelets (24, 25) and lymphocytes (26), suppressed LPS-augmented adherence (Fig. 2). Neither agent affected basal PMN

TABLE II  
Exogenous PG and PMN Adherence to Nylon

PG added	Concentration  $\mu\text{g/ml}$	Additions	
		None	LPS
			%
Control		3.0 $\pm$ 1	40.0 $\pm$ 3
PGE <sub>1</sub>	0.1	1.2 $\pm$ 0.2	36.0 $\pm$ 7
	0.3	2.2 $\pm$ 1.2	33.7 $\pm$ 4
	3.0	2.0 $\pm$ 1.4	25.0 $\pm$ 7
PGE <sub>2</sub>	0.1	2.2 $\pm$ 1.4	35.4 $\pm$ 5
	0.3	8.0 $\pm$ 2.0	43.4 $\pm$ 7
	3.0	1.6 $\pm$ 1.5	32.0 $\pm$ 10
PGF <sub>2</sub> $\alpha$	0.3	2.4 $\pm$ 1.4	ND*

$3 \times 10^6$  granulocytes were incubated in 10% autologous serum with PG and with or without LPS, 5  $\mu\text{g/ml}$ , for 1 h at 37°C. Results are presented as mean  $\pm$  SEM of four experiments.

\* ND, not done.

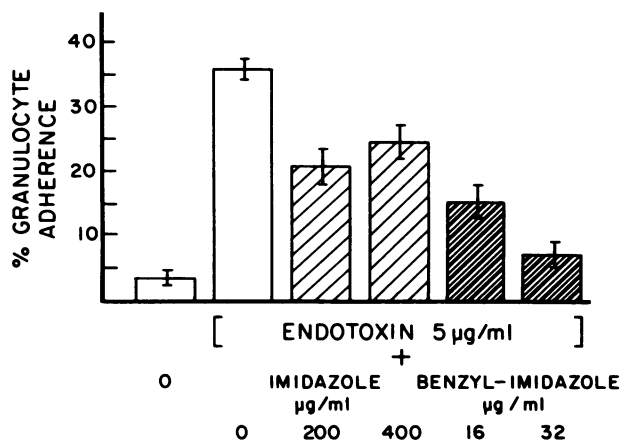


FIGURE 2 The effect of Tx synthetase inhibitors on LPS augmentation of PMN (granulocyte) adherence.  $3 \times 10^6$  PMN were preincubated with various concentrations of inhibitor for 10 min at 37°C before addition of LPS, 5 µg/ml (endotoxin). PMN adherence is expressed as mean percent  $\pm$  SEM of five separate experiments. The effect of imidazole and 1-benzylimidazole significantly differed from LPS alone ( $P < 0.01$ ,  $t$  test).

adherence or viability (estimated by exclusion of trypan blue dye). On a molar basis, 1-benzylimidazole was the more effective, reducing adherence by 83% at 32 µg/ml (0.2 mM) compared with imidazole (31% at 400 µg/ml [5.9 mM]). Azo analogue 1 at 5 and 10 µg/ml also suppressed LPS-augmented adherence significantly by 26 and 66%, respectively, in five experiments (data not shown). Imidazole and 1-benzylimidazole markedly suppressed arachidonic acid enhancement of adherence (Fig. 3), suggesting that a metabolite of ara-

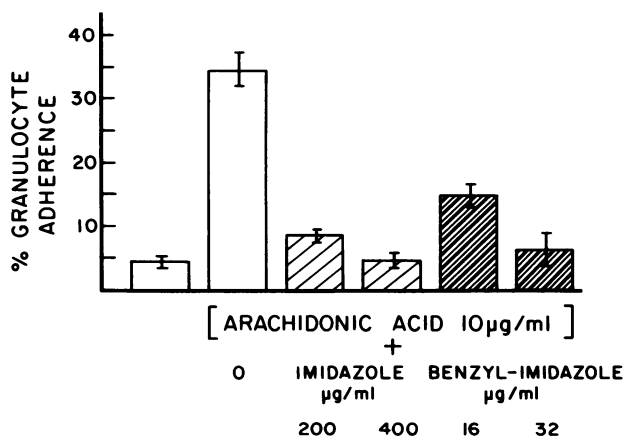


FIGURE 3 Tx synthetase inhibitors block PMN (granulocyte) adherence to nylon induced by arachidonic acid.  $3 \times 10^6$  PMN were preincubated with inhibitor before addition of 10 µg/ml arachidonic acid for 120 min at 37°C. Adherence is represented as mean  $\pm$  SEM of four experiments. Imidazole and 1-benzylimidazole significantly reversed the activity of arachidonic acid. ( $P < 0.01$ ,  $t$  test.)

chidonic acid, resulting from Tx synthetase activity, increased PMN adherence. In contrast to the increased adherence with arachidonic acid, 8,11,14-eicosatrienoic acid (1–10 µg/ml, 60–120 min incubation), the precursor of monoenoic PG that forms little TxA<sub>1</sub> in platelets (27), did not augment PMN adherence in five experiments (data not shown).

The results thus far presented do not preclude the possibility that LPS causes significant PMN aggregation and that the increase in nylon adhesiveness, induced by LPS, results from entrapment of larger PMN aggregates. Light microscopic examination of PMN after 60 min incubation with LPS revealed occasional large clumps; however, aggregation reduced the number of PMN by <10%. To evaluate further the extent of PMN aggregation, we assessed PMN adherence and aggregation to plastic, an assay that could be monitored by light microscopy. 5 µg/ml LPS increased PMN adhesiveness to plastic >twofold (Table III). PMN failed to aggregate on the surface of the plastic following exposure to LPS. 1 µg/ml indomethacin and 400 µg/ml imidazole significantly inhibited LPS-stimulated adherence of PMN to plastic by 44 and 59%, respectively.

PGI<sub>2</sub> inhibits platelet aggregation induced by a variety of physiologic and pharmacologic agents (28). This effect is thought to be mediated by increases of intracellular cyclic AMP concentrations. Since cyclic AMP has also been proposed to be a potential regulator of PMN function, including adherence and aggregation, we hypothesized that PGI<sub>2</sub> might be an inhibitor of LPS-stimulated PMN adherence. PGI<sub>2</sub> at concentrations of 2.5 and 5.0 ng/ml inhibited LPS-induced aug-

TABLE III  
The Actions of Indomethacin and Imidazole  
on PMN Adherence to Plastic

	Experiment		
	1	2	3
	( $\times 10^{-3}$ cells/well)		
Base-line adherence	51	53	52
LPS 5 µg/ml	107	113	116
Indomethacin 1 µg/ml	51	52	51
LPS + indomethacin*	70	91	94
Imidazole 400 µg/ml	49	52	51
LPS + imidazole†	74	72	81

$10^6$  PMN were preincubated with indomethacin, 1 µg/ml, or imidazole, 400 µg/ml, 10 min before addition of buffer or LPS, 5 µg/ml. Results are presented as mean number of cells per well of triplicate determinations.

\* Differs from LPS,  $P < 0.005$ ,  $t$  test.

† Differs from LPS,  $P < 0.025$ ,  $t$  test.

mentation of PMN adherence by 17 and 55%, respectively (data not shown). Basal adherence of PMN was not altered at either PGI<sub>2</sub> concentration.

Next, we sought to determine whether a factor such as Tx was released by LPS-stimulated PMN and was capable of enhancing the adhesiveness of unstimulated PMN. We used parabiotic chambers separated by a low molecular weight cutoff cellulose membrane (3,500 daltons) that excluded substances having higher molecular weights such as C5a ( $1.12 \times 10^4$  daltons [29]), which are generated by the interaction of LPS with plasma (30), and LPS itself ( $>10^5$  daltons [31]). Incubation of PMN with 10% autologous plasma on both sides of the membrane resulted in basal nylon adherence of 6% (Table IV). If 5  $\mu$ g/ml LPS was incubated with PMN on one side of the chamber, the nylon adherence of PMN in both compartments was enhanced significantly, despite exclusion of C5a and LPS by the membrane (2A, 2B). This finding was consistent with generation of a low molecular weight substance capable of enhancing adhesion to nylon. This substance was labile, as suggested by experiments in which supernates of PMN exposed to LPS for 60 min were incubated at 37°C for 5 min. When placed in one compartment of a chamber, the supernates were now in-

capable of enhancing the adherence of fresh PMN in the other compartment (4, 2B<sub>1</sub>).

Exposure of the PMN in chamber B to imidazole did not prevent the stimulation of adherence in these PMN in response to LPS addition to chamber A (4A, B). 5  $\mu$ g/ml LPS and PMN were preincubated for 10 min before addition to compartment A. Simultaneously, 400  $\mu$ g/ml imidazole and PMN were preincubated 10 min before addition to compartment B. Adherence of both stimulated and unstimulated PMN was increased. Furthermore, preincubation of PMN in chamber A with 400  $\mu$ g/ml imidazole for 10 min blocked the enhancement of adherence in both A and B chambers. Thus, imidazole suppressed the generation of a low molecular weight diffusible factor, but did not inhibit its adherence-augmenting activity. These data are consistent with the release of TxA<sub>2</sub> by LPS-stimulated PMN, which can directly increase the adherence of unstimulated PMN.

Using a radioimmunoassay for TxB<sub>2</sub>, a 10-fold increase in immunoreactive TxB<sub>2</sub> was detected following exposure of PMN to LPS for 120 min (Fig. 4). This increment was blocked by preincubation of PMN with 32  $\mu$ g/ml 1-benzylimidazole, a concentration that nearly completely reversed LPS augmentation of PMN ad-

TABLE IV  
Activity of Diffusible Factors Released by PMN on Adherence

Experiment	Chamber side	Additions to chambers		PMN adherence	No. of experiments
		LPS (5 $\mu$ g/ml)	Imidazole (400 $\mu$ g/ml)		
				%	
1	A	—	—	6.6 $\pm$ 1.5	6
	B	—	—	6.8 $\pm$ 1.3	
2	A	+	—	47.2 $\pm$ 1.0	10
	B	—	—	26.0 $\pm$ 1.0*	
	B <sub>1</sub> †	—	—	6.2 $\pm$ 3.0	
3	A	+	+§	6.4 $\pm$ 1.0	6
	B	—	—	6.3 $\pm$ 1.0	
4	A	+	—	47.8 $\pm$ 1.5	6
	B	—	+	27.0 $\pm$ 4.0*	

$3 \times 10^6$  PMN in 10% autologous plasma were added to A and B sides of parabiotic chambers separated by dialysis membrane with a molecular mass cutoff of 3,500 daltons. PMN adherence to nylon was determined following 60 min incubation on a shaking platform. Values are mean $\pm$ SEM.

\* Differs from 1B,  $P < 0.001$ ,  $t$  test.

† A supernate obtained from PMN exposed to LPS, 5  $\mu$ g/ml, for 60 min was incubated at 37°C for 5 min, then added to one compartment of a second chamber, and the adherence of fresh PMN in the other compartment (B<sub>1</sub>) was determined after 60 min.

§ Imidazole was preincubated for 10 min with PMN before addition of LPS.

|| Imidazole was preincubated for 10 min with PMN in B side of chamber before addition of plasma, PMN, and LPS preincubation mixture to A side.

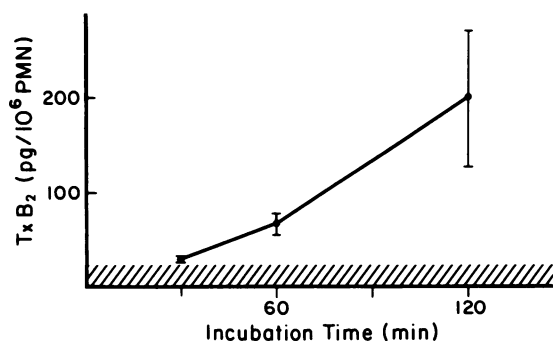


FIGURE 4 Biosynthesis of immunoreactive  $\text{TxB}_2$  by LPS-stimulated PMN.  $3 \times 10^6$  PMN in 10% autologous plasma were incubated with  $5.0 \mu\text{g/ml}$  LPS for varying periods of time.  $\text{TxB}_2$  concentrations are corrected for basal levels of  $\text{TxB}_2$  in unconditioned plasma. Hatched area represents  $\text{TxB}_2$  production by unstimulated PMN during 60 min incubation. In five experiments (mean  $\pm$  SEM) LPS-stimulated PMN produced significantly greater amounts of  $\text{TxB}_2$  at 60 and 120 min incubation than unstimulated PMN ( $P < 0.05$ ,  $t$  test).

herence (Fig. 2). The role of  $\text{TxA}_2$  as mediator of LPS-stimulated PMN adherence was further evaluated using several rabbit antibodies against  $\text{TxB}_2$ . Preincubation of PMN with a 1/100 and 1/200 dilution of anti- $\text{TxB}_2$  plasma, prepared by us, significantly inhibited LPS enhancement of PMN adherence by 56 and 32%, respectively (data not shown). PMN ad-

herence was not affected by the antibody alone nor by serum obtained from a rabbit immunized with tetanus toxoid. Two additional antibodies from different laboratories (see Methods) also significantly inhibited LPS-augmented PMN adherence by 50% ( $P < 0.002$ ) and 75% ( $P < 0.005$ ) at dilutions of 1/10,000 and 1/4,000, respectively. The inhibition of LPS-stimulated adherence presumably occurred by cross-reaction of  $\text{TxA}_2$  with anti- $\text{TxB}_2$ . This is supported by the observation that exogenous  $\text{TxB}_2$  (at concentrations of 100–500  $\mu\text{g/ml}$ ; data not shown) did not enhance PMN adherence.

The aforementioned experiments do not unambiguously identify the source of  $\text{TxA}_2$  because of the possibility of platelet release of  $\text{TxA}_2$  in response to LPS. Experiments using platelet preparations having PMN ratios of 10:1 and 1:1 showed similar enhancement of PMN adherence after exposure to  $5 \mu\text{g/ml}$  LPS (data not shown), suggesting that PMN rather than platelet production of  $\text{TxA}_2$  were responsible for the increase in adherence. To evaluate further the role of platelet  $\text{TxA}_2$ , we incubated  $2 \times 10^7$  platelets (equal to the maximal number of platelets present in our PMN preparations) on one side with purified PMN on the other side of parabolic chambers (Table V). Platelets alone or exposed to  $5 \mu\text{g/ml}$  LPS did not increase PMN adherence. Thrombin-stimulated platelets in chamber A

TABLE V  
Activity of Diffusible Factors Released by Platelets on PMN Adherence to Nylon

Experiment	Chamber side	LPS ( $5 \mu\text{g/ml}$ )	Thrombin (0.2 U/ml)	Anti- $\text{TxB}_2$ antibody (1/100 dilution)	PMN adherence %	No. of experiments
1	A	—	—	—	—	6
	B	—	—	—	$6.8 \pm 2$	
2	A	+	—	—	—	5
	B	—	—	—	$5 \pm 2$	
3	A	—	+	—	—	7
	B	—	—	—	$20 \pm 2^*$	
	B <sub>1</sub> †	—	—	—	$5 \pm 2$	
4	A	—	+	—	—	4
	B	—	—	+§	$9.4 \pm 1^{\text{II}}$	

In each series of experiments,  $2 \times 10^7$  platelets in 10% heterologous normal plasma were added to the A side of parabolic chambers separated by a low molecular mass cutoff membrane. Simultaneously,  $3 \times 10^6$  PMN in 10% autologous plasma were added to the B side and PMN adherence to nylon was determined after 60 min incubation at  $37^\circ\text{C}$  on a shaking platform.

\* Differs from 1B,  $P < 0.0005$ ,  $t$  test.

† A supernate obtained from platelets exposed to thrombin for 60 min was incubated at  $37^\circ\text{C}$  for 5 min, then added to the compartment of a second chamber, and the adherence of fresh PMN in the B<sub>1</sub> compartment was determined after 60 min.

§ Anti- $\text{TxB}_2$  antibody (final dilution 1/100) was preincubated with PMN for 10 min on B side of chamber before addition of platelets and thrombin to A side.

<sup>II</sup> Differs from 3B,  $P < 0.0025$ ,  $t$  test.

produced a labile, diffusible factor, presumably  $\text{TxA}_2$ , that increased PMN adherence in chamber B. This effect was blocked by preincubation of PMN in chamber B with anti- $\text{TxB}_2$  antibody (3B, 4B).

## DISCUSSION

These studies demonstrate that PMN release  $\text{TxA}_2$  when stimulated by *E. coli* LPS in the presence of plasma. Complement-derived peptides (probably C5a, as suggested by Snyderman et al. [30]), rather than LPS directly, function as the membrane stimulus. The blockade of PMN adherence by both cyclo-oxygenase and Tx synthetase inhibitors is consistent with mediation by  $\text{TxA}_2$ . Furthermore, the greater effect of indomethacin added to PMN 10 min before LPS suggests that  $\text{TxA}_2$  is important primarily in the initial stages of the biological events leading to increased adherence.

Additional evidence supporting a role for thromboxane in PMN adhesiveness was obtained through evaluation of the addition of arachidonic acid. Exogenous arachidonic acid, when added to platelets, has been shown by others (32, 33) to stimulate aggregation and platelet degranulation coincident with its metabolism to various bioactive endproducts. Our studies demonstrate that arachidonic acid increases PMN adherence, an effect that can be blocked by cyclo-oxygenase and Tx synthetase inhibitors. Furthermore, the lack of effect of 8,11,14-eicosatrienoic acid, which is converted by the platelet to  $\text{PGH}_1$  and ultimately to monoenoic PG but not  $\text{TxA}_1$  (27), strongly suggests that metabolism of arachidonic acid to  $\text{TxA}_2$  is a requirement for LPS stimulation of PMN adherence.

Finally, we have shown that LPS-stimulated PMN release a low molecular weight factor that increases the adherence of unstimulated PMN. Imidazole blocks the generation but not the action of this factor. Parallel measurements of immunoreactive  $\text{TxB}_2$  in the supernates of PMN incubated with LPS revealed a 10-fold increase. These studies show that not only does  $\text{TxA}_2$  mediate increases in adherence induced by LPS, but LPS-stimulated PMN release sufficient  $\text{TxA}_2$  to affect other PMN as well.

The precise nature of the biologically active Tx and its source require further consideration. We found that  $\text{TxB}_2$  is produced by PMN after stimulation with LPS. In most other cell systems, including the platelet and lymphocyte,  $\text{TxA}_2$  is much more biologically active than  $\text{TxB}_2$  (34); however,  $\text{TxA}_2$  rapidly hydrolyzes to  $\text{TxB}_2$  ( $t_{1/2} = 40$  s) (34) making difficult its direct measurement. The demonstrated labile nature of the low molecular weight substance that stimulates PMN adhesiveness supports a role for  $\text{TxA}_2$  as the relevant factor. The lack of effect of  $\text{TxB}_2$  on PMN adhesiveness

at concentrations equal to those measured in vitro further supports a role for  $\text{TxA}_2$  as the active mediator of changes in adherence. Moreover, the inhibition of increased PMN adherence in the presence of anti- $\text{TxB}_2$  antibody presumably reflects cross-reactivity of the antibody with  $\text{TxA}_2$ . The significant reversal of augmented adherence by three different anti- $\text{TxB}_2$  antibodies argues against a primary role for endoperoxides. Inhibition of LPS-induced adherence by imidazole, 1-benzylimidazole, and Azo analogue 1, which reduce Tx generation, makes a major role for the endoperoxides unlikely.

Platelets produce Tx when stimulated to aggregate (25, 33). Conceivably, contaminating platelets might have contributed to the generation of  $\text{TxA}_2$  by PMN on exposure to LPS. However, removal of 90% of contaminating platelets by differential centrifugation did not diminish enhancement of PMN adherence stimulated by LPS. In addition, LPS did not stimulate platelets, at concentrations equal to that present in PMN preparations. With the appropriate stimulus, namely thrombin, platelets could be shown to produce a labile substance that increased adherence of unstimulated PMN, and this effect was blocked by anti- $\text{TxB}_2$  antibody.

Other products of arachidonic acid metabolism may modulate PMN inflammatory responses. Products of the lipoxygenase pathway, 12-L-hydroperoxy- and hydroxy-5,8,11,14-eicosatetraenoic acid, are chemotactic for human PMN (35), but their role in PMN adhesiveness is undefined.  $\text{PGE}_2$  is capable of enhancing both random and directed PMN migration (10). However, Bryant et al. (36), using an assay of PMN adherence to glass beads, demonstrated that  $\text{PGE}_1$  and cyclic AMP inhibited basal adhesiveness. In addition, O'Flaherty et al. (37) recently demonstrated that  $\text{PGE}_1$ ,  $\text{PGE}_2$ , and  $\text{PGF}_2$  in concentrations of 0.3–3  $\mu\text{g/ml}$  partially inhibited PMN aggregation or cell-to-cell adhesiveness induced by a synthetic chemotactic polypeptide. Although we could not confirm PG inhibition of base-line adhesiveness to nylon, our assay technique was most sensitive to enhancement of adherence. Unstimulated or basal adhesiveness was low, and we may not have detected small changes. Regardless, PG were not produced in significant concentrations by LPS-stimulated PMN and did not contribute to increases in adherence.

More recently, O'Flaherty et al. (38, 39) have shown that arachidonic acid stimulates PMN aggregation, a measure of cell-to-cell adhesiveness, although this observation could not be confirmed by Hammerschmidt et al. (40). In general, PMN adhesiveness to nylon and aggregation in response to complement-related chemotactic factors appear to be parallel phenomena (41); however, the PMN aggregation response is short-lived and particles disaggregate within 15–30 min (41,

42). Enhanced adhesiveness to nylon in our system persisted to 60 min and, in the instance of arachidonic acid stimulation, increased at 120 min. However, occasional cellular aggregates were present in incubation mixtures after 60 min. Using an assay of PMN adhesion to plastic surfaces, we were able to monitor aggregation microscopically; LPS did not cause significant aggregation of PMN but increased their adhesion to plastic, an effect that was inhibited by both indomethacin and imidazole. Our data cannot exclude conclusively an effect of aggregated PMN on the adherence assay, but the assay of PMN adhesiveness to plastic confirmed the observations obtained with the nylon adherence assay, i.e., a primary augmentation of adherence.

PGI<sub>2</sub> also inhibited LPS-induced PMN adherence. McGillen et al. (43) have recently shown that PGI<sub>2</sub> inhibits PMN base-line and complement-induced adhesiveness to nylon. We theorize that PGI<sub>2</sub> increases cyclic AMP in PMN, thereby decreasing PMN adherence (36), an action similar to the effects of PGI<sub>2</sub> on platelet cyclic AMP and aggregation-adherence (28). Vascular endothelium, which synthesizes PGI<sub>2</sub>, may alter the inflammatory response of PMN through these actions. These findings suggest a startling similarity between leukocyte and thrombocyte biochemistry and function; that is, although TxA<sub>2</sub> is proaggregatory and proadhesive in platelets and PMN, PGI<sub>2</sub> is antiaggregatory and antiadhesive. We speculate that this concept, originally proposed by Vane and co-workers (44), may be applicable in blood and other organs.

The current studies suggest a major role for TxA<sub>2</sub> as a mediator of increased PMN adhesion to surfaces. It appears that inflammatory stimuli such as LPS, whose effects are mediated by C5a (30), release significant quantities of TxA<sub>2</sub> from PMN with little concomitant release of PGE<sub>2</sub> or PGF<sub>2</sub>. TxA<sub>2</sub> presumably enhances PMN adhesiveness and thus facilitates PMN margination and migration to local inflammatory sites.

## ACKNOWLEDGMENTS

The authors wish to acknowledge the expert technical assistance of Christine Santoro and the help of Laraine Croson in typing this manuscript. We also thank Dr. Roger Herzig for assistance in the preparation of platelets used in this study.

This work was supported by grants from the Veterans Administration (P.J.S.) and the U. S. Public Health Service (M.J.D., HL-20224).

## REFERENCES

- McCall, E., and L. J. F. Youlten. 1973. Prostaglandin E<sub>1</sub> synthesis by phagocytosing rabbit polymorphonuclear leukocytes: its inhibition by indomethacin and its role in chemotaxis. *J. Physiol.* **234**: 98P.
- Adkinson, N. F. 1977. Prostaglandin production by human peripheral blood cells in vitro. *J. Lab. Clin. Med.* **90**: 1043-1053.
- Zurier, R. B., and D. M. Sayadoff. 1975. Release of prostaglandins from human polymorphonuclear leukocytes. *Inflammation*. **1**: 93-101.
- Bunting, S., G. A. Higgs, S. Moncada, and J. R. Vane. 1976. Generation of thromboxane A<sub>2</sub>-like activity from prostaglandin endoperoxides by polymorphonuclear leukocyte homogenates. *Br. J. Pharmacol.* **58**: 296P.
- Higgs, G. A., S. Bunting, S. Moncada, and J. R. Vane. 1976. Polymorphonuclear leukocytes produce thromboxane A<sub>2</sub>-like activity during phagocytosis. *Prostaglandins*. **12**: 749-757.
- Davidson, E. M., A. W. Ford-Hutchinson, M. J. H. Smith, and J. R. Walker. 1978. The release of thromboxane B<sub>2</sub> by rabbit peritoneal polymorphonuclear leukocytes. *Br. J. Pharmacol.* **63**: 407P.
- Goldstein, I. M., C. L. Malmsten, H. Kindahl, H. B. Kaplan, O. Radmark, B. Samuelsson, and G. Weissmann. 1978. Thromboxane generation by human peripheral blood polymorphonuclear leukocytes. *J. Exp. Med.* **148**: 787-792.
- Morley, J., M. A. Bray, R. W. Jones, D. H. Nugteren, and D. A. van Dorp. 1979. Prostaglandin and thromboxane production by human and guinea pig macrophages and leukocytes. *Prostaglandins*. **17**: 730-736.
- Williams, T. J., and J. Morley. 1973. Prostaglandins as potentiators of increased vascular permeability in inflammation. *Nature (Lond.)*. **246**: 215-217.
- Till, G., E. Kownatzki, M. Seitz, and D. Gerns. 1979. Chemokinetic and chemotactic activity of various prostaglandins for neutrophil granulocytes. *Clin. Immunol. Immunopathol.* **12**: 111-118.
- Rabson, A. R., R. Anderson, R. Lomnitz, and J. H. Koornhof. 1974. *In vitro* effects of prostaglandins on polymorphonuclear leukocyte function. *S. Afr. Med. J.* **48** (Suppl.): 44-50.
- Kaley, G., and R. Weiner. 1971. Prostaglandin E<sub>1</sub>: a potential mediator of the inflammatory response. *Ann. N. Y. Acad. Sci.* **180**: 338-350.
- Svensson, J., M. Hamberg, and B. Samuelsson. 1975. Prostaglandin endoperoxides. IX. Characterization of rabbit aorta contracting substances (RCS) from guinea pig lung and human platelets. *Acta Physiol. Scand.* **94**: 222-228.
- Kitchen, E. A., J. R. Boot, and W. Dawson. 1978. Chemotactic activity of thromboxane B<sub>2</sub>, prostaglandin and their metabolites for polymorphonuclear leukocytes. *Prostaglandins*. **16**: 239-244.
- Allison, F., M. R. Smith, and W. B. Wood. 1955. Studies on the pathogenesis of acute inflammation. *J. Exp. Med.* **102**: 669-676.
- MacGregor, R. R., E. Macarak, and N. A. Kefalides. 1978. Comparative adherence of granulocytes to endothelial monolayers and nylon fiber. *J. Clin. Invest.* **61**: 697-702.
- Spagnuolo, P. J., and J. J. Ellner. 1979. Salicylate blockade of granulocyte adherence and the inflammatory response to experimental peritonitis. *Blood*. **53**: 1018-1022.
- Flower, R. J., H. S. Cheung, and D. W. Cushman. 1973. Quantitative determination of prostaglandins and malondialdehyde formed by the arachidonate oxygenase system of bovine seminal vesicle. *Prostaglandins*. **4**: 325-341.
- MacGregor, R. R., P. J. Spagnuolo, and A. L. Lentnek. 1974. Inhibition of granulocyte adherence by ethanol, prednisone, and aspirin, measured with an assay system. *N. Engl. J. Med.* **291**: 642-647.
- Gallin, J. L., D. G. Wright, and E. Schiffmann. 1978. Role of secretory events in modulating human neutrophil chemotaxis. *J. Clin. Invest.* **62**: 1364-1374.



21. Dray, F., B. Charbonnel, and J. Maclof. 1975. Radioimmunoassay of prostaglandins  $F\alpha$ ,  $E_1$ , and  $E_2$  in human plasma. *Eur. J. Clin. Invest.* 5: 311-318.
22. Sors, A., P. Pradelles, F. Dray, M. Rigaud, J. Maclof, and P. Bernard. 1978. Analytical methods for thromboxane  $B_2$  measurement and validation of radioimmunoassay by gas-liquid chromatography-mass spectrometry. *Prostaglandins*. 16: 277-289.
23. Gaut, Z. N., H. Baruth, and L. O. Randall. 1975. Stereoisomeric relationships among anti-inflammatory activity, inhibition of platelet aggregation and inhibition of prostaglandin synthetase. *Prostaglandins*. 10: 59-66.
24. Tai, H. H., and B. Yuan. 1978. On the inhibitory potency of imidazole and its derivatives on thromboxane synthetase. *Biochem. Biophys. Res. Commun.* 80: 236-242.
25. Needleman, P., A. Raz, J. A. Ferendelli, and M. Minkes. 1977. Application of imidazole as a selective inhibitor of thromboxane synthetase in human platelets. *Proc. Natl. Acad. Sci. U. S. A.* 74: 1716-1720.
26. Kelly, J. P., M. C. Johnson, and C. W. Parker. 1979. Effect of inhibitors of arachidonic acid metabolism on mitogenesis in human lymphocytes: possible role of thromboxanes and products of the lipoxygenase pathway. *J. Immunol.* 122: 1563-1571.
27. Diczfalussy V., and S. Hammarström. 1979. A structural requirement for the conversion of prostaglandin endoperoxides to thromboxanes. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 105: 291-295.
28. Gorman, R. R. 1979. Modulation of human platelet function by prostacyclin and thromboxane  $A_2$ . *Fed. Proc.* 38: 83-88.
29. Fernandez, H. N., and T. E. Hugli. 1978. Primary structural analysis of the polypeptide portion of human C5a anaphylatoxin. Polypeptide sequence determination and assignment of the oligosaccharide attachment site on C5a. *J. Biol. Chem.* 253: 6955-6964.
30. Snyderman, R., H. S. Shin, and J. K. Philips, H. Gewurz, and S. E. Mergenhagen. 1969. A neutrophil chemotactic factor derived from C5 upon interaction of guinea pig serum with endotoxin. *J. Immunol.* 103: 413-422.
31. Morrison, D. C., and R. J. Ulevitch. 1978. The effects of bacterial endotoxins on host mediation systems. *Am. J. Pathol.* 93: 527-617.
32. Hansberg, M., J. Swensson, T. Wakabayashi, and B. Samuelsson. 1974. Isolation and structure of two prostaglandin endoperoxides that cause platelet aggregation. *Proc. Natl. Acad. Sci. U. S. A.* 71: 345-349.
33. Feinstein, M. B., E. L. Becker, and C. Fraser. 1977. Thrombin, collagen and A23187 stimulated endogenous platelet arachidonate metabolism: differential inhibition by  $PGE_1$ , local anesthetics and a serine-protease inhibitor. *Prostaglandins*. 14: 1075-1093.
34. Hamberg, M., J. Swensson, and B. Samuelsson. 1975. Thromboxanes: a new group of biologically active compounds derived from prostaglandin endoperoxides. *Proc. Natl. Acad. Sci. U. S. A.* 72: 2994-2998.
35. Goetzl, E. J., J. M. Woods, and R. R. Gorman. 1977. Stimulation of human eosinophil and neutrophil polymorphonuclear leukocyte chemotaxis and random migration by 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid. *J. Immunol.* 59: 179-183.
36. Bryant, R. E., and M. C. Sutcliffe. 1974. The effect of 3'5'-adenosine monophosphate on granulocyte adhesion. *J. Clin. Invest.* 54: 1241-1244.
37. O'Flaherty, J. T., D. L. Kruetzer, P. A. Ward. 1979. Effect of prostaglandin  $E_1$ , and  $F_2\alpha$  on neutrophil aggregation. *Prostaglandins*. 17: 201-210.
38. O'Flaherty, J. T., H. J. Showell, E. L. Becker, and P. A. Ward. 1979. Neutrophil aggregation and degranulation. Effect of arachidonic acid. *Am. J. Pathol.* 95: 433-444.
39. O'Flaherty, J. T., H. J. Showell, P. A. Ward, and E. L. Becker. 1979. A possible role of arachidonic acid in human neutrophil aggregation and degranulation. *Am. J. Pathol.* 96: 799-809.
40. Hammerschmidt, D. E., J. G. White, P. R. Craddock, and H. S. Jacob. 1979. Corticosteroids inhibit complement-induced granulocyte aggregation. A possible mechanism for their efficacy in shock states. *J. Clin. Invest.* 63: 798-803.
41. O'Flaherty, J. T., D. L. Kreutzer, and P. A. Ward. 1978. The influence of chemotactic factors on neutrophil adhesiveness. *Inflammation*. 3: 37-48.
42. O'Flaherty, J. T., D. L. Kreutzer, and P. A. Ward. 1977. Neutrophil aggregation and swelling induced by chemotactic agents. *J. Immunol.* 119: 232-239.
43. McGillen, J., R. Patterson, and J. Phair. 1980. Adherence of polymorphonuclear leukocytes to nylon: modulation by prostacyclin ( $PGI_2$ ), corticosteroids, and complement activation. *J. Infect. Dis.* 141: 382-388.
44. Moncada, S., and J. R. Vane. 1979. Pharmacologic and endogenous roles of prostaglandin endoperoxides, thromboxane  $A_2$ , and prostacyclin. *Pharmacol. Rev.* 30: 293-331.