Brief Definitive Report

THROMBOXANE GENERATION BY HUMAN PERIPHERAL BLOOD POLYMORPHONUCLEAR LEUKOCYTES*

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Thromboxane A_2 (TXA_2) is an unstable, yet biologically active, byproduct of a biosynthetic pathway which is initiated by the action of cyclo-oxygenase upon the polyunsaturated fatty acid substrate, arachidonic acid (1). Another limb of this pathway leads to generation of stable prostaglandins. TXA_2 is derived from the cyclic endoperoxides, prostaglandin G_2 (PGG_2) and prostaglandin H_2 (PGH_2). After a half-life of only 30–40 s in aqueous media at physiologic temperature and pH, it is primarily transformed to the biologically inactive and stable thromboxane B_2 (TXB_2) (1). At nanomolar concentrations, TXA_2 causes rapid, irreversible platelet aggregation and the associated platelet release reaction (1, 2); it also provokes contraction of vascular and tracheal smooth muscle (3). Its role in provoking other phenomena associated with inflammation is currently under investigation (4). Since polymorphonuclear leukocytes (PMN) of human peripheral blood have recently been shown capable of synthesizing stable prostaglandins (of the E and F series) upon exposure to phagocytic stimuli (5), we have examined whether generation of thromboxanes occurs under similar conditions. For these studies we have employed a sensitive radioimmunoassay for the stable end-product, TXB_2 (6). The formation of [^{14}C]TXB_2 from [^{14}C]arachidonic acid in suspensions of PMN was demonstrated by means of thin-layer radiochromatography (TLC), radio-gas chromatography, and mass spectrometry.

Materials and Methods

Preparation of PMN Suspensions and Serum-Treated Zymosan (STZ). Leukocyte suspensions containing approximately 98 ± 1% PMN were prepared from heparinized (10 U/ml) venous blood obtained from healthy adult donors by employing Hypaque/Ficoll gradients (7) and dextran sedimentation. Erythrocytes were removed by hypotonic lysis. The cells were suspended in phosphate (10 mM)-buffered saline, pH 7.4, supplemented with 0.6 mM $CaCl_2$ and 1.0 mM $MgCl_2$. This buffer was used throughout. The platelet: PMN ratio in the cell suspensions was generally in the range of 1:10. This ratio never exceeded 2:5. For some experiments, PMN were obtained from a patient with untreated idiopathic thrombocytopenia whose peripheral platelet count was 16,000/mm^3. The platelet:PMN ratio in the cell suspension prepared from this patient's blood was less than 1:40. For other experiments, suspensions of washed platelets (containing fewer than 1×10^5 PMN/ml) were prepared from normal platelet-rich plasma (8).

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STZ was prepared as previously described (9) and suspended in buffer at concentrations ranging from 5.0 to 50 mg/ml.

Reaction Mixtures and Radioimmunoassay. Portions of cell suspensions containing 1×10^7 PMN were dispensed into 12 × 75-mm polypropylene tubes (BioQuest, BBL, and Falcon Products, Cockeysville, Md.) before addition of appropriate compounds and stimuli. Some cells were preincubated with cytochalasin B (5.0 μg/ml) (ICI Research Laboratories, Alderley Park, Cheshire, England) in 0.1% dimethyl sulfoxide (Matheson Gas Products, East Rutherford, N. J.) at 37°C for 10 min. Further incubations at 37°C were terminated by addition of indomethacin (5×10^{-5} M) (Sigma Chemical Co., St. Louis, Mo.) and by placing the tubes in dry ice-acetone. Samples were stored at −70°C before radioimmunoassay. Radioimmunoassay of TXB_2 was performed as previously described by Granström et al (6). Results are expressed as picograms/10^6 PMN.

TLC, Radio-Gas Chromatography, and Mass Spectrometry. Reaction mixtures for analysis by TLC contained 5–8 × 10^7 PMN. These were preincubated for 2 min at 37°C before addition of 0.03 mM (1.5 μCi) [^{14}C]arachidonic acid (The Radiochemical Centre, Amersham, England), appropriate compounds and stimuli (STZ, 5.0 mg/10^7 PMN). Further incubations at 37°C were terminated after 15 min by adding 10 ml cold ethanol. Crude incubation/ethanol mixtures were diluted with an equal volume of H_2O, acidified to pH 3.0, and extracted twice with diethyl-ether. The combined organic phases were washed with H_2O until neutral and dried with $MgSO_4$. The ether was evaporated and the residue treated with diazomethane in methanol before separation with ether:methanol (99:1) on TLC plates coated with Silica gel G (0.25 mm) (Analtech, Inc., Newark, Del.). A Berthold Dünnschichtscanner II was employed to identify radiolabeled fractions on the TLC plates.

Compounds that on TLC appeared at the same R_f value as the methyl-ester of a reference standard of TXB_2 (i.e., between methylesters of PGE_2 and PGA_1) were extracted with hexane. The yields were 0.8–2.1 ng/10^6 PMN. Hexane was removed and the samples were dissolved in 0.2 ml pyridine containing 5.0 mg of methoxyamine hydrochloride (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N. Y.). The solutions were stored in a stoppered test tube overnight in a desiccator before addition of 20 μl of trimethylchlorosilane and 40 μl of hexamethyldisilazane (Merck, Sharp & Dohme, Quebec, Canada). After an additional 1 h in the desiccator, the samples were evaporated to dryness, extracted with ether and finally dissolved in hexane before injection into the radio-gas chromatograph (Barber Colman Co., Rockford, Ill.). Chromatography was performed at a temperature of 225°C with a 6.0 mm × 185 cm column packed with 1.0% SE 30 on Chromosorb W 80/100. Carrier gas (He) flow was 60 ml/min. The flash heater and detector were kept at a temperature 15°C above that of the column (10). The gas chromatogram of the methoxyamine trimethylsilyl derivatives of the samples showed characteristic peaks at the equivalent chain length C-24.9.

Mass spectra of samples (methoxyamine trimethylsilyl derivatives) from several incubations were obtained with a combined gas chromatograph-mass spectrometer (LKB 9000) equipped with a 210-cm long 1.0% SE 30 column on Chromosorb W 100/120. Column inner diameter was 3 mm and the carier gas (He) flow was 20 ml/min.

Other Compounds and Reagents. Unlabeled arachidonic acid was purchased from Nu-Chek Co., Elysian, Minn. TXB_2 (as a standard) was prepared according to Hamberg and Samuelsson (11). PGE_2 and PGA_1 were kindly supplied by Dr. G. L. Bundy of the Upjohn Co., Kalamazoo, Mich. Indomethacin was dissolved in ethanol before being diluted in buffer. The final concentration of ethanol in the reaction mixtures never exceeded 0.05%. All solvents were either reagent grade (Eastman Kodak Co.) or Fisher-certified (Fisher Scientific Co., Pittsburgh, Pa.). Some were redistilled before use.

Results

PMN incubated for up to 60 min in buffer alone generated minimal amounts of TXB_2 (15.7 ± 1.9 pg/10^6 PMN, n = 10). However, when PMN were exposed to STZ, which they ingest avidly, up to 10-fold increments in TXB_2 generation were observed. The mean ± SE for 13 experiments was 65.8 ± 12.2 pg/10^6 PMN. This response varied with the particle-to-cell ratio and with time. Representative experi-

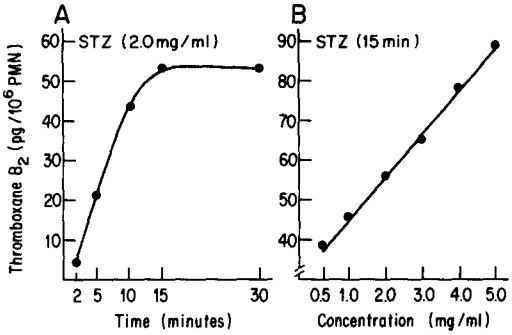

FIG. 1. Thromboxane B_2 generation by human PMN exposed to STZ: (A) versus duration of incubation, (B) versus concentration of STZ. Reaction mixtures contained 1×10^7 PMN in a 1.0-ml vol.

TABLE I

Generation of Thromboxane B_2 (TXB_2) by Human PMN

PMN exposed to:		TXB_2*
	(*n*)	*picograms/10^6 PMN/15 min*
Buffer alone	(10)	15.7 ± 1.9
+ Indomethacin (5×10^{-6} M)	(6)	9.9 ± 1.4
Cytochalasin B + buffer‡	(6)	16.2 ± 4.2
+ Indomethacin	(4)	10.8 ± 1.8
STZ (2.0 mg/ml)	(13)	65.8 ± 12.2
+ Indomethacin	(9)	13.5 ± 0.9
Cytochalasin B + STZ	(9)	59.9 ± 3.4
+ Indomethacin	(5)	13.5 ± 1.4

* TXB_2 measured by radioimmunoassay. Mean ± SE. *n* = number of experiments.
‡ PMN were preincubated with cytochalasin B (5.0 μg/ml) for 10 min at 37°C.

ments are shown in Fig. 1. Maximum TXB_2 generation usually occurred before 15 min and an almost linear response was noted with concentrations of STZ between 0.5 and 5.0 mg/ml.

To determine whether phagocytosis was a prerequisite for TXB_2 generation, experiments were performed employing the fungal metabolite, cytochalasin B. Cytochalasin B-treated PMN are unable to ingest particles such as STZ but nevertheless increase their oxidative metabolism and degranulate (release lysosomal enzymes) upon contact of the particles with their cell surfaces (9, 12). PMN treated with cytochalasin B and then exposed to STZ generated amounts of TXB_2 that were comparable to amounts generated by normal cells (Table I). Generation of TXB_2 by both normal and cytochalasin B-treated PMN in response to stimulation was inhibited appropriately by the cyclo-oxygenase inhibitor, indomethacin (1, 2) (Table I). Indomethacin neither interfered with particle-to-cell contact nor with other responses of these cells to surface stimulation (i.e., lysosomal enzyme release and superoxide anion generation) (data not shown). Furthermore, these concentrations of indomethacin did not influence the radioimmunoassay.

Generation of TXB_2 in these experiments could not be attributed to platelet contamination of the PMN suspensions. Addition of washed platelets in 10-fold excess of normal platelet contamination did not influence the amount of TXB_2 measured in reaction mixtures containing either resting cells or cells stimulated by the addition of STZ. Stimulated PMN generated comparable amounts of TXB_2 in the presence or

absence of added platelets (65.0 ± 5.2 and 59.8 ± 7.1 pg/10^6 PMN, respectively; n = 4). Moreover, no increase in TXB_2 generation was observed in suspensions of washed platelets (without PMN) after addition of STZ. Zymosan has previously been shown to be incapable of provoking platelet aggregation in the absence of plasma (13). Finally, generation of TXB_2 by cells obtained from the patient with thrombocytopenia (54.6 pg/10^6 PMN, platelet: PMN ratio <1:40) was comparable to that measured when cells obtained from normal donors were exposed to identical amounts of STZ.

The ability of PMN to form [^{14}C]TXB_2 from precursor [^{14}C]arachidonic acid was demonstrated by means of TLC, gas chromatography, and mass spectrometry. The compounds which on radio-TLC showed the same R_f values as a reference standard of authentic TXB_2 also appeared at the same retention as authentic TXB_2 after radio-gas chromatography. When purified fractions of these samples were analyzed by gas chromatography-mass spectrometry, the mass spectra were in accordance with that of authentic TXB_2 (11). Characteristic peaks were at: m/e 311, 201 (base peak), 191, 174, 173. Whereas some of the TXB_2 demonstrated by these procedures could have been produced by the platelets that contaminated the PMN suspensions, considerably more TXB_2 was recovered than could be accounted for by platelets alone. With a maximum platelet:PMN ratio of 2:5 and with a conversion of approximately 10% by platelets (11), the recovery of TXB_2 produced by platelets alone should have been less than 0.4 ng/10^6 PMN in suspension. The amount of TXB_2 actually isolated on TLC was in the range of 0.8–2.1 nanograms/10^6 PMN.

Discussion

Previous studies have established that human peripheral blood PMN are capable of producing stable prostaglandins of the E and F series when exposed to phagocytic stimuli (5). Recently, Higgs et al. (14) reported that homogenates of rabbit peritoneal PMN that had been permitted to ingest killed bacteria generated rabbit aorta contracting activity (attributable to TXA_2) when incubated with the prostaglandin endoperoxides, PGG_2 or PGH_2. TXA_2 activity was not generated by homogenates prepared from unstimulated cells. It is of interest in this regard that Smolen and Shohet (15) were able to demonstrate a marked decrease during phagocytosis of the arachidonic acid content of phospholipids in the membranes of phagocytic vacuoles isolated from human PMN. In another recent report (16), unstimulated rabbit peritoneal PMN were found to produce monohydroxy acids (by the action of lipoxygenases) upon exposure to arachidonic acid and homo-γ-linoleic acid.

We have previously demonstrated that intact, viable human PMN release and/or generate inflammatory materials and substances capable of provoking tissue injury when they encounter appropriate cell surface stimuli even in the absence of phagocytosis. These include hydrolases and non-enzymatic substances ordinarily contained within azurophil or specific granules, as well as oxygen-derived free radicals (9). The studies reported here enable us to add thromboxanes to this list. The results of our studies demonstrate that stimulation of intact, viable human peripheral blood PMN leads to the generation of thromboxanes. Generation of TXB_2 by PMN varied with the concentration of the stimulus employed and with time, but occurred independently of phagocytosis. The studies with cytochalasin B-treated PMN suggest that surface stimulation, in the absence of particle ingestion, is sufficient to provoke thromboxane synthesis by these cells.

Summary

Human peripheral blood polymorphonuclear leukocytes were stimulated to generate thromboxane B_2 in a time- and concentration-dependent fashion upon exposure to serum-treated zymosan particles. Conversion by stimulated PMN of [^{14}C]arachidonic acid to [^{14}C]thromboxane B_2 was confirmed by thin-layer radiochromatography, radio-gas chromatography, and mass spectrometry. Generation of thromboxane B_2 was independent of platelet contamination and could be inhibited by the cyclooxygenase inhibitor, indomethacin. Cells rendered incapable of ingesting particles by treatment with cytochalasin B generated comparable amounts of thromboxane B_2. These results suggest that human peripheral blood polymorphonuclear leukocytes synthesize thromboxanes in response to surface stimulation independently of phagocytosis.

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References

1. Hamberg, M., J. Svensson, 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.
2. Svensson, J., M. Hamberg, and B. Samuelsson. 1976. On the formation and effects of thromboxane A_2 in human platelets. *Acta Physiol. Scand.* **98:**285.
3. 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.
4. Kuehl, F. A., Jr., J. L. Humes, R. W. Egan, E. A. Ham, G. C. Beveridge, and C. G. Van Arman. 1977. Role of prostaglandin endoperoxide PGG_2 in inflammatory processes. *Nature (Lond.).* **265:**170.
5. Zurier, R. B., and D. M. Sayadoff. 1975. Release of prostaglandins from human polymorphonuclear leukocytes. *Inflammation.* **1:**93.
6. Granström, E., H. Kindahl, and B. Samuelsson. 1976. Radioimmunoassay for thromboxane B_2. *Anal. Lett.* **9:**611.
7. Böyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. *Scand. J. Clin. Lab. Invest.* **21**(Suppl. 97)**:**77.
8. Hamberg, M., J. Svensson, 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.
9. Goldstein, I. M., D. Roos, H. B. Kaplan, and G. Weissmann. 1975. Complement and immunoglobulins stimulate superoxide production by human leukocytes independently of phagocytosis. *J. Clin. Invest.* **56:**1155.
10. Green, K. 1969. Gas chromatography-mass spectrometry of O-methyl-oxime derivatives of prostaglandins. *Chem. Phys. Lipids.* **3:**254.
11. Hamberg, M., and B. Samuelsson. 1974. Prostaglandin endoperoxides. Novel transformations of arachidonic acid in human platelets. *Proc. Natl. Acad. Sci. U. S. A.* **71:**3400.
12. Zurier, R. B., S. Hoffstein, and G. Weissmann. 1973. Cytochalasin B: effect on lysosomal enzyme release from human leukocytes. *Proc. Natl. Acad. Sci. U. S. A.* **70:**844.
13. Zucker, M. B., and R. A. Grant. 1974. Aggregation and release reaction induced in human

blood platelets by zymosan. *J. Immunol.* **112:**1219.

14. Higgs, G. A., S. Bunting, S. Moncada, and J. R. Vane. 1976. Polymorphonuclear leukocytes produce thromboxane A_2-like activity during phagocytosis. *Prostaglandins.* **12:**749.
15. Smolen, J. E., and S. B. Shohet. 1974. Remodeling of granulocyte membrane fatty acids during phagocytosis. *J. Clin. Invest.* **53:**726.
16. Borgeat, P., M. Hamberg, and B. Samuelsson. 1976. Transformation of arachidonic acid and homo-γ-linoleic acid by rabbit polymorphonuclear leukocytes. *J. Biol. Chem.* **254:**7816.