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The Effects of Aspirin on Megakaryocyte Prostaglandin Production (40716)

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Prostaglandins are believed to play a central role in the platelet release reaction (1). During platelet aggregation, prostaglandin E (PGE) and thromboxane A_2 (TXA_2) are released. This event coincides with the concomitant release of ADP (2). The release of prostaglandins by the platelets during aggregation can be readily inhibited by acetylsalicylic acid (aspirin) (3). This effect of aspirin has been shown to be due to its effect on the cyclooxygenase enzyme. Acetylation of this enzyme by aspirin renders it incapable of promoting the synthesis of prostaglandin endoperoxides from fatty acid substances (4, 5). Ingestion of aspirin abolishes the ability of platelets to produce prostaglandins. This effect can be demonstrated as early as 2 hr following aspirin ingestion and lasts for the entire duration these platelets are in the circulation (6).

A 2-day lag phase has been shown to occur before any detectable release of prostaglandins can be elicited from human platelets following ingestion of a single dose of aspirin. Recovery of prostaglandin production to preaspirin treatment levels is not complete until 8 days after cessation of oral aspirin intake (6).

The 2-day lag phase in platelet prostaglandin synthesis following aspirin ingestion suggests that the platelet precursor cell, the megakaryocyte may be similarly affected by aspirin and that this lag phase represents the maturation time during which new megakaryocytes are produced from earlier precursors. This study was designed to determine the effects of aspirin on the rat megakaryocyte, as the information thus obtained may significantly alter the design of clinical trials with antiplatelet drugs.

Megakaryocytes were enriched from rat bone marrow suspensions. This system was used to characterize the effect of aspirin on the capacity of megakaryocytes to synthesize prostaglandins.

Methods and materials. Animals. Twenty male 70- to 100-g SD strain rats were purchased from Charles River Animal Breeding Farm, Charles River, Massachusetts. All animals were maintained on a regular diet with water *ad libitum.*

Velocity sedimentation. Megakaryocyte-rich cell suspensions were obtained according to the method developed by Pretlow and Stinson (7). Rats were anesthetized with ether and sacrificed immediately. Marrow was obtained by perfusion of each femoral shaft with 3 ml Joklik's modification of minimum essential medium (Grand Island Biological Company, Grand Island, N.Y.) containing 10% fetal calf serum (Grand Island Biological) and heparin (2 units/ml). The material was gently aspirated through an 18-gauge needle into a 3-ml plastic tuberculin syringe. The suspension was transferred to a 5-ml plastic test tube and incubated in a 37°C water bath for 10 min. At the end of this period, either 0.6 ml of 1.0 mm NEM in saline or 0.6 ml saline was added to the cell suspension. The suspension was again incubated at 37°C for 10 min, after which the tubes were placed in an ice bath.

Linear gradients of Ficoll (polysucrose, average molecular weight 400,000, Pharmacia Fine Chemicals, Piscataway, N.J.) in Joklik's modification of minimum essential medium were constructed and collected using a two-chambered, cam-operated gradient maker (Beckman, Palo Alto, Calif.). The gradients were contained in 50-ml plastic centrifuge tubes (Corning Glass Works, Corning, N.Y.). The 30-ml gradients varied from 1.0% (w/w) Ficoll at the sample–gradient interface to 6.0% (w/w)

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Ficoll at the gradient–centrifuge cushion interface.

One milliliter of cell suspension (containing approximately 3.0×10^7 cells) was carefully layered over the gradient just prior to centrifugation. It was determined that the best separation was accomplished after centrifugation for 10 min at 4°C using a centrifugal force of 15*g* measured at the sample–gradient interface.

Gradients were collected (from bottom to top) in three fractions: 2, 15, and 10 ml, respectively (Fig. 1). The middle fraction was found to be relatively megakaryocyte-rich, the upper fraction megakaryocyte-poor. The lowest fraction (2 ml) was discarded. Fractions obtained were then placed in 50-ml centrifuge tubes and centrifuged for 10 min (4°C) at 400*g* to form a pellet of cells. After centrifugation, the supernatant was aspirated from each tube, leaving the pellet intact. This was subsequently resuspended in 2 ml Joklik's medium. A sample of 0.2 from each tube was applied to a hemacytometer. The remaining material was quick frozen in a dry ice–ethyl acetate bath and stored at −20°C for prostaglandin assay. Binary differential cell counts (megakaryocytes vs nonmegakaryocytes) were performed on all fractions using pair of hemacytometers. Four thousand cells were counted per sample (Table 1).

Acetylsalicyclic acid level. Aspirin (20 mg/kg), dissolved in 0.3 ml distilled water, was injected subcutaneously into each rat. Blood samples were obtained 2 hr prior to sacrifice, from the jugular vein while the animal was under ether anaesthesia. The serum salicylate levels were then measured using the ferric nitrate-complexometric method performed on a Dupont automatic clinical analyzer. Results were reported as milligrams of salicylate per deciliter of serum.

PGE determination. Prostaglandins were determined in the samples using radioimmunoassay as described previously (6). Upon thawing, the cells in suspension were disrupted by sonification (Branson Ultrasonics, Danbury, Conn.) for 20 sec. Following prostaglandin recovery trace addition, the samples were extracted into ethyl acetate and applied to silicic acid chromatography to separate the E, A, and F series prostaglandins. The E fraction was collected and evaporated to dryness under nitrogen and reacted with specific antiserum to PGE raised in rabbits against an albumin–PGE conjugate. Tritium-labeled prostaglandins (NEN, Waltham, Mass.) competed with standard or unknown prostaglandins for binding to the antibody. Unknown results were determined from a standard curve, utilizing PGE standards obtained from the Upjohn Company (Kalamazoo, Mich.). Dextran-coated charcoal was used to separate the antibody bound from free labeled prostaglandins. All calculations were performed using the Rodbard statistical program for logit B/B_0 transformation. Results were expressed as picograms of prostaglandins per 10^6 cells.

Statistics. The significance of the results were calclated using Student's t test. Results with values of P less than 0.05 were considered to represent significant differences between the groups.

Calculation of PG yield by megakaryocyte fractions. The use of velocity sedimentation in an isokinetic gradient yielded cell suspensions containing 0.0077 to 0.1173% megakaryocytes as shown in (Fig. 1). The megakaryocytes appeared morphologically normal following separation in the gradient.

The two values to be obtained from the data were (i) the PGE production by the megakaryocytes and (ii) the PGE production by the background cells. It was assumed that every cell type, other than the substantially larger megakaryocyte, had a parallel distribution in the gradient. Thus the background group of cells was considered to have the same proportion of all cell types regardless of sample. A set of linear equations yields the results given in Tables II and III.

Each sample was characterized by four values (i and j represent individual samples):

a_i = number of megakaryocytes (/ml) in sample i, as determined by hemocytometer,

b_i = number of background cells (total cells-megakaryocytes), as determined by hemacytometer,

c_i = PGE (pg) in Joklik's medium and fetal calf serum (in sample i), an unknown due to possible cell–medium interactions,
n_i = PGE (pg) in sample i, as determined by radioimmunoassay.

The marrow from each rat was layered on two (2) isokinetic gradients, yielding two megakaryocyte-rich and two megakaryocyte-poor fractions per rat.

If
X = PGE/megakaryocyte (pg/cell),
Y = PGE/background cell (pg/cell)

then each animal generated four equations of the form

$$a_i X + b_i Y + c_i = N_i,$$

where $C_i = c_j$ for all i and j. Three such equations generate two equations of the form

$$(a_i - a_j) X + (b_i - b_j) Y = N_i - N_j,$$

which are independent of c, an unknown. These two resulting equations can be solved for the unknowns X and Y to yield PGE levels in the samples containing megakaryocytes and background cells.

Results. Table I shows the results of the enrichment of megakaryocytes by cellular velocity sedimentation on an isokinetic Ficoll gradient. It was noted that a 15-fold enrichment of megakaryocytes, was obtained from the top to the bottom of the gradient (Fig. 1). This enriched fraction was used to determine the production of PGE by the megakaryocytes. As shown in Table II, when megakaryocytes were exposed to NEM, there was a significant augmentation of PGE production from these isolated cells. Background cells (bg) were obtained from the megakaryocyte-poor fraction. No significant increase in PG synthesis occurred in these background cells (bg) when they were treated with NEM. When megakaryocytes were obtained from rats that were pretreated with aspirin, the expected augmentation of PGE production by NEM was abolished (Fig. 2). Aspirin blood levels of these rats were monitored to ensure adequacy of aspirin challenge. No significant change in PGE production by the bg cells was noted even with NEM treatment (Table III).

Discussion. The importance of prostaglandins in platelet aggregation and thrombosis have become well recognized. Prostaglandins of the E series are formed during the aggregation of human blood platelets whether induced by thrombin, collagen, ADP, or epinephrine (8). It is now apparent that PGE synthesis by the human platelet results from a secondary pathway via the PG endoperoxides. The major pathway of PG metabolism in platelets leads to thromboxane A_2 synthesis (9–11). Thromboxane A_2 is believed to be the biologically active prostaglandin moiety which participates in platelet aggregation and in the release reaction accompanying this process.

Aspirin has been shown to be extremely effective in abolishing the synthesis of PGE and TXA_2 by either pathway in platelets due to its ability to acetylate the cyclooxygenase enzyme (4, 5). This enzyme promotes the formation of the prostaglandin endoperoxide intermediates in the prostaglandin synthesis pathway. Aspirin therefore inhibits platelet aggregation in response to collagen, ADP, or epinephrine (12). We have demonstrated that *N*-ethylmaleimide (NEM) *in vitro* is capable of augmenting significantly (10-fold) the PG production by platelet suspension (6). NEM is believed to "turn on" the oxygenation

TABLE I. DIFFERENTIAL CELL COUNTS OBTAINED USING THE FICOLL ISOKINETIC GRADIENT

	N	Total cells (/ml)	Meg[a] (/ml)	%Meg
Top (10 ml) fraction	40	$1.91 \pm 0.06 \times 10^{7}$[b]	$1.48 \pm 0.14 \times 10^{3}$	0.0077
Bottom fraction	40	$5.44 \pm 0.47 \times 10^{6}$	$6.38 \pm 0.54 \times 10^{3}$	0.1173

[a] Meg refers to megakaryocytes.
[b] Results are expressed as mean ± standard error of mean.

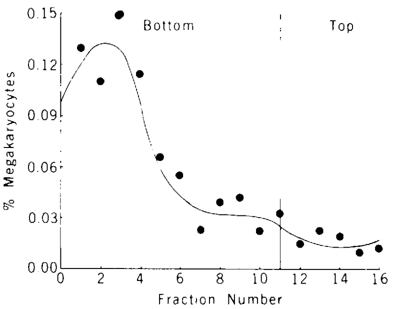
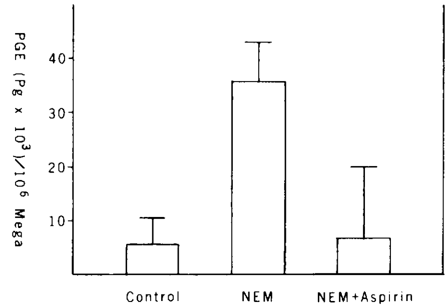


FIG. 1. Enrichment of megakaryocytes on linear gradients of Ficoll using cellular velocity sedimentation. The enrichment (top to bottom) ranged from 0.0077 to 0.1173% of megakaryocytes in the cell suspensions.



Results = Mean + S.E.M.

FIG. 2. PGE production by megakaryocytes. Control group = PGE production by megakaryocytes from control animals. NEM group = PGE production by megakaryocytes exposed to NEM, from control animals. NEM + ASA group = PGE production by megakaryocytes from animals pretreated with aspirin.

process in prostaglandin synthesis by depleting the cell of sulfhydryl activator through an inhibition of glutathione peroxidase activity in the cell (13). Glutathione peroxidase is an inhibitor of the prostaglandin cyclooxygenase enzyme (14). Platelets exposed to aspirin *in vivo* have a marked decrease in PG production as tested by the above method. This inhibitory effect of aspirin was noted as little as 2 hr following exposure to aspirin and persisted for approximately 8 days. In these studies it was noted that a linear recovery in terms of prostaglandin synthesis occurred following removal from aspirin which was preceded by an initial 2-day delay in recovery.

This suggests that the delayed recovery phase may be a consequence of megakaryocyte acetylation by aspirin since this cell is the immediate precursor cell leading to the mature platelet. These findings suggest that megakaryocyte prostaglandin cyclooxygenase system is also susceptible to inhibition by aspirin. Aspirin has also been shown to prevent the platelet lipid peroxidation normally induced by aggregating agents (15, 16).

It appears that once the megakaryocyte is acetylated by aspirin, this acetylation defect is transmitted to the mature platelet. This may explain the 2-day delay in platelet PGE synthesis which follows the ingestion of aspirin in humans (6). The recovery of PGE synthesis by peripheral platelets after this period may represent new platelet synthesis from early megakaryocyte precursors which may have been free of the acetylation defect due to the immaturity of their cytoplasm.

Stuart and Murphy have proposed that the recovery of platelet lipid peroxidation after a single dose of aspirin may provide a simple nonradioisotopic method for the determination of platelet life span (16). This explanation may prove to be too simplistic as it does not take into account the effect of aspirin on similar enzyme systems within the megakaryocyte cytoplasm. Platelets de-

TABLE II. PGE LEVELS IN MEGAKARYOCYTE AND BACKGROUND (BG) CELLS

Treatment	*N*	PGE/10^6 bg cells (pg)	PGE/10^6 meg (pg)
Control	7	0.6 ± 6.8^a	$5.8 \pm 7 \times 10^3$
NEM[b]	7	0.0 ± 16	$35.9 \pm 6.8 \times 10^3$*

[a] Results are expressed as mean ± standard error of mean.
[b] NEM, *N*-Ethyl maleimide treatment of cells *in vitro*.
* Significant at $P < 0.05$ level from control.

TABLE III. NEM-INDUCED PRODUCTION OF PGE IN MEGAKARYOCYTES FOLLOWING ASPIRIN ADMINISTRATION *IN VIVO*

Treatment	*N*	Aspirin (mg/dl)	PGE/10^6 bg cells (pg)	PGE/10^6 meg (pg)
Aspirin	6	2.2	0.2 ± 4.1[a]	6.7 ± 13.3 × 10^3*
Control	7	—	0.0 ± 16.0	35.9 ± 6.8 × 10^3

[a] Results are expressed as mean ± standard error of mean.

* Significant at the $P < 0.05$ level from the control.

rived from mature megakaryocytes within the bone marrow may be produced with the aspirin defect and therefore such techniques may not be able to provide a true assessment of platelet survival. An error, which would tend to artificially prolong platelet survival is already built in and inherent to these techniques. Therefore, the ^{51}Cr platelet survival still remains a better way to judge platelet survival accurately.

Since the larger megakaryocytes in the bottom fraction seem to have been affected most by the aspirin treatment, this may suggest that at least in rats this is the cell which is capable of PG synthesis and therefore susceptible to the effects of aspirin. It has been determined that platelet production from the earliest recognizable megakaryocytes takes 43–75 hr in rats (18). The cessation of DNA synthesis by rat megakaryocytes occurs at the level of Stage I of maturation (19). At this time these megakaryocytes mature rapidly and enter the recognizable megakaryocyte compartment (20). Maturation is associated with cytoplasmic growth and differentiation as well as an increase in cell size and nuclear segmentation (21). Sometime during this maturation process these megakaryocytes develop the ability to synthesize PG from precursor fatty acids. From this step onward the PG synthetic ability of the megakaryocyte can be affected by aspirin treatment *in vivo*. The exact nature of this cell, its state of maturity, and the level at which PG production is bestowed upon these megakaryocytes must await further experimentation.

Aspirin has a lasting and potent inhibitory effect on the prostaglandin synthesizing capacity of both the precursor megakaryocyte and the mature platelet. These studies demonstrate that this acetylation effect can occur in the bone marrow at the level of the megakaryocyte and lasts for the entire life cycle of the platelet produced by that particular cell.

Further studies are necessary to pinpoint the exact site at which human megakaryocytes are capable of PG synthesis and therefore the time required for recovery from aspirin inhibition. Such studies will critically effect the design of future clinical trials with anti-prostaglandin and antiplatelet drugs.

Summary. Aspirin inhibits the enzyme cyclooxygenase in platelets by irreversibly acetylating it. Since platelets are derived from the fragmentation of megakaryocyte cytoplasm and contain similar organelles and enzymes, we attempted to study the inhibitory effect of aspirin on the prostaglandin synthesis of isolated rat megakaryocytes. Megakaryocytes were isolated from rat bone marrow using cellular velocity sedimentation in an isokinetic Ficoll gradient. The isolated megakaryocytes were found capable of significant prostaglandin E synthesis as measured by radioimmunoassay when exposed to *N*-ethylmaleimide (NEM). Aspirin pretreatment *in vivo* abolished the ability of rat megakaryocytes to synthesize prostaglandin E. It seems likely that aspirin can also acetylate irreversibly the megakaryocyte cyclooxygenase enzyme and that this defect is carried over to the newly formed platelets. The implications of these results may significantly influence the dose schedules of antiplatelet drugs currently being used in clinical trials.

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