Platelet Recovery From Aspirin Inhibition In Vivo; Differing Patterns Under Various Assay Conditions

By Patricia M. Catalano, J. Bryan Smith, and Scott Murphy

The linear return of platelet malondialdehyde (MDA) production after aspirin ingestion as described by Stuart et al. has been widely accepted as a nonisotopic method for the determination of platelet survival. However, using assays for platelet prostaglandin production and cyclooxygenase activity, a number of authors have now reported a 24 to 48-hr delay in recovery after aspirin. This delay has been interpreted as evidence that aspirin inhibits megakaryocyte as well as platelet cyclooxygenase. The discrepancy in these findings has not been explained. We have measured the return of production of MDA and thromboxane B2 (TXB2) by platelets of normal volunteers after a single dose of aspirin using a variety of assay conditions. We find striking differences in the pattern of recovery depending upon the presence of protein in the medium in which the platelets are stimulated. Production of both MDA and TXB2 returns in a linear fashion with time when platelets are washed, resuspended in buffer and stimulated with either NEM or thrombin. A 48-hr lag in recovery is seen when platelets are stimulated in whole blood or platelet-rich plasma, or when washed platelets are resuspended in either platelet-poor plasma or in buffer containing 4 g % albumin. This lag is present regardless of stimulus or metabolite measured. It does not appear to be an artifact resulting from binding of the arachidonic acid or its metabolic products to the protein in the medium, as we are able to detect TXB2 production by even small numbers of aspirin-free platelets in whole blood mixtures. Therefore, if megakaryocyte cyclooxygenase is inhibited by aspirin, this effect is obscured when the platelets are suspended in protein-free medium. We have also studied a number of patients with short platelet survivals as judged by Cr51 labeling. Survivals using the aspirin method and washed platelets in these patients are consistently longer than those determined simultaneously by Cr51. This leads us to postulate that there is a lag effect even in the washed platelet system, and that this effect is more evident in states of increased platelet turnover. This complex effect must be better understood before aspirin platelet survivals can be accurately interpreted.

EXPOSURE of platelets to aspirin in vitro or in vivo prevents the production of prostaglandins, cyclic endoperoxides, and thromboxanes by irreversibly inhibiting platelet cyclooxygenase. Stuart et al. first proposed that this inhibitory effect of aspirin could be used as a nonisotopic marker for the determination of platelet survival in vivo. Their technique used malondialdehyde (MDA) as the marker of platelet prostaglandin production after stimulation of platelets with N-ethyl-maleimide (NEM). Results of studies using this technique or modifications of it have now been reported by other groups.

The theoretical and practical advantages of the nonisotopic technique are numerous. The survival is always autologous. Aspirin does not label a selected population of platelets as does a method that uses platelets obtained for labeling by differential centrifugation. It does not expose the subject under study to radiation and, therefore, would be particularly useful in children and during pregnancy. In addition, it does not require the facilities necessary to perform platelet pheresis and isotopic measurements.

There are also several disadvantages to the technique as originally described. These include the fact that MDA is a nonspecific breakdown product of lipids, and therefore not specific for the prostaglandin pathway. The colorimetric assay for MDA is relatively insensitive; it does not accurately detect concentrations of MDA below 100 pmole/ml. Consequently, washed concentrates of platelets must be used for each sample. Since these must be prepared by differential centrifugation, this reintroduces the possibility that the platelets studied may be selected by size or density. It also requires relatively large blood samples (as much as 10 ml/day), a practical consideration in the pediatric population. The most important disadvantage, however, is that the insensitivity of the MDA assay excludes from study that population of severely thrombocytopenic patients (<50,000 platelets/cu mm) in whom the study is of great interest. Sufficient numbers of platelets cannot be obtained from such patients to accurately determine the small amounts of MDA produced in the first few days after aspirin administration. In addition to these technical considerations, some authors have stressed that recovery from aspirin effect in fact measures platelet production. To use such a technique to determine platelet survival one must not only assume that the rate of platelet production equals the rate of destruction, but also that the megakaryocyte is not affected and that the platelets released from the marrow immediately after aspirin administration are completely free of the aspirin...
effect. Some investigators have presented evidence that suggests that the return of cyclooxygenase activity after a single dose of aspirin may be delayed for as much as 48 hr.\(^2\)\(^9\)\(^11\) This has been explained by postulating that aspirin may also inhibit megakaryocyte cyclooxygenase. If this were true, the technique could not be used to measure platelet survival per se. The discrepancies between these results and those of Stuart et al.\(^4\) have not been explained.

The current studies were originally designed to evaluate thromboxane B\(_2\) (TXB\(_2\)) as the marker for platelet prostaglandin metabolism in nonslotis platelet survivals. A radioimmunoassay for TXB\(_2\) is available in our laboratory that can detect as little as 0.5 pmole/ml TXB\(_2\). Because the assay is so sensitive, we hypothesized that platelets could be stimulated in whole blood. Thus there would be no selection of the platelet population studied. Very small volumes of blood would be necessary even from thrombocytopenic patients. When we compared this technique with the MDA method, we found differences in recovery patterns that have important implications regarding the interpretation and clinical application of aspirin platelet survivals.

**MATERIALS AND METHODS**

Individuals studied were either normal volunteers or adult patients referred for autologous Cr\(^{51}\) platelet survival. All subjects were instructed not to ingest aspirin for at least 14 days prior to study. Blood was drawn, and the subjects were then given 600 mg ASA (Wyeth, Philadelphia, Pa.) by mouth. Blood was again obtained at 2 hr and then at 24-hr intervals after aspirin ingestion. Written informed consent was obtained. This study was approved by the Thomas Jefferson University Human Studies Committee.

**MDA Measurement**

Fifteen milliliters of whole blood was anticoagulated with 2 ml of 0.1 M NaEDTA, brought to 20 ml by addition of 3 ml of saline, and then centrifuged at 220 g for 5 min at 22°C. Platelet-rich plasma was removed using a plastic pipette; the remaining cells were resuspended in 8 ml of saline and again spun at 280 g for 5 min. The platelet containing supernatant was then pooled with that from the first spin, and the total volume of the platelet suspension was brought to 20 ml with saline. From this, a platelet count was obtained using a Coulter Counter Model F, and two 9-ml aliquots were then spun at 1920 g and 22°C for 20 min in polypropylene tubes (Falcon, Oxnard, Calif.). The supernatants were decanted, the tubes were inverted for 2 min, and the residual plasma was removed with a cotton swab. The resultant platelet buttons were resuspended in 0.9 ml of 0.14 m M tris-buffered saline, pH 7.4, containing 3 mM EDTA and 5 mM glucose (TSGE). MDA production was determined by the following modification of the method described by Smith et al.\(^{12}\) The washed platelets were warmed to 37°C for 5 min as were solutions of N-ethylmaleimide (Eastman) 50 nM (NEM) and thrombin (Parke Davis) 500 U/ml. The platelets were stimulated by addition of 0.1 ml of the NEM solution to one tube and thrombin to the duplicate. Incubation at 37°C was continued for 15 min and the reaction was stopped by addition of an equal volume of cold 20% trichloroacetic acid in 0.6 N HCl. In some experiments, a 0.1 ml aliquot of the reaction mixture was removed for assay of TXB\(_2\) before addition of the acid. This mixture was then centrifuged at 3400 g for 15 min at 4°C. One milliliter of the resultant supernatant was added to 0.2 ml of 0.12 M 2-thiobarbituric acid and the pink color was developed by incubation at 70°C for 30 min. Optical absorption was measured at 532 nM in a split beam spectrophotometer against a blank of TSGE prepared exactly as in the platelet suspensions. The concentration of MDA was calculated using a molar extinction coefficient of 1.56 x 10\(^5\). Results were calculated in nmole/10\(^6\) platelets and expressed as percent inhibition of preaspirin values.

In one series of experiments 1 of the 2 platelet buttons was resuspended in autologous platelet-poor plasma (PPP) obtained prior to aspirin ingestion. The other button was resuspended in TSGE containing 4 g% bovine serum albumin (fraction V Sigma) which had been adjusted to pH 7.4 (TSGE-albumin). These samples were then stimulated with NEM, and the remainder of the procedure carried out as above. Samples were read in the spectrophotometer against the appropriate blank prepared with PPP or TSGE-albumin.

**TXB\(_2\) Measurement**

TXB\(_2\) was measured on unextracted specimens by radioimmunoassay as previously described.\(^{13}\) The detection limit of the assay is 0.5 pmole TXB\(_2\)/ml.

Serum specimens were prepared by thrombin stimulation of anticoagulated whole blood. Blood (4.5 ml) was collected into 0.5 ml of 3.8% Na citrate. Samples were equilibrated at 37°C for 5 min as was a stock solution of thrombin, 500 U/ml. The thrombin solution (0.1 ml) was then added, the tubes were gently mixed by inversion, and the blood was then allowed to clot for 30 min at 37°C. Serum was removed and frozen for assay at the end of the study.

TXB\(_2\) was also measured from platelets that had been resuspended in a variety of media. When 0.1 ml samples were removed from specimens prepared and stimulated for MDA assay, they were diluted 1:10 by addition to 0.9 ml of TSGE, vortexed, and centrifuged at 12,000 g for 2 min to remove platelet debris. The supernatant was then frozen for TXB\(_2\) assay at the end of the experiment.

In some experiments, platelet buttons prepared as for MDA assay were resuspended in autologous PPP obtained prior to aspirin ingestion. In other experiments, the platelet buttons were resuspended in TSGE-albumin. In all instances duplicate samples were stimulated in parallel, 1 with NEM and 1 with thrombin. In these experiments, at the end of the 37°C incubation, the entire specimen was centrifuged and the supernatant frozen for TXB\(_2\) assay.

TXB\(_2\) was also measured in platelet-rich plasma that was stimulated directly with NEM or thrombin. One milliliter aliquots were brought to 37°C as in all other experiments and then stimulated by incubation with NEM (10 mM, final concentration) or thrombin (10 U/ml final concentration) for 30 min at 37°C. Again the entire specimen was centrifuged and the supernatant assayed for TXB\(_2\).

Results were expressed as percent inhibition of pre-aspirin levels.

**Cr\(^{51}\) Platelet Survivals**

Cr\(^{51}\) platelet survivals were performed as previously described.\(^{14}\) Results were expressed at t ½ where t ½ is that time at which circulating platelets contain 50% of the radioactivity present at the start of the study.

**RESULTS**

The return of MDA and TXB\(_2\) production after aspirin ingestion in 7 normal volunteers is illustrated in Fig 1. Samples for determination of MDA and TXB\(_2\) were obtained simultaneously. In MDA studies,
ASPIRIN INHIBITION

Fig. 1. Return of MDA and TXB₂ production after a single dose of aspirin in normal volunteers. Results are expressed as percent inhibition of pre-aspirin values. MDA recoveries were determined from platelets resuspended in protein free buffer and stimulated with NEM. Fifty percent recovery ranged from 3.1 to 4.0 days, with 9.6% recovery in the first 24 hr. TXB₂ recoveries were measured from thrombin stimulated whole blood. Fifty percent recovery ranged from 5.8 to 7.9 days, and there was less than 1% recovery in the first 24 hr.

the platelets were suspended in TSGE buffer and stimulated with NEM. The mean pre-aspirin level was 6.4 nmole MDA/10⁹ platelets ± 0.62 (SEM). Results are expressed as percent inhibition of pre-aspirin level. The time required for the return of 50% of the MDA activity ranged from 3.1 to 4.0 days, with a mean of 3.5 days. The normal range in our laboratory for platelet survival after conventional ⁵¹Cr labeling is 3.2–5.4 days. We found no alteration in this pattern whether the volunteer took aspirin on one occasion or daily for up to 1 wk prior to measuring return of activity. These results agree with those obtained by Stuart et al.¹ in normals.

The return of TXB₂ production was measured from whole blood samples stimulated with thrombin (serum TXB₂). The mean pre-aspirin value was 438 pmole TXB₂/ml of serum ± 135 (SEM) and again the results are expressed as percent inhibition. In these experiments, a delay in return of activity during the first 48 hr was consistently seen. In addition to this initial lag, statistically significant differences in the percent inhibition between the two methods persisted for 7 days. We found no difference in the degree of inhibition or in recovery pattern of serum TXB₂ when as little as 80 mg of aspirin was administered.

MDA and serum TXB₂ recoveries were also studied in a number of patients referred for diagnostic ⁵¹Cr platelet survival. All of the aspirin studies were performed simultaneously with the ⁵¹Cr studies. The results from all patients in whom the ⁵¹Cr survival was short are illustrated in Figs. 2 and 3. In the patient illustrated in figure 2A, the t½'s determined by MDA and ⁵¹Cr are in close agreement at 2.9 and 2.8 days, respectively. In patients with shorter ⁵¹Cr survivals, however, this was not the case. In the 3 patients in figure 2B, t½'s determined with ⁵¹Cr were 0.8–1.2 days. The t½'s determined simultaneously using MDA, however, were 2.3–2.9 days. Although these are shorter than our normal range for the MDA method, they are more than twice as long as the simultaneous ⁵¹Cr t½'s. In the patients in figure 2C.

Fig. 2. Simultaneous ⁵¹Cr and MDA platelet survivals in patients. MDA was measured from platelets resuspended in protein free buffer and stimulated with NEM. Normal range for t½ using ⁵¹Cr in our laboratory is 3.2–5.4 days. Using MDA the normal range is 3.1–4 days. (A) A patient with t½ by ⁵¹Cr 2.8 days, had t½ determined using MDA, 2.9 days, and the survival curves were essentially coincident. (B) Patients with t½'s determined by ⁵¹Cr 0.8, 1.1, and 1.2 days. t½'s determined by MDA were 2.3, 2.5, and 2.9 days, respectively. These MDA survivals were shorter than our normal range but significantly longer than the ⁵¹Cr survivals. (C) Patients with t½'s by ⁵¹Cr 1.4, 1.8, and 2.2 days. MDA t½'s were 3.1, 3.8, and 4 days, respectively. Although the ⁵¹Cr survivals were shorter than normal, the MDA survivals were within normal limits.
the $t_{1/2}$'s determined by Cr$^{51}$ were shorter than normal at 1.5–2.2 days. Those determined using MDA were 3.1–4 days, which are within the normal limits for MDA.

Serum TXB$_2$ recovery in the patients with Cr$^{51}$ $t_{1/2}$ less than 2.5 days showed a pattern similar to that in normals, with a 48-hr delay in recovery in most patients, but the return to baseline was in all but one instance faster than for normal individuals (Fig. 3). In these patients, platelet radioactivity at 24 hr was 51.4 ± 11.1 (SEM)% of platelet radioactivity immediately after infusion. This emphasizes the marked discrepancy in results using the two techniques.

A series of experiments were carried out in normals in an attempt to elucidate the cause of the differences in recovery patterns between the two methods (Fig. 1). We first examined the possibility that cellular or other whole blood elements might interfere with our ability to detect the TXB$_2$ produced by a small number of normal, uninhibited platelets. Both arachidonic acid and TXA$_2$ have been reported to bind to albumin.$^{15,16}$ Therefore, it was possible that blood elements inhibited the production of TXB$_2$ by small numbers of normal platelets. Varying percentages of aspirin-free whole blood were mixed with blood from an ABO-compatible donor who had ingested 600 mg of ASA 4 hr before donation. These mixtures were stimulated by the addition of thrombin and the sera were assayed for TXB$_2$ as in Fig. 1. The results are illustrated in Fig. 4. They demonstrated that the amount of TXB$_2$ produced and measured was proportional to the percent aspirin-free blood (and consequently platelets) in the mixture. Therefore, if the 10% recovery of MDA production 24 hr after aspirin ingestion shown in Fig. 1 is due to the presence of 10% aspirin-free platelets, we should have been able to detect 10% of the baseline TXB$_2$ activity in our whole blood system.

To explain this discrepancy, it was necessary to examine the individual differences in technique between the two systems. These included: the metabolite measured, the stimulus to platelet arachidonic acid metabolism, the medium in which the platelets were suspended, and the effect of centrifugation.

To document that both metabolites were measuring the same sequence of events, it was necessary to measure both when platelets were collected and stimulated under identical conditions. The relative insensitivity of the MDA assay already mentioned precluded following MDA recovery in thrombin stimulated whole blood because 10% or even 20% of baseline values would be below the limits of detection of the assay. Consequently, production of both MDA and TXB$_2$ was measured in the supernatant of washed, NEM-stimulated platelets. A representative illustration of results is shown in Fig. 5. The patterns of recovery of MDA and TXB$_2$ production were essentially identical. To substantiate this finding, TXB$_2$ and MDA were measured in washed, NEM-stimulated platelets obtained from 7 normal volunteers at 24 and 48 hr after aspirin. (Table 1). No statistically significant difference was found between the percent inhibition of MDA and TXB$_2$ at each of these intervals.

The possibility that the discrepancy could be due to the two different stimuli used was tested by stimulating washed platelets with thrombin and measuring both MDA and TXB$_2$ in the supernatant (Table 1).
Although the recovery towards normal was slightly more rapid when NEM was the stimulus used, there was again no statistical difference between MDA and TXB₂. Simultaneous whole blood samples stimulated with thrombin showed significantly less return of TXB₂ activity at 24 and 48 hr than washed platelet samples stimulated with either thrombin or NEM (p < 0.005).

The effects of centrifugation and of the composition of the suspending medium were studied in four normal volunteers. Platelet-rich plasma, washed platelets resuspended in platelet-poor plasma obtained prior to aspirin ingestion (PPP), and washed platelets resuspended in buffer containing 4% albumin (TSGE-albumin) were all stimulated both with NEM and thrombin before, 2 hr after, and then at 24, 48, and 72 hr after aspirin. The results when TXB₂ was measured are illustrated in Fig. 6. Essentially, identical results were obtained when NEM-stimulated MDA return was measured from platelets that had been resuspended in PPP and TSGE-albumin. MDA production in platelet-rich plasma was not studied because of the limitation of the sensitivity of the assay. In all instances, the 48-hr lag was seen, regardless of stimu-

**Table 1.**

<table>
<thead>
<tr>
<th>Hours After ASA</th>
<th>Stimulus</th>
<th>Protein-Free Buffer</th>
<th>Whole Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>MDA*</td>
<td>TXB₂*</td>
</tr>
<tr>
<td>24</td>
<td>NEM</td>
<td>9.6 ± 1</td>
<td>9.7 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>Thrombin</td>
<td>5.5 ± .9</td>
<td>3.9 ± .7</td>
</tr>
<tr>
<td>48</td>
<td>NEM</td>
<td>24 ± 1.3</td>
<td>29.4 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>Thrombin</td>
<td>17.9 ± 1.6</td>
<td>16.7 ± 2.6</td>
</tr>
</tbody>
</table>

*Percent return of activity ± SEM.

Fig. 5. Return of TXB₂ production by platelets resuspended in protein-free buffer and stimulated with NEM. The stippled area represents the normal range of MDA production by such platelets. The results illustrate the similarity of recovery patterns for the two metabolites under these conditions.

Fig. 6. The effect of suspending medium on the return of TXB₂ production after aspirin ingestion. The normal NEM stimulated MDA range from Fig. 1 is indicated by the stippled area. Platelets in (A) platelet-rich plasma or suspended in (B) platelet-poor plasma or (C) albumin-containing buffer were stimulated with NEM (Δ) or thrombin (■). Each point is the mean of four experiments. A significant delay in return of activity is seen when platelets are stimulated in the presence of plasma or albumin. There is no significant difference seen between NEM and thrombin stimulation, nor is there a significant difference between platelets which have been centrifuged and resuspended in plasma and those left in their original platelet-rich plasma.

**DISCUSSION**

In an attempt to develop a simple, sensitive, nonisotopic method for the determination of platelet survival, we measured the return of TXB₂ production in whole blood after a single dose of aspirin. When we compared these results to those obtained using the previously described method of measuring the return of MDA produced by washed platelets, we observed striking discrepancies in the recovery patterns between those two methods. It was therefore apparent that further investigation was needed to better understand the significance of these differences. When MDA was measured after NEM stimulation of washed platelets, return of activity was approximately linear, as one would expect if production of newly made, presumably aspirin-free platelets exactly equaled destruction of aspirin-labeled (inhibited) platelets. However, the return of TXB₂ activity from thrombin-stimulated whole blood was significantly delayed, with little return during the first 48 hr. This is a pattern of recovery that other investigators have observed when measuring other prostaglandin metabolites or acety-
latable cyclooxygenase, and it has been attributed to inhibition of megakaryocyte cyclooxygenase by aspirin. Because aspirin inhibits production of both MDA and TXB₂ by acetylation of the enzyme cyclooxygenase, we had anticipated that the return of activity after aspirin, with or without a megakaryocyte effect, would be the same for both metabolites. The many technical differences between the two methods, and reports that the presence of albumin can bind arachidonic acid or TXA₂, preventing its conversion to TXB₂ prompted us to design a series of experiments to evaluate the effect of each of the variables that differed in the two methods.

In experiments in which whole blood mixtures were stimulated by clotting with thrombin, we could detect appropriate amounts of TXB₂ produced by even small numbers of aspirin-free platelets. Consequently, it seemed unlikely that the delay in recovery of TXB₂ in our whole blood system after aspirin was due to artifactual inhibition of production due to other blood elements. Rather, this evidence supported the theory that the delay in recovery we saw in the whole blood method was due to inhibition of cyclooxygenase at the megakaryocyte level. If megakaryocyte cyclooxygenase were inhibited, the production of MDA should also be inhibited at 24 and 48 hr, unless it was coming from some other source which recovered more quickly than cyclooxygenase activity. To evaluate this possibility, we examined the return of TXB₂ in the identical system to that used to measure MDA. TXB₂ is more specific for platelet prostaglandins metabolism than MDA (that can result from nonspecific fatty acid degradation) and we would anticipate that we would see the same pattern of TXB₂ recovery in washed platelets as in whole blood. However, this was not the case, and we found that the recovery of TXB₂ in the washed platelet system was coincident with that of MDA. The different stimuli, NEM and thrombin were also evaluated and, although there were slight differences (Table 1), they were not great enough to explain the discrepancy observed.

Upon examining the suspending medium, however, we found that the readdition of platelet-poor plasma or the addition of 4% bovine serum albumin to washed platelets obtained 24 to 48 hr after aspirin decreased the ability of these platelets to produced TXB₂ and MDA, thus reinducing the lag seen in whole blood. In these systems there was no difference between thrombin or NEM stimulation when both were measured. At this time, we do not know the mechanism by which albumin blunts the production of TXB₂ and MDA by platelets newly-released after aspirin ingestion. However, these results led us to conclude that there is a difference between platelets that are newly released from the marrow after a single dose of aspirin and those platelets (such as the aspirin-free ones in our mixing experiments. Fig. 5), which have not been exposed to aspirin during their development in the marrow. The fact that the expected amount of MDA or TXB₂ production can be measured when the platelets produced after aspirin ingestion are washed free of protein demonstrates that this difference can be masked in some settings. Thus, the results in normals suggest an in vivo effect of aspirin that is quite complex.

A megakaryocyte effect could explain our results in patients with short platelet survivals as determined by Cr³¹ labeling. In the original report of Stuart et al., two patients with short platelet survivals by Cr³¹ labeling were found to have equally short survival by the MDA method. In the current, larger group of patients, with Cr³¹ t½ less than 2.5 days, there are major differences between the isotopic measurements and either the washed platelet-MDA method (Fig. 2) or the whole blood-TXB₂ method (Fig. 3). Although accelerated rate of turnover is in general suggested by the aspirin methods relative to the normal range, the extent of change is far less than with Cr³¹ labeling. In fact, in three patients, the MDA survivals were within normal limits while simultaneous Cr³¹ survivals were short. The discrepancy between MDA and Cr³¹ methods was greatest in those patients with the shortest Cr³¹ survivals. This delay in recovery in states of increased platelet turnover suggests to us an effect at the megakaryocyte level.

Our data document that recovery from the aspirin effect is complex. The patterns obtained when aspirin is used to determine platelet survival vary markedly with the environment in which the platelets are stimulated. At the present time, platelet survivals after aspirin ingestion must be interpreted with great caution.

REFERENCES

5. Roncucci R, Deperon R, Destaillieur J, Doumont J, Lambelin


Platelet recovery from aspirin inhibition in vivo; differing patterns under various assay conditions

PM Catalano, JB Smith and S Murphy