Preliminary Communications

Inhibition by Aspirin of Release of Antiheparin Activity from Human Platelets

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Summary: Both in vitro and in vivo, aspirin inhibited the adenosine diphosphate and collagen-induced release of platelet factor 4 (antiheparin factor). The release induced by adrenaline and thrombin was not affected. The in-vivo effect in normal persons lasted for at least three days. Platelet uptake of acetyl-^{14}C-aspirin was significantly greater than that of carboxyl-^{14}C-aspirin.

Introduction

Aspirin (acetylsalicylic acid) can affect platelet function in several ways. It can inhibit the aggregation of platelets by collagen (Evans, Nishizawa, Packham, and Mustard, 1967; Weiss and Aledort, 1967) and can prevent the secondary aggregation, serotonin and adenosine diphosphate (A.D.P.) release, and platelet factor 3 activation which normally follow the addition of collagen or small concentrations of A.D.P. (Weiss, Aledort, and Kochwa, 1968; Zucker and Peterson, 1968). The secondary aggregation of platelets by critical amounts of adrenaline is also abolished (O'Brien, 1968a; Weiss, 1968).

We have also noted another effect of aspirin on platelets. In the course of studies on the release of the heparin-neutralizing principle or platelet factor 4 (P.F.4) from human platelets by collagen and A.D.P. (Youssef and Barkhan, 1968) we observed that aspirin would inhibit this release (Barkhan and Youssef, 1968), and in the present paper we provide the experimental evidence for this action of aspirin, both in vitro and in vivo; we also present the results of work carried out with radioactive aspirin which aimed at finding out how the effect of aspirin on platelet function is mediated.

Materials and Methods

Tests for release of P.F.4 in response to A.D.P., collagen, thrombin, and adrenaline were carried out as described previously (Youssef and Barkhan, 1968). For the in-vitro test 0·1 ml. of aspirin solution was incubated with the platelet-rich plasma for 10 minutes at 37° C. *before* adding the aggregating agents. The basis of the test is the determination of the thrombin time of platelet-rich plasma in the presence of a critical amount of heparin; the release of P.F.4 leads to neutralization of heparin and a shortening of the thrombin time, which is inversely proportional to the amount of P.F.4 liberated.

For the in-vivo test normal adult volunteers were given a test dose of three tablets (900 mg.) of acetylsalicylic acid, *B.P.* (British Drug Houses Ltd.). Blood samples were collected into 1/10 volume of 3·8% sodium citrate immediately before and at intervals after aspirin ingestion: in five subjects samples were collected at three and five hours, and in another five subjects at 2, 24, 48, 72, and 96 hours after taking aspirin. None of the volunteers had been taking aspirin or any other drug for at least 10 days before the test.

Aspirin solutions for in-vitro testing were prepared as follows: acetylsalicylic acid powder (B.D.H.) was suspended in phosphate-buffered normal saline (*p*H 7) to give an initial concentration of 1 ng./ml. The suspension was shaken vigorously for about 15 minutes and then centrifuged at about 4,000 *g* for an hour. Doubling dilutions in buffered saline were made from the upper two-thirds of the centrifuged fluid and were used for testing.

Sodium salicylate and salicylic acid (B.D.H.) were prepared for testing as described for aspirin.

^{14}C-aspirin.—Two preparations were used. *Carboxyl-^{14}C*-acetylsalicylic acid (Radiochemical Centre, Amersham), specific activity 3·26 mCi/mM and radiochemical purity 98%. *Acetyl-1-^{14}C*-salicylic acid (New England Nuclear Corporation, Boston, Massachusetts), specific activity 1·62 mCi/mM and radiochemical purity greater than 99%.

The ^{14}C-aspirin compounds were suspended in buffered saline as described for unlabelled aspirin. For the experiments a concentrated platelet suspension was prepared by resuspending the platelet button obtained from 10 ml. of citrated platelet-rich plasma in 0·3 ml. of platelet-poor plasma. Then 0·1 ml. of the labelled aspirin compound was added to the concentrated platelet suspension, which was incubated for 20 minutes at 37° C., with gentle mixing at intervals. After incubation the mixture was made up to 5 ml. with platelet-poor plasma containing unlabelled aspirin, and 0·1 ml. of this mixture was taken for counting of radioactivity; the remainder was centrifuged at about 2,600 *g* for 30 minutes to deposit the platelet button, which was subsequently washed twice in buffered saline and finally resuspended in 5 ml. of "cold" platelet-poor plasma. Of this final suspension 0·1 ml. was taken for counting of radioactivity. For counting, the platelet samples were mixed with 9·9 ml. of Buhler's (1962) solution and the radioactivity was measured in a liquid scintillation counter (Nuclear Chicago) with a counting efficiency of 78% (background count 45 c.p.m.).

Results

In-vitro Effect of Aspirin

The test was carried out on blood obtained from 20 normal individuals, and in all cases aspirin inhibited almost completely the A.D.P. and collagen induced release of P.F.4—that is, the shortening of the thrombin time which normally takes place when heparinized platelet-rich plasma is mixed with A.D.P. or collagen failed to occur if aspirin was first added to the plasma.

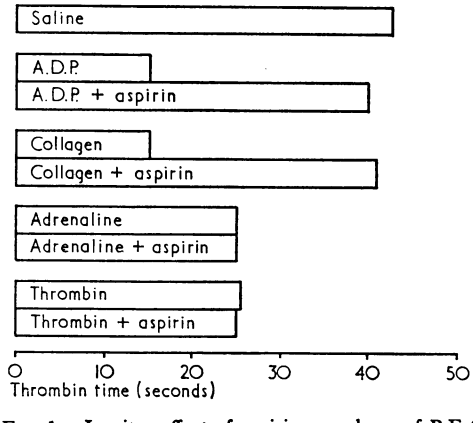

FIG. 1.—In-vitro effect of aspirin on release of P.F.4 in the presence of A.D.P., collagen, adrenaline, and thrombin as measured by changes in thrombin time.

In contrast the release of P.F.4 after platelet aggregation by thrombin or adrenaline was not affected by aspirin. A typical set of results is shown in Fig. 1. The effect was consistently

reproducible to within one to three seconds on the same blood sample and also on samples taken at different times from the same individual. Aspirin was maximally effective down to a final concentration in the test system of 3 to 5 μg./ml.; lower concentrations were progressively less effective, but detectable inhibition was still present at concentrations of 0·05 μg./ml.

There are two possible explanations for the inhibitory effect of aspirin: direct neutralization of P.F.4 or inhibition of release from the platelet. Aspirin in the concentration used was not a direct antagonist of P.F.4 in that there was no inhibition when it was added after the platelets had been aggregated by A.D.P. or collagen, and, furthermore, it did not affect the P.F.4 activity released from platelets by freezing and thawing. It therefore seems reasonable to ascribe the aspirin effect, so far as A.D.P. and collagen are concerned, to interference with the release of P.F.4 from the platelets.

Sodium salicylate and salicylic acid were inactive at concentrations comparable to those at which aspirin was found to be effective.

In-vivo Effect of Aspirin

Inhibition of release of P.F.4 by A.D.P. and collagen was evident between two and three hours after aspirin ingestion. The effect was maximal at three hours and persisted for at least 72 hours; the inhibitory effect had disappeared completely by 96 hours. All 10 subjects showed a complete response, and a typical result is shown in Fig. 2. Release of P.F.4 by adrenaline and thrombin was not affected.

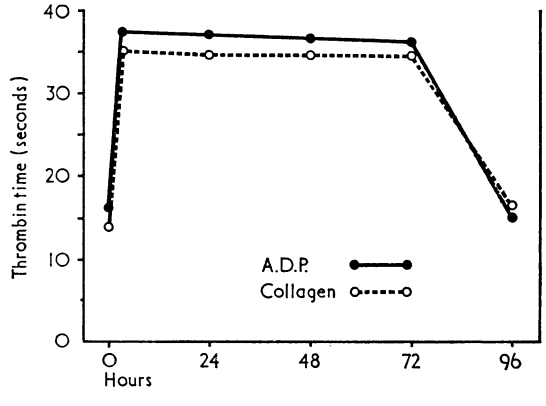

FIG. 2.—Effect of aspirin after ingestion on release of P.F.4 in response to A.D.P. and collagen as measured by changes in thrombin time.

Platelet-poor plasma prepared from blood taken four hours after aspirin ingestion in three subjects was tested for its effect on release of P.F.4 from normal platelet-rich plasma in the presence of A.D.P. and collagen. The plasma of one subject blocked the release completely (Table I), but no effect was observed with the plasma of the other two subjects.

TABLE I.—*Inhibitory Effect on Release of P.F.4 of Plasma Taken from a Subject Four Hours After Aspirin Ingestion*

Aggregating Agent	Thrombin Time (seconds)	
	With Saline	With Plasma
A.D.P.	21	36
Collagen	17	30
Adrenaline	23	21
Thrombin	19	18
Saline control	40	35

Uptake by Platelets

Six tests of the uptake of acetyl-^{14}C- and carboxyl-^{14}C-aspirin by platelets were carried out on blood samples obtained from five normal subjects. The platelet buttons were counted for 40 minutes. The total counts above background recorded for the platelets incubated with acetyl-^{14}C-aspirin were between 13,000 and 37,000; for the platelets incubated with carboxyl-^{14}C-aspirin the corresponding counts were between 1,800 and 2,500. In all cases the percentage uptake of the acetyl-^{14}C-aspirin was greater than that of the carboxyl-^{14}C-aspirin (Table II). Statistical analysis of the results showed that the observed values in each of the aspirin groups lay within acceptable confidence limits (1·73 S.D. from the mean). The t test with correction for departure from unity of the ratio of the variances of the means (Aspin–Welch correction) gave $t=6{\cdot}0858$; corrected number of degrees of freedom $=5{\cdot}23$. In these conditions $P=<0{\cdot}005$, and therefore the difference between the means is highly significant.

TABLE II.—*Uptake by Platelets of ^{14}C-aspirin Labelled in the Acetyl and the Carboxyl Groups*

Test No.	% Uptake of ^{14}C-Aspirin by Platelets	
	Acetyl-^{14}C-Aspirin	Carboxy-^{14}C-Aspirin
1	3·5	0·04
2	5·1	0·2
3	1·95	0·49
4	2·23	0·38
5	4·04	0·58
6	4·74	0·38
Mean	3·59	0·34

The method which we have used to investigate the uptake of ^{14}C-aspirin by platelets was evolved from the study of several different experimental conditions and gave the more conclusive results.

Discussion

These studies provide evidence for another effect of aspirin on platelet function—namely, the suppression of release of the platelet antiheparin principle (platelet factor 4) after aggregation of platelets by A.D.P. or collagen. The effect is found both in vitro and in vivo. Aspirin prevents the release of intrinsic platelet A.D.P. by collagen and added A.D.P. (Weiss *et al.*, 1968), and it is possible that the inhibition of release of P.F.4 by aspirin is related to the inhibition of release of platelet A.D.P. We have found that the release of P.F.4 by added A.D.P. and collagen is detectable within 30 seconds of adding these agents, and this would seem to occur earlier than the release of intrinsic platelet A.D.P. The precise relation between A.D.P. release and P.F.4 release requires clarification. Our observation that aspirin did not affect the adrenaline- or thrombin-induced release of P.F.4 suggests that the mechanism of release by these agents is different from that of A.D.P. or collagen. Presumably aspirin affects platelet membrane permeability, but the exact site of action remains to be established.

The onset and duration of the in-vivo action of aspirin on the release of P.F.4 is similar to that observed for the inhibition of the secondary phase of platelet aggregation by adrenaline (O'Brien, 1968a) or by critical concentrations of A.D.P. (Weiss *et al.*, 1968). It has been suggested by O'Brien (1968a) and Weiss *et al.* (1968) that aspirin after absorption into the blood permanently damages the platelets in the circulation, since the effect persists long after aspirin has disappeared from the plasma. Under our experimental conditions the release of P.F.4 by collagen and by A.D.P. had returned to normal values by the fourth day after aspirin ingestion, and this implies that the new population of platelets which had entered the blood in the intervening period had accumulated in sufficient number by the fourth day to normalize the test. It is not easy, however, to explain the plateau effect of aspirin in our in-vivo tests and the relatively rapid disappearance of the inhibition of release of P.F.4 after three days in terms of the foregoing hypothesis. It seems equally possible that the effect of aspirin

on the platelet is reversible and that the active group of aspirin is metabolized or removed from the platelet in three or four days with a return of normal platelet function. Whatever the explanation of the peculiar pattern of the in-vivo response which we have found, our observations would suggest that a dose of aspirin taken every three days would result in a constant inhibition of release of P.F.4.

Our experiments with ^{14}C-aspirin show that platelets can take up some aspirin but that the uptake of the acetyl-^{14}C compound was significantly greater than that of the carboxyl-^{14}C compound. The percentage uptake of the acetyl-^{14}C-aspirin was, however, relatively small despite the use of a concentrated platelet suspension. It is likely that much of the added aspirin was taken up by the proteins of the plasma in which the platelets were suspended. The problem obviously requires further study, and we need to know much more about the conditions and the kinetics of aspirin-binding by platelets in plasma and in other suspension media. Our findings would suggest that when aspirin becomes attached to the platelet it does so via the acetyl radical, and, to explain the failure to detect binding of the carboxyl-^{14}C compound, it must be assumed that the carboxyl radical is split off when aspirin binds to the platelet. These observations might explain why sodium salicylate does not affect platelet function—in the absence of the acetyl radical the salicylate cannot bind to the platelet. Pinckard, Hawkins, and Farr (1968) reported that aspirin acetylates a number of plasma proteins, and it is therefore possible that acetylation by aspirin of certain protein components in the platelet, perhaps in the membrane, is responsible for its observed effects on platelet function.

We have tested a number of other anti-inflammatory agents, including phenylbutazone, prednisolone, indomethacin, and paracetamol, as well as some local anaesthetics (procaine and lignocaine) and the antihistaminic compound chlorpheniramine (Piriton). All these agents were able to block the in-vitro release of P.F.4 induced by A.D.P. or collagen, but they were less active than aspirin in terms of dose-response relationship (to be published). Other anti-inflammatory agents will also abolish the secondary wave of adrenaline-induced platelet aggregation (O'Brien, 1968b). Mills and Roberts (1967) reported that imipramine compounds, such as chlorpromazine, will inhibit collagen-induced platelet aggregation, will abolish the secondary wave of A.D.P.-induced platelet aggregation, and will reduce the aggregation, especially the second phase, induced by 5-hydroxytryptamine (serotonin). An effect of non-steroidal anti-inflammatory drugs has been shown on other cells: human erythrocytes were protected against hypotonic haemolysis by low concentrations of these drugs, and this was believed to involve stabilization of the cell membrane (Inglot and Wolna, 1968).

Aspirin will prolong the bleeding-time in man (Quick, 1966), and when administered to rabbits in doses sufficient to block collagen-induced platelet aggregation will impair haemostasis, decrease platelet deposition in extracorporeal shunts, and prolong platelet survival (Evans, Packham, Nishizawa, and Mustard, 1968). It is therefore conceivable that the platelet changes induced by aspirin may be of benefit in the management of occlusive vascular disease, and O'Brien (1968a) commented on its possible therapeutic value in thrombosis.

The physiological role of P.F.4 remains to be clarified. Apart from its antiheparin activity, P.F.4 obtained from pig platelets neutralizes the anticoagulant activities of fibrinogen breakdown products (Niewiarowski, Farbiszewski, and Poplawski, 1965). It has also been found to cause paracoagulation (that is, coagulation of soluble fibrin monomer complexes): this effect was correlated with the release of P.F.4 during platelet aggregation (Niewiarowski, Lipinski, Farbiszewski, and Poplawski (1968). Enhancement of platelet aggregation in vitro by P.F.4 has been described (Niewiarowski, Poplawski, Lipinski, and Farbiszewski, 1968), and the intravenous injection of P.F.4 concentrates in rabbits reduced the platelet count and the fibrinogen concentration (Farbiszewski, Lipinski, Niewiarowski, and Poplawski, 1968). It has been suggested that the release of P.F.4 may alter the surface properties of platelet membranes and that it may induce coagulation of soluble fibrin monomer complexes in the plasmatic atmosphere of the platelet—the formation of fibrin threads between platelets may then promote their mutual attachment (Niewiarowski, Lipinski, *et al.*, 1968). It is therefore possible that the physiological and perhaps pathological effects of P.F.4 are related to properties other than that of heparin neutralization, but it remains to be shown whether these mechanisms hold for human blood under physiological conditions.

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