Effect of aggregating agents and their inhibitors on the mean platelet shape

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SYNOPSIS The 'swirling' seen when platelet-rich plasma is stirred is caused by the average asymmetry of the platelets and a technique for recording the swirling is reported. After the addition of adenosine diphosphate, 5-hydroxytryptamine, thrombin, and collagen, platelets become rounded and more symmetrical immediately before they become sticky. Monoiodo-acetate and adenosine prevent both the change in shape and sticking whereas E.D.T.A. and p-tosyl arginine methylester prevent sticking but the shape still changes. Adrenaline produces sticking but no change in shape. The effects of temperature and E.D.T.A. are also reported. All these findings are discussed and diagrammatic representations of some reactions are tentatively proposed.

Under the light microscope a wet preparation of platelets usually shows them to be predominantly disc-shaped but with some pleomorphism; electron microscope pictures of platelets fixed *in vivo* suggest that they are normally flattened discs with a smooth outline. It is reported (Bull and Zucker, 1965) that platelets become more spherical and increase in volume when adenosine diphosphate sodium salt (A.D.P.) is added but that no change occurs when adrenaline is added. A technique to be described permits a second-by-second evaluation of the mean platelet shape. The change in shape in a number of situations will be reported and discussed. Preliminary findings have already been reported (O'Brien, 1965, 1966).

REAGENTS

Adenosine diphosphate sodium salt (A.D.P.), adenosine, and p-tosyl arginine methylester (T.A.M.E.) were obtained from Sigma Chemicals, 5-hydroxytryptamine creatinine sulphate (5-HT) from Roche Products Ltd., and adrenaline acid tartrate from British Drug Houses; human thrombin from the Lister Institute, and sodium monoiodo-acetate (M.I.A.) from L. Light and Co. Ltd. These were made up in barbitone saline, buffered to pH 7.35. The adrenaline solution was made up daily from the powder. Human tendon was ground up with sand and saline, and the liquid decanted and centrifuged slowly. This saline extract will be referred to as collagen.

METHOD

The method is based on the well-known finding that

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asymmetrical particles (*e.g.*, bacilli) shaken in a test tube can be seen to 'swirl' because they become orientated across a velocity gradient. Symmetrical particles (*e.g.*, cocci) cannot become orientated and on shaking no swirl is seen.

Two ml. citrated human platelet-rich plasma is placed in a small cuvette which is partially immersed in a Perspex water bath on the stage of a modified E.E. titrometer. The plasma is stirred at constant speed by a special constant-speed motor with a plastic-coated magnet in the cuvette. The temperature of the water bath is kept constant by circulating water through it from a large reservoir at the appropriate temperature, usually 37°C, unless otherwise stated. On stirring the platelet-rich plasma, swirling is seen and the light transmitted to the photocell fluctuates rapidly. The signal from the photocell is made to activate a ten millivolt pen recorder with a rapid response time and chart speed 2 in. per minute. This rapid fluctuation will be called the amplitude of the tracing. When platelets aggregate, more light reaches the photocell and the mean level of the tracing goes down. However, when sizable aggregates form, these interfere with the light path and cause rapid fluctuations. In the early stages of aggregation this does not occur and any change in the amplitude of the tracings at this time reflects a change in the degree of average symmetry or asymmetry of the platelets.

RESULTS

BENADRYL If citrated or heparinized platelet-rich plasma is stirred and 0.1 ml. of cocaine 3 mg./ml. (Fig. 11) or benadryl 10 mg./ml. is added, the amplitude of the tracing decreases markedly in the next minute and microscopic inspection confirms that initially the platelets were predominantly disc-

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FIGS. 1-15. Citrated platelet-rich plasma, 2.0 ml., stirred at 37°C. Additions of 0.1 ml. containing the aggregating agent were added where indicated by the arrow or where there is a sudden jump in the tracing level. Figs. 1-10, 0.1 of saline or adenosine $M \times 10^{-3}$ or M.I.A. $M \times 10^{-3}$, or E.D.T.A. 5%, or T.A.M.E. $5M \times 10^{-1}$ were added as indicated 3 min. before the tracing was begun. The tracings of Figs. 1-5 record the effect of then adding ADP $5M \times 10^{-5}$. In Figs. 6-10 a 'collagen' solution was added.

FIG. 11. 0.1 ml. of 0.3% cocaine was added at the arrow.

FIG. 12. 0.1 ml. of 5-H.T. $M \times 10^{-4}$ was added.

FIG. 13. 0.1 ml. of thrombin 1.25 units/ml.

FIG. 14. 0.1 ml. of adrenaline $M \times 10^{-5}$.

FIG. 15. 0.1 ml. of T.A.M.E. added 3 min. before the tracing began: adrenaline 0.1 ml. of $M \times 10^{-5}$ then added.

FIG. 16. One sample of plasma warmed up from 37°C. and stirred when the temperature had equilibrated. Another sample of plasma cooled with sequential tracings at the temperatures indicated.

shaped with some pleomorphism and asymmetrical: after the addition they are spherical and therefore symmetrical.

ADENOSINE DIPHOSPHATE If 0.1 ml. of A.D.P. $M \times 10^{-5}$ is added to 2 ml. of platelet-rich plasma, the addition causes an immediate slight increase in transmission by simple dilution which is reflected in a displacement downwards of the mean level of the tracing (Fig. 1). The amplitude then decreases rapidly and at the precise moment it reaches its minimum the mean level of the tracing starts going down which indicates that aggregation is beginning. Thus, the change in shape from asymmetrical to a more symmetrical form occurs in the two or three seconds before the platelets become sticky enough to begin sticking together. If 0.1 ml. adenosine or adenosine monophosphate (A.M.P.) $M \times 10^{-2}$ is added to platelet-rich plasma it has no effect on the amplitude; if A.D.P. is then added, neither the change in amplitude nor aggregation occur (Fig. 2). If weaker A.M.P. or adenosine (*e.g.*, $M \times 10^{-5}$) is added before the A.D.P. partial aggregation subsequently occurs and also some diminution of the amplitude. No strength inhibits aggregation without a parallel inhibition of the amplitude.

Sodium monoiodo-acetate, 0.1 ml. of $M \times 10^{-2}$, incubated at 37°C. with 2 ml. of platelet-rich plasma, completely inhibits both the decrease in amplitude and aggregation when A.D.P. is added (Fig. 3) but by incubating the platelet-rich plasma with monoiodo-acetate for a shorter time, or using a weaker solution or stronger A.D.P. it was repeatedly shown that an apparently full decrease in amplitude could be obtained with an almost complete inhibition of aggregation. The addition of 0.1 ml. of E.D.T.A. over a concentration range from 2 to 10% added to citrated or heparinized platelet-rich plasma produces no change in amplitude. If A.D.P. is now added, the amplitude decreases normally (Fig. 4) but aggregation is inhibited. P-tosyl arginine methylester over a considerable range of strengths and even at a concentration of $5M \times 10^{-1}$ (initial concentration) itself produces no change in amplitude. When A.D.P. $M \times 10^{-5}$ was added the amplitude decreased normally but aggregation was totally inhibited (Fig. 5). Thus no strength of p-tosyl arginine methylester studied prevented the decrease in amplitude.

5-HT, THROMBIN, AND COLLAGEN The addition of 0.1 ml. of 5-HT $M \times 10^{-4}$ to platelet-rich plasma (Fig. 12) produces rapid and marked diminution in amplitude and then aggregation. The results of adding adenosine, A.M.P., monoiodoacetate, p-tosyl arginine methylester, and E.D.T.A. before 5-HT are similar to those with A.D.P.

The addition of thrombin, 0.1 ml. of 1.25 units/ml., causes no change in amplitude for up to 10 seconds, then the amplitude decreases and aggregation begins (Fig. 13). If p-tosyl arginine methylester, 0.1 ml. $M \times 10^{-2}$, is added to platelet-rich plasma and then thrombin, no diminution in amplitude, no aggregation, and no clotting occur, even if very strong thrombin is used: otherwise the effect of the inhibitors on aggregation and amplitude were identical with their effect on subsequently adding adenosine diphosphate.

When 0.1 ml. collagen is added to platelet-rich plasma the opacity of the collagen is so great that it increases the optical density of the plasma and the tracing is displaced upwards (Fig. 6): after a delay of about 60 seconds the amplitude, hitherto unaffected by the addition, decreases rapidly and is immediately followed by aggregation. As with A.D.P., the prior addition of adenosine or A.M.P. inhibits both the decrease in amplitude and aggregation (Fig. 7). Monoiodo-acetate, 0.1 ml. of $M \times 10^{-1}$, prevents the decrease in amplitude and inhibits aggregation (Fig. 8). Unlike the situation with A.D.P., any strength of monoiodo-acetate that inhibits aggregation also inhibits the decrease in amplitude. Both E.D.T.A. (Fig. 9) and p-tosyl arginine methylester (Fig. 10) inhibit aggregation but the decrease in amplitude occurs normally.

ADRENALINE On adding adrenaline, $M \times 10^{-2}$, aggregation is rapid, giving a steep slope on the tracing, and it is difficult to study any change in amplitude. When adrenaline, 0.1 ml. $M \times 10^{-5}$, is added no change in amplitude occurs but the platelets aggregate (Fig. 14). The prior addition of p-tosyl arginine methylester inhibits aggregation, and the absence of change in amplitude is clear (Fig. 15).

TEMPERATURE Citrated platelet-rich plasma prepared at room temperature was warmed to 37°C. for 5 min. and the amplitude recorded. The temperature was then lowered stepwise and immediately after equilibration the plasma was again stirred and the amplitude recorded (Fig. 16). The amplitude remains fairly constant till 20°C. is reached; on further cooling it decreases markedly and is abolished at 10°C. This plasma was then warmed to 37°C.; six minutes later the amplitude had reappeared and it reverted to normal after 40 minutes. Another sample of platelet-rich plasma was warmed above 37°C.; the amplitude decreased at 43°C. and was almost abolished at 45°C. Protein precipitation occurred at 47°C.

E.D.T.A. If blood was collected into liquid E.D.T.A. in a final concentration of 1 mg./ml. the platelets

were seen to be spherical and a tracing of minimal amplitude was obtained compared with the tracing obtained with citrated or heparinized plasma. If E.D.T.A. was added to heparinized or citrated platelet-rich plasma no change occurred. No explanation of these findings is apparent.

DISCUSSION

The decrease in amplitude following the addition of cocaine or benadryl is clearly related to the spherizing of platelets which can be seen under the microscope. A decrease in amplitude would also occur if an asymmetrical form were changed to any symmetrical form such as a star shape. Microscopic inspection of platelets exposed to A.D.P. shows predominantly rounded forms but some spicules may remain, and the platelets are not nearly as uniform in shape as in the presence of cocaine. Nevertheless a decrease in amplitude must indicate that on average the platelets have changed to a more symmetrical rounded form.

Probably the only attribute of the platelet that influences the amplitude of the tracings is its shape. Bull and Zucker (1965) have shown that on adding A.D.P. not only is the platelet shape changed but the platelet volume increases by about 20%. The changes in shape and in volume presumably occur simultaneously and indicate a radical change in the membrane permeability reflected in the uptake of water. There is no information available whether water was taken in in all circumstances in which a decrease in amplitude was recorded above, but this seems probable.

Following the addition of A.D.P. or 5-HT the change in shape immediately precedes the development of stickiness; and with thrombin and collagen, these two phenomena always occur sequentially after the considerable delay following the addition of the aggregating agent. If the effect of adrenaline (aggregation and no change in amplitude) is ignored for the moment, it is possible to suggest that the change in shape is a prerequisite of the development of 'stickiness' and that the two phenomena are closely linked since one is always immediately followed by the other. In the presence of E.D.T.A. the platelets do not stick together but the shape changes. This may be due to removal of calcium ions if they in fact act

as a cationic ligands, or E.D.T.A. may have other actions. Salzman and Chambers (1964) reported that p-tosyl arginine methylester inhibits A.D.P. induced aggregation competitively which suggests that it is interfering with an enzymatic process concerned with the development of stickiness. However, it has no effect on the change in shape and must act after this process is complete. Adenosine and A.M.P. inhibit both aggregation and the change in shape and are competitive and must act earlier in the chain of events or at a site common to both change in shape and aggregation. If it is assumed that 5-HT and thrombin and collagen eventually cause stickiness and the change in shape by activating an intrinsic A.D.P. system then it is possible to construct a hypothetical diagram (Fig. 17) of the sequence of changes and to indicate the sites of action of these three inhibitors. The site of action of monoiodo-acetate is more speculative. Unlike A.M.P. is it not immediately effective which suggests an indirect, more complex action. When incubated in high concentration for 30 minutes it regularly inhibits both the change in shape and aggregation suggesting that it acts early in the chain of reactions but added before A.D.P. and thrombin on occasion it can be shown almost completely to inhibit aggregation while having little effect on the normal change in shape. Thus it may also have some action late in the sequence of events.

The arguments above have ignored the action of adrenaline and noradrenaline that produce stickiness with no change in shape or volume (Bull and Zucker, 1965). The kinetics of adrenaline-induced aggregation have important differences from those resulting from the other aggregating agents (O'Brien, 1964). Perhaps the catecholamines cause platelets to stick together through binding forces completely different from those produced by the other aggregating agents, in which case the suggestions of Fig. 17 may stand. If, however, the kind of stickiness induced is the same for all aggregating agents, then clearly stickiness does not always follow the change in shape with its implication of causality. Figure 17 can, however, be redrawn with the development of stickiness, and the change in shape, being independent results of the intrinsic A.D.P. system and sites of action of the inhibitors, can still be suggested.

FIG. 17. Possible sites of action of inhibitors (in boxes) on aggregation and on change of shape, if adrenaline-induced stickiness is an independent phenomenon: ————— indicates probable reactions: - - - - - indicates alternative reactions where the evidence is inconclusive.

FIG. 18. Possible sites of action of inhibitors (in boxes) on aggregation and on change of shape, if adrenaline-induced stickiness is similar to that considered above. — indicates probable reactions: - - - - indicates alternative reactions where the evidence is inconclusive.

compatible with the reported observations (Fig. 18).

Platelets are more rounded at low temperatures and are most asymmetrical at 29 to 40°C. The rounded form is the configuration requiring least energy to maintain and oval flattened discs, the usual shape in the body, will require more energy. This energy is almost certainly derived from enzymatic metabolism *in vivo* and it will decrease at low temperature. The same explanation may apply to the change in shape above 43°C.

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ADDENDUM

Since the preparation of this paper it has been found that if 0.7 g. of fine glass beads 0.5 mm. in diameter is added to 2.0 ml. of stirred heparinized platelet-rich plasma at 24°C. there is an immediate decrease in amplitude indicating a rounding up of the platelets. The tracing continues horizontally for 20 to 30 seconds and then goes down, indicating platelet aggregation which can be confirmed microscopically. These two glass-induced reactions have different characteristics from those considered in the body of this paper.