Platelet impedance aggregation in whole blood and its inhibition by antiplatelet drugs

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SUMMARY Platelet aggregation was studied in citrated whole blood by an electrical impedance method. Blood samples from normal volunteers were studied with the aim of finding a suitable method for the routine study of samples from patients. An erratic tracing and low maximum aggregation were seen in samples with a high normal haematocrit. Optimal aggregation was seen when blood was diluted to a haematocrit of $\cdot 300$; isotonic saline was a better diluent than platelet poor plasma. No appreciable differences were seen when the platelet count was diluted down to $50 \times 10^9/l$, after which there was a progressive reduction in response. Dose response curves were obtained, and normal ranges for ADP, collagen, and sodium arachidonate were determined. Acetylsalicylic acid had a more pronounced effect on ADP aggregation than on collagen. Prostacyclin (Epoprostenol) and the synthetic prostacyclin analogue ZK 36,374 both showed dose dependent inhibition of aggregation, but the duration of effect of the latter was much longer (> 6 h).

For the last 20 years platelet aggregation has been studied extensively by optical methods using platelet rich plasma and turbidometric devices.¹ Although of proved diagnostic value, these systems have certain limitations. Centrifugation is required for sample preparation so that red cells, white cells, and some of the heavier platelets are removed from the final platelet suspension to be tested. These other blood cells are probably important modulators of platelet function *in vivo* since they actively take up adenine nucleotides, synthesise and release regulatory prostaglandins, and may preferentially bind prostacyclin.²⁻⁴ Substances that regulate platelet function such as prostacyclin and cyclic AMP may degrade to biologically inactive products in the time required for platelet rich plasma preparation.

The recent innovation of electronic aggregation by the impedance method in whole blood circumvents most of these problems.⁵ With this system, blood may be analysed immediately after sampling without centrifugation and aggregation can be investigated in the presence of all blood elements. Most reports describe whole blood platelet aggregation in blood from laboratory animals; we have studied the application of this method to human blood in a routine coagulation laboratory.

Material and methods

Blood was obtained from apparently healthy adult human volunteers, who had not taken any anti platelet drugs for at least two weeks before sampling. Blood was anticoagulated with 3.13% tri-sodium citrate, nine parts blood to one part anticoagulant. A full blood count and platelet count were performed using a Coulter S Plus IV (Coulter Electronics Ltd, Luton, Beds). Normal subjects were also studied after ingesting varying doses of acetylsalicylic acid.

WHOLE BLOOD PLATELET AGGREGATION

Whole blood platelet aggregation was performed in citrated blood using the Chronolog Model 540 whole blood aggregometer (Coulter Electronics Ltd, Luton, Beds). A $975 \mu l$ sample of blood (neat or diluted with platelet poor plasma or isotonic saline) was warmed for 2-3 min at $37^\circ C$ in the machine heater block and then stirred with a Teflon coated stir bar in a non-siliconised glass cuvette 10×44 mm after inserting the impedance electrode. The calibration and gain controls were used to calibrate a Rikadenki R302 chart recorder in ohms. Twenty five microlitres of aggregating reagent was added and the change in impedance (ohms) recorded at a paper speed of 2 cm/min, giving an aggregation

trace. The following aggregating reagents were studied (final concentrations in whole blood): 1–25 μM ADP (Sigma Chemical Co) 2.5–125 μM adrenaline (Immuno Ltd), 1–25 $\mu\text{g/ml}$ collagen (Hormon Chemie, Munchen), 0.5–1 mM sodium arachidonate (Sigma Chemical Co). Dose response curves were produced for each reagent. The possibility of spontaneous aggregation was also studied by stirring undiluted blood at 37°C for 15 min and recording any change in impedance. Aggregation was performed at stirrer speeds varying between 100 and 1200 rpm on the same sample of blood, using a fixed final concentration of one agonist. After completion of impedance aggregation the electrodes were scrupulously cleaned to remove all aggregated cells using isotonic saline and by gentle cleaning with a tissue.

QUANTITATION

Rate of aggregation was assessed by measuring the angle between the base line and the linear portion of the aggregation curve; thus increased aggregation rate was shown by an increase in angle. Extent of aggregation was measured as the maximum height of response in chart paper units. This could also be expressed in ohms, since 10 units equalled 5 ohms.

INFLUENCE OF HAEMATOCRIT

Aggregation was also performed on undiluted whole blood and after diluting with isotonic saline or platelet poor plasma to reduce the haematocrit. Both normal and polycythaemic samples were studied to create a wide range of haematocrits between .100 and .600.

Fig. 1 Influence of haematocrit and diluent. Whole blood platelet aggregation to 5 $\mu\text{g/ml}$ collagen at various haematocrits prepared by dilution with saline (a) .300, (b) .100, (d) .350, (e) .420 (undiluted), (f) .050, or with platelet poor plasma (c) .100.

PLATELET COUNT

Two thrombopenic subjects (platelets $<25 \times 10^9/l$) were bled to study the effect of platelet count. Whole blood platelet aggregation in these subjects was barely detectable using collagen or ADP. Varying amounts of platelet rich plasma from normal (blood group compatible) donors were diluted in citrated whole blood from thrombopenic patients and the volume corrected with platelet poor plasma to give a range of final platelet counts, at a constant haematocrit of .300. Alternatively, a 975 μl aliquot of thrombopenic blood was allowed to settle for 20 min and a volume of platelet rich plasma was removed and replaced with platelet rich plasma from a normal donor.

REPRODUCIBILITY

Precision was studied using normal blood and repeating aggregation to one reagent on the same sample.

INHIBITORS

The effects of adding prostacyclin (Epoprostenol, Wellcome Pharmaceuticals) and the stable prostacyclin analogue ZK 36,374 (Schering Chemicals Ltd) were studied by adding varying concentrations to whole blood before the addition of aggregating reagents. The dose (ID_{50}) causing a 50% reduction in response to agonists was estimated.

Results

As expected, low stirring speeds produced poor aggregation responses; optimal aggregation occurred at about 600 rpm. Higher stirring speeds showed a gradual reduction in the total impedance change.

Changes in haematocrit produced dramatic effects on impedance (Fig. 1). High haematocrits caused erratic tracings and often minimal aggregation with poor precision, while large dilutions with saline also caused a reduction in platelet count, which also decreased the aggregation response. Dilution to a haematocrit of .300 appeared to be optimal; this gave a better wave of aggregation and a less erratic trace than seen with neat whole blood while causing a minimal decrease in platelet numbers. This also enabled standardisation of whole blood aggregation from different subjects. Saline appeared to be the diluent of choice, giving a steeper and more regular trace than platelet poor plasma.

Reduction of the platelet count by dilution of platelet rich plasma in thrombopenic blood showed no significant changes in aggregation down to platelet counts of about $50 \times 10^9/l$. Below this value a progressive reduction in the response was seen (Fig. 2).

Fig. 2 Effect of platelet count. Aggregation to 5 µg/ml collagen at a constant haematocrit of .300 prepared by dilution with platelet poor plasma and platelet rich plasma to give various platelet counts: (a) 50, (b) 100, (c) 25, (d) $13 \times 10^9/l$.

Reproducibility testing showed a coefficient of variation between 15% and 20%, depending on the sample used and the dose of aggregating reagent; poor aggregation waves tended to be less reproducible.

Good aggregation responses could be obtained with collagen at concentrations of 1–5 µg/ml (Fig. 3). Aggregation with ADP required doses about 10 times higher than those routinely used for platelet rich plasma aggregation (Fig. 3). Only a single wave of aggregation was observed at ADP concentrations causing any impedance change; concentrations of 12.5 and 25 µM appeared to be most useful for study. Aggregation with adrenaline was extremely poor, giving low impedance changes even at 125 µM. Normal ranges for ADP, collagen, and sodium arachidonate were established and are shown in the Table. Normal donor blood showed no appreciable differences (other than normal variation) when the

Fig. 3 Dose response curves to collagen and ADP. Citrated whole blood was diluted to a haematocrit of .300 with saline and aggregated with various concentrations of (a) ADP or (b) collagen.

same sample was tested immediately after venepuncture or up to 6 h after sampling. Ingestion of acetylsalicylic acid (600 mg 24 h before sampling) appeared to have only a small effect on collagen aggregation, while ADP responses were abolished or greatly reduced (Fig. 4).

The addition of prostacyclin immediately before aggregation led to a dose dependent inhibition of aggregation with an S-shaped dose response curve, giving an ID_{50} of about 1 ng/ml for 5 µg/ml collagen.

Normal values for whole blood platelet aggregation

	ADP (µM)		Collagen (µg/ml)		Arachidonate (mM)
	12.5	25	1	5	1
Aggregation slope					
Mean	45.0	48.6	45.1	61.4	46.1
SD	18.1	10.9	10.2	8.1	11.8
Range	13–77	31–75	27–62	45–73	27–69
Aggregation extent					
Mean	13.5	15.2	13.3	22.6	20.6
SD	7.6	6.2	4.2	4.6	12.0
Range	6.5–40	9–32	6.5–20	13.5–31	2–38

Citrated whole blood from 20 normal subjects was diluted with isotonic saline to a haematocrit of .300. Aggregation was performed to 12.5 and 25 µM ADP, 1 and 5 µg/ml collagen, and 1mM arachidonate. Results are expressed as aggregation slope (degrees) and aggregation extent (ohms).

Fig. 4 Inhibition by acetylsalicylic acid. Whole blood platelet aggregation at a haematocrit of 300 prepared with saline before (a) and 24 h after (b) taking 600 mg of soluble acetylsalicylic acid.

Fig. 5 Inhibition with prostacyclin. Prostacyclin (Epoprostenol) was added to citrated whole blood to give a final concentration of 1 ng/ml. Aggregation was tested (b) in the absence of prostacyclin and (a) 1 h, (c) 30 min, (d) 0 min after adding it.

Addition of ZK 36,374 to whole blood produced a similar dose dependent inhibitory response, giving an ID_{50} of about 1.75 ng/ml for 5 µg/ml collagen. Neither inhibitor was able to abolish completely the response to collagen, even with very high doses, whereas both agents could completely block ADP aggregation using relatively low inhibitor concentrations. Time course studies showed that the inhibitory effect of prostacyclin disappeared rapidly within 30–60 min after incubation with whole blood (Fig. 5), whereas the inhibitory effect of ZK 36,374 was stable in whole blood for at least 6 h.

Discussion

Many of the studies of whole blood impedance aggregation have used blood from laboratory animals.^{5,6} A detailed study using human blood, was therefore necessary to establish a suitable method and normal range with various aggregating reagents. One of our first observations was the dramatic influence of haematocrit on whole blood aggregation. This is of great importance owing to the wide variation in haematocrit found in clinical samples. Subjects are studied either for the diagnosis of bleeding disorders, the investigation of a thrombotic state, or the influence of antiplatelet agents. Based on our results, it seems reasonable to standardise the haematocrit to 300, which does not cause excessive platelet dilution and suits most cases. It is of interest that a small dilution of whole blood leads to an increase in platelet aggregation by impedance, whereas in platelet rich plasma a decrease in optical aggregation results. This increase remains fairly constant as the haematocrit is lowered, until the platelet count becomes the limiting factor. There was no correlation between response and starting haematocrit or platelet count. This effect is probably a combination of the removal of impeding red cells and the difference in conductivity between saline and platelet poor plasma.

The diluent of choice appears to be isotonic saline. This is immediately available and gives a better response than platelet poor plasma, which also requires centrifugation thus delaying analysis. Dilution with the patient's own platelet poor plasma, however, may be advisable in the investigation of certain ex-vivo inhibitory or pro-aggregatory plasma substances present in trace amounts, the effect of which may disappear due to dilution with saline.

Whole blood aggregation appeared to be more suitable than conventional aggregation for low platelet counts, giving good responses at platelet counts of about $50 \times 10^9/l$; the optical technique cannot be effectively used as a diagnostic method below $100 \times 10^9/l$ and in our experience is best at $200\text{--}400 \times 10^9/l$. The method may therefore have important applications in the study of patients with mild thrombopenia who are bleeding.

As reported by Ingberman-Wojenski *et al.*,⁷ we could not detect a reversible wave of aggregation with ADP even when a wide dose response curve was tested, and biphasic curves were not seen. Therefore responses to ADP are probably related to the nucleotide salvage pathways of red and white blood cells, which are efficient at taking up and utilising adenine nucleotides which would prevent their use by platelets. The poor response to adrenaline is difficult to explain but could also be

due to an uptake mechanism or competition by receptors on other cells.

The weak effect of acetylsalicylic acid on collagen aggregation has been described previously⁸ and is believed to be due to the ability of the impedance method to detect even very small platelet aggregates which may still form in the presence of acetylsalicylic acid. Prostacyclin and the stable prostacyclin analogue ZK 36,374, however, caused a marked inhibition of both ADP and collagen aggregation, at doses of the same order of magnitude as those active in platelet rich plasma. Prostacyclin was only effective in whole blood for short incubation periods, showing the usefulness of this technique for ex-vivo studies of substances with short half lives. The discrepancy between duration of effect of prostacyclin and ZK 36,374 is probably related to the non-enzymatic degradation of prostacyclin at pH 7.4 to biologically inactive metabolites⁹; ZK 36,374 is chemically stable and although possibly adsorbed to the red cell surface does not appear to be appreciably inactivated in whole blood ex vivo.¹⁰

In summary, we recommend the following for routine clinical work: collection of blood into citrate and dilution to a haematocrit of .300 with isotonic saline; stirrer speed 600 rpm; 975 μ l blood plus 25 μ l agonist (12.5 and 25 μ M ADP, 1 and 5 μ g/ml collagen, 0.5 and 1mM sodium arachidonate); chart speed 2 cm/min; and 5 ohm equal to 2.5 cm of chart paper.

The value of whole blood aggregation in the clinical laboratory remains to be seen. There may be important uses in the study of hypercoagulable states; antiplatelet treatment; and disorders of red and white cells, such as sickle cell crisis, infection, and the leukaemias. These possible applications are currently under study in our laboratory and will be reported elsewhere.

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