Aggregation of human platelets by commercial preparations of bovine and porcine antihaemophilic globulin


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SYNOPSIS Infusion of commercial preparations of porcine and bovine antihaemophilic globulin into three haemophilic patients produced transient thrombocytopenia. This platelet-aggregating activity has been shown to be present in a wide range of animal plasmas and is related to the fibrinogen fraction. The mechanism of platelet aggregation by animal fibrinogen is discussed and some inhibitors of the reaction are described.

Antihaeomophilic globulin (AHG) prepared from bovine and porcine plasma is of proven value in the management of haemorrhage in haemophilia (Biggs, 1967). It has been effectively used in human patients (Macfarlane, Biggs, and Bidwell, 1954; Macfarlane, Mallam, Witts, Bidwell, Biggs, Fraenkel, Honey, and Taylor, 1957; Macfarlane and Biggs, 1959; Biggs, 1960; Rizza and Biggs, 1969), in haemophilic dogs (Sharp and Dike, 1963), and in a haemophilic horse (Nossel, Archer, and Macfarlane, 1962). Bovine AHG at concentrations of 0.5 mg/ml produces platelet aggregation both in vitro (Sharp and Bidwell, 1957) and invariably in vivo (Macfarlane and Biggs, 1959). Thrombocytopenia was also recorded in the haemophilic horse treated with bovine AHG (Nossel et al, 1962).

The effect of porcine AHG preparations in vivo and in vitro is not so well defined. Sharp and Bidwell (1957) showed no effect on human platelets in vitro when the porcine preparation was used at 0.5 mg/ml but when the concentration was raised to 5 mg/ml platelet aggregation could be produced. Occasional cases of transient thrombocytopenia have been recorded in human haemophilic patients during therapeutic administration of porcine AHG (Macfarlane et al, 1957; Rizza and Biggs, 1969).

The present investigation was undertaken in an attempt to identify the platelet aggregating agent and its mechanism of action.

Material

COLLECTION OF BLOOD

All samples were collected by clean venepuncture from healthy donors using plastic syringes and a mixture made of 9 volumes of blood with 1 volume of 3.8% sodium citrate or 2% ethylenediaminetetraacetic acid. All glassware was siliconized using Siliclad (Clay Adams Inc, New Jersey, USA).

PLATELET-RICH PLASMA

This was prepared from citrated whole blood by centrifugation at 600 g for five minutes at room temperature.

PORCINE AND BOVINE ANTIHAEMOPHILIC GLOBULIN

These preparations were purchased from S. Maw & Son, Barnet, England. The contents of each ampoule was dissolved in 50 ml of normal saline and stored at −20°C. The average weight of dry material per vial of bovine AHG was 577 mg and of porcine material was 407 mg. The dilutions referred to in the text were prepared from this stock solution. The fibrinogen content of this material was (bovine) 576 mg/100 ml and (porcine) 350/100 ml.

ADENOSINE DIPHOSPHATE (ADP) (SIGMA CHEMICAL COMPANY, ST LOUIS, MISSOURI)

A stock solution of 100 µg ADP per ml was prepared and kept at −20°C. Dilutions referred to in the test were made from this.
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ANIMAL PLASMAS
These were purchased from the Colorado Serum Company (Colorado, USA) and were kept frozen at -20°C. Plasma from a range of primates was obtained from the Laboratory for Experimental Medicine and Surgery in Primates (LEMSIP). All samples were obtained deep frozen in 4% sodium citrate.

ADENOSINE (SIGMA CHEMICAL COMPANY LIMITED, LONDON)
This was made up in normal saline and used at a final concentration of 50 \( \mu \text{g/ml} \) of platelet-rich plasma.

CELITE 512
Celite 512 was obtained from the Johns Manville Company, Celite Division, New York, N.Y.

SEPHADEX G-200
Sephadex (Pharmacia, Fine Chemicals, Uppsala, Sweden) was expanded in barbitone-saline buffer (0-15M, pH 7-3).

CLINICAL STUDY
Three haemophilic patients were given porcine AHG therapeutically. Two underwent major surgery and one, with an AHG inhibitor, was bleeding from the gastrointestinal tract. Standard dosage schedules as recommended by the manufacturers were given except in the third patient in whom twice the standard dose was eventually given in an attempt to overcome the inhibitor and provide a haemostatic level of AHG.

Methods

PLATELET AGGREGATION
Platelet aggregation was measured by the turbidimetric method of Born (1962). The apparatus used in this study consisted of an EEL titrator connected to a galvanometer (EEL type 20, Evans Electro-selenium Limited, England). A perspex cuvette was fitted onto a titrator above a magnetic stirrer and in the light path from the photoelectric cell. Platelet-rich plasma, 2 ml amounts, was added to the cuvette together with a small stirring rod. When the stirring rod was rotating at a uniform speed, the optical density reading of the plasma sample on the galvanometer scale was adjusted to an arbitrary value of 0-600. The plasma was agitated for 30 seconds to ensure that no change in optical density occurred before the addition of 0-1 ml aliquots of aggregating reagent. Changes in light transmission, at a wavelength of 492 nm, were recorded at 30-second intervals over a period of 10 minutes from the addition of the AHG. Control samples were handled in an identical fashion except that normal saline replaced the test solution.

PLATELET ADHESIVENESS
Platelet adhesiveness was measured by the glass bead column method using the principles described by Hellem (1960). The glass bead column was made by filling a length of vinyl tubing (NT/13, Portland Plastics, Kent) with 2-5 g Ballotini glass beads (0-57 mm in diameter) to give a column 6 cm in length. The glass beads were held in the column by a filter of fine nylon gauze fitted at each end of the column. Two ml of platelet-rich plasma was incubated for 10 minutes at 37°C with 0-1 ml aliquots of porcine or bovine AHG then drawn into a graduated plastic syringe which was then fitted to an electrically operated mechanical pump. The plasma was pushed through the column of beads at a constant rate. The apparatus used in the study gave a mean contact time between plasma and glass of 30 ± 1 seconds. Platelet counts were performed on the plasma samples before and after passage through the column. The difference between the two counts was expressed as a percentage of the initial platelet count and this value taken as an index of platelet adhesiveness in the sample. This was repeated in seven different plasma samples.

CHANDLER TUBE TECHNIQUE
This was described by Chandler (1958) and modified by Cunningham, McNicol, and Douglas (1965). The transparent vinyl tubing, 12-3 mm bore, 71 cm in length, was made into loops by means of a nylon adaptor. The loops were washed in cold water and rinsed in 0-9% sodium chloride. Citrated platelet-rich plasma, 5 ml aliquots, was added to the two loops and the volume made up to 15 ml with saline. AHG solution or saline (0-25 ml) was added to the system and the loops were rotated on the turntable of a blood cell suspension mixer (Matburn Limited, London) at 28-5 rpm in a glass-fronted incubator at 37°C. The platelet aggregation was assessed by the length of time taken for the appearance of a 'snow-storm' effect. This was repeated on seven different plasmas.

To determine if the aggregating agent was a protein, an equal volume of 10% trichloroacetic acid (TCA) was added to the AHG preparations and the resulting precipitate was centrifuged at 2 000 rpm for 10 minutes. A control tube was set up in which an equal volume of saline was added to the AHG preparation. Aliquots (0-1 ml) were then added to platelet-rich plasma in the aggregometer.

Adsorption was performed with calcium phosphate or celite 512, 10 mg/ml of AHG preparation.
at room temperature. The adsorbent was removed by centrifugation and eluted with 10% sodium chloride. The eluate was dialysed overnight against barbitone-saline buffer. The aggregating activity of the supernatant and the eluate was tested.

**EFFECT OF TEMPERATURE**

Aliquots of the AHG preparations were kept for 10 minutes in a waterbath at 40°C, 50°C, 60°C, 70°C. Any precipitate which formed was removed by centrifugation at 2000 rpm for 10 minutes. Samples of stock AHG solutions were also kept at −20°C for two years and at room temperature for one week. Of these materials, 0.1 ml amounts were added to 2 ml of platelet-rich plasma in the aggregometer.

**EFFECT OF INHIBITORS OF PLATELET AGGREGATION**

Adenosine or heparin was incubated with platelet-rich plasma at 37°C before the addition of bovine or porcine AHG. The effect of 'aspirin' on AHG-induced platelet aggregation was determined in vitro on the plasma of one subject, the blood having been collected before and two hours after ingestion of four 300 mg tablets.

**ASSOCIATION WITH ANTIHAEMOPHILIC GLOBULIN**

Bovine and porcine AHG preparations were diluted with saline to give approximately 100% AHG activity as compared with a standard human plasma pool. Aliquots of 2 ml were then incubated for two hours at 37°C with 0.25 ml of plasma from a haemophiliac with a high titre of inhibitor active against human bovine and porcine AHG, or 0.25 ml of haemophilic plasma without inhibitor. Assay of the residual AHG and aggregating agent in the tubes was then performed.

**ASSOCIATION OF THE AGGREGATING AGENT WITH FIBRINOGEN**

Gel filtration of the AHG preparations was carried out on 2.5 × 40 cm columns of Sephadex G-200 pre-swollen with barbitone-saline. Three ml samples of the bovine and porcine AHG preparations were applied in 10% sucrose and 3 ml volumes of effluent were collected and measured for AHG activity, aggregating activity, and fibrinogen.

Plasma from the four animal species which produced maximum platelet aggregation was clotted by the addition of 0.025 M CaCl₂ and the serum incubated at 37°C for three hours to remove any residual thrombin. Platelet aggregation of the starting plasma was then compared with the incubated serum.

Platelet counts were performed by the method of Dacie (1956) using formol citrate as the diluting fluid.

Antihaemophilic globulin was assayed by the method of Margolis (1958) as modified by Breckenridge and Ratnoff (1962).

Dialyses were performed at 4°C in cellophane casings (Visking Corporation, Chicago, Illinois).

Ultracentrifugation was carried out in a Beckman model L ultracentrifuge, the samples being spun at 60000 g for 24 hours.

**Results**

**PLATELET AGGREGATION IN VIVO BY PORCINE AHG**

Infusion of porcine AHG into haemophilic patients resulted invariably in thrombocytopenia. In the first two patients (Fig. 1A) thrombocytopenia was transient and the platelet count returned to normal within 24 hours. However, in patient 3 (Fig. 1B), severe, prolonged, thrombocytopenia was produced with exacerbation of gastrointestinal bleeding and a rise in blood transfusion requirements. No rise in AHG levels could be detected in this patient.

**PLATELET AGGREGATION IN VIVO BY BOVINE AND PORCINE AHG PREPARATIONS**

Bovine and porcine AHG preparations are potent platelet aggregating agents in vitro (Fig. 2). When either was added to human platelet-rich plasma in concentrations equivalent to those obtained during therapeutic administration irreversible platelet aggregation occurred within two minutes. At lower concentrations the effect was less marked and aggregation became reversible. On a weight-for-weight basis the bovine material was approximately four times more potent in producing platelet aggregation than the porcine material. Platelet aggregation was produced by samples from 10 separate batches of bovine and porcine AHG preparations.

**IN VITRO EFFECT OF VARIOUS ANIMAL PLASMAS ON AGGREGATION OF HUMAN PLATELETS**

The results are shown in Figure 3. Plasma from goat, sheep, pig, cow, and horse produced aggregation of human platelets in vitro. Plasma from a range of primates (chimp, rhesus monkey, black ape, baboon, gibbon, and squirrel monkey) produced very slight, reversible aggregation and plasma from a wide range of animals (dogs, cat, rat, opossum, rabbit, hamster, guinea pig, turkey, pigeon, chicken, paddlefish, and lobster) produced no aggregation.
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**Fig. 1a.**

- **Patient 1**
  - Platelet count ($\times 10^3$)
  - Time (hours)

- **Patient 2**
  - Platelet count ($\times 10^3$)
  - Time (hours)

**Fig. 1b.**

- Blood transfused (pints)
- Haemoglobin (gm/100ml)
- Time (days)

**Fig. 2.**

- **Bovine AHG preparation**
  - 5 μg/ml plasma
  - 50 μg/ml plasma
  - 240 μg/ml plasma

- **Porcine AHG preparation**
  - 50 μg/ml plasma
  - 100 μg/ml plasma
  - 200 μg/ml plasma
  - 800 μg/ml plasma

**Fig. 3.**

- **HORSE PLASMA**
- **COW PLASMA**
- **PIG PLASMA**
- **SHEEP PLASMA**
- **GOAT PLASMA**

**Fig. 1A** Effect on the platelet counts of infusion of 8000 unit doses (X) of porcine AHG in two patients with haemophilia. B Effects on platelet count, haemoglobin, and the blood transfusion requirements of the infusion of porcine AHG; 8000 units were given twice, 16000 units were given three times.

**Fig. 2** Aggregation of human platelets by different concentrations of commercial porcine and bovine AHG preparations.

**Fig. 3** Aggregation of human platelets by various mammalian plasmas. Aliquots of 0.1 ml of animal plasma were added to 2 ml of human platelet-rich plasma.
PLATELET ADHESIVENESS BY THE METHOD OF HELLEM
The mean adhesiveness of the seven control samples using platelet-rich plasma is 12% ± 8% and the seven test samples containing animal AHG 79% ± 4%. This difference is highly significant (t = 22, P < 0.001).

EFFECT ON THE CHANDLER TUBE SYSTEM
The average time for the appearance of the 'snowstorm' in the seven tests was 141 seconds with a standard deviation of 17 seconds. The 'snowstorm' consisted of aggregates of 50 to 100 platelets. No snowstorm appeared in the control tubes and no fibrin formation was seen in either tube.

EFFECT OF INHIBITORS OF PLATELET AGGREGATION
Aggregation by animal AHG is dependent on the presence of ionic calcium. The addition of ethylene diamine tetracetic acid (EDTA) to the platelet-rich plasma resulted in inhibition of aggregation.

Similarly pre-incubation of platelet-rich plasma with adenosine at a concentration of 50 μg/ml of plasma resulted in inhibition of aggregation.

Heparin produced no inhibition of aggregation even in concentrations of 25 units/ml of plasma.

The effect of salicylic acid is shown in Figure 4. The lower curve shows the biphasic response of platelet aggregation to porcine AHG. The upper curve is the response in the same subject's platelet-rich plasma two hours after the ingestion of acetylsalicylic acid.

PHYSICAL PROPERTIES OF THE AGGREGATING AGENT
Effect of temperature
The results indicate progressive destruction of the platelet aggregating factor as the temperature is raised, with complete destruction of aggregating activity when the preparations were kept at 70°C for 10 minutes (Figs. 5a and b).

The aggregating factor is stable when kept at room temperature, for a week and also at −20°C for two years.

Dialysis
After 24 hours' dialysis no significant change could be detected in aggregating ability.

Effect of trichloroacetic acid
Precipitation with 10% trichloracetic acid resulted in complete loss of platelet aggregating activity.
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Absorption with calcium phosphate or Celite 512
This resulted in no loss of platelet aggregating activity. No activity was found in the eluates.

Ultracentrifugation
Ultracentrifugation of the preparation had no effect on aggregating ability which was present to the same degree at the top and bottom of the tube.

Association with AHG content of the material
Reduction of the level of AHG activity in the preparations from 100 to 1% produced no difference in the platelet aggregating material.

Association with the fibrinogen fraction
The results of Sephadex G-200 gel filtration of bovine and porcine AHG preparations showed that in both preparations the peak of AHG activity aggregating ability and fibrinogen coincided.

Comparison of the aggregating action of four animal plasmas with their sera is shown in Figure 6. In all four cases removal of fibrinogen by clotting resulted in a marked diminution of aggregating ability though this did not disappear entirely.

Discussion

Both bovine and porcine AHG preparations cause aggregation of human platelets in vivo and in vitro. This action is intrinsic to the native plasma from which the AHG is prepared and is also found in the plasma of goat, sheep, and horse. Aggregating activity is found at low levels in primates but is absent from many other mammals and all fish and fowl tested.

The aggregating activity is precipitated by 10% trichloracetic acid and is inactivated by heating at 60°C for 10 minutes. It is not adsorbed by Celite 512 or calcium phosphate and is therefore not related to the contact or vitamin K-dependent coagulation factors. Aggregation was not inhibited by heparin which inhibits thrombin-induced platelet aggregation (Clayton and Cross, 1963).

The pattern of platelet aggregation is biphasic, aggregation starting on addition of AHG without the delay seen with collagen (Zucker and Borrelli, 1962; Hovig, 1963a and b). The reaction is dependent on calcium ions and is blocked by pre-incubation of the platelet-rich plasma with adenosine, a competitive inhibitor of endogenous ADP release. The second phase of aggregation can be blocked by prior ingestion of acetylsalicylic acid which is thought to produce inhibition of release of endogenous platelet ADP (Zucker and Peterson, 1968).

The action of the antihaemophilic globulin in the AHG preparations is not clear. It is closely related to fibrinogen fractions and is inactivated during the clotting of plasma. However, when the AHG activity of a sample was reduced from 100% of normal to 1% by incubating with a potent AHG inhibitor no change in platelet aggregation occurred.

The aggregating agent is probably fibrinogen or some protein intimately related to the fibrinogen fraction. Clotting of animal plasma produces a marked reduction in the level of aggregating agent and gel filtration of porcine and bovine AHG through Sephadex G-200 produced coincidental peaks of AHG activity, fibrinogen, and platelet-aggregating activity.

Figure 6. Comparison of plasma and serum from various animal plasma in the aggregation of human platelets.

These findings agree with those of Solum (1968), who showed that highly purified bovine platelet fibrinogen aggregated human platelets and with those of LeRoy, Mason, and Brinkhous (1960), Brinkhous, Read, and Mason (1965), and Mason and Read (1967) who described an activity present in the fibrinogen fraction of various animal species which they called thrombocyte agglutinating activity (TAg).

The mechanism by which animal fibrinogens aggregate human platelets is unknown but may reflect species differences in the fibrinogen molecule (Doolittle and Blombäck, 1964). Bovine fibrinogen has been used as a platelet aggregating agent by Caen, Castaldi, Leclerc, Incemar, Larrieu, Probst, and Bernard (1966a) and by Caen, Vainer, and Gautier (1966b) in patients with thrombasthenia whose platelets were not aggregated by ADP, adrenaline, noradrenaline, 5-hydroxytryptamine, thrombin, purified collagen, crude connective tissue, or human fibrinogen. However, bovine fibrinogen at a concentration of 1.5 mg/ml produced aggregation in five of the six cases tested. They also showed that the aggregating activity could be destroyed on incuba-
tion with plasmin and the reaction does not consume complement. Falcão, Probst, Gautier, Vainer, Michel, and Caen (1967), using the electron microscope, showed that the intercellular cement in the external membranes was unaltered in platelets aggregated by bovine fibrinogen.

Infusion of animal AHG preparations in haemophilic patients gives rise to thrombocytopenia which is transient and is related to the dose given. Thrombocytopenia is probably due to trapping of the large platelet aggregates in the capillaries, a phenomenon which is similar to the retention of platelet aggregates in the Hellem glass bead columns. Bleeding as a result of the low platelet count has been occasionally documented (Macfarlane and Biggs, 1959; Hall, Handley, and Webster, 1962; Rizza and Biggs, 1969), despite a normal or high level of AHG. In one of our patients (case 3) there was no rise in the plasma level of AHG due to inactivation by inhibitor but there was evidence of increased gastrointestinal blood loss with a rise in blood transfusion requirements and a fall in haemoglobin which corresponded in time to the thrombocytopenic episode.

All three of our patients experienced pain along the line of the vein through which the AHG solution was administered and also suffered thrombosis in the veins at the infusion site. This thrombogenic effect has been recorded previously (Biggs, 1960) and may be due to adherence of platelet aggregates to the endothelium of the vein with production of platelet thrombi as suggested by Berman and Fulton (1961) and Mustard, Murphy, Rowsell, and Downie (1962). Such platelet thrombi may stimulate formation of fibrin with resultant occlusion of the vessel.

Examination of a blood film in one of our patients during infusion of porcine AHG showed multiple platelet aggregates consisting of 30 to 50 platelets, measuring 100-200 μ in diameter. Such platelet aggregates could have pathological consequences but this has not been proven in man. However, in experimental situations, a similar picture can be produced by infusion of adenosine diphosphate intravenously. Transient thrombocytopenia has been produced by this means (Davey and Lander, 1964), rats (Nordøy and Chandler, 1967), cats (Born and Cross, 1963), swine (Mustard, Rowsell, and Murphy, 1964; Murphy, Mustard, Rowsell, and Downie, 1964), and in rabbits (Regoli and Clark, 1963). In experimental animals, platelet aggregates induced by ADP can be seen histologically blocking capillaries in the lung (Nordøy and Chandler, 1967) where they may produce irreversible tissue changes even though the platelet thrombus itself is transient. Mustard et al (1964) produced myocardial infarction in swine by infusion of ADP, and in the rat infusion of ADP may result in respiratory arrest with platelet thrombi in the pulmonary capillaries, a reaction that can be produced under experimental conditions by the injection of other embolizing particles (De Takats, Beck, and Fenn, 1939). In the rabbit, if platelet aggregates are formed within the renal circulation, focal acute glomerulonephritis is produced with areas of cortical necrosis (Glynn, Jørgensen, and Buchanan, 1966). In these experiments fibrin thrombi were found in the renal arteries and glomerular capillaries 24 hours after ADP infusion. This finding was interpreted by the authors as indicating that the fibrin thrombi were secondary to the platelet aggregates occluding blood flow.

In the original toxicity studies by Sharp and Bidwell (1957) injection of AHG preparations into rabbits produced no histological changes of note. This was probably because rabbit platelets are not aggregated by bovine or porcine AHG preparations. This is in keeping with the finding of LeRoy et al (1960) that there is marked interspecies differences to a range of aggregating agents.

Despite wide usage of these preparations there has been no clinical evidence of myocardial or pulmonary complications in patients receiving bovine or porcine AHG. In one case (Wardle, 1967), proteinuria developed during a course of bovine AHG which also produced thrombocytopenia. Proteinuria and biochemical evidence of renal damage was present in this patient three months later.

With the increased availability of human concentrates of AHG the need for animal AHG preparations will diminish, but they will still be of value in patients with inhibitors, perhaps in combination with exchange transfusion (Roberts, Scales, Madison, Webster, and Penick, 1965). In particular modern fractionation methods may permit large scale separation of the potent AHG fraction from the platelet-aggregating fibrinogen fraction.

References
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