METHOD FOR MEASURING PLATELET AGGREGATION

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8 Claims

ABSTRACT OF THE DISCLOSURE

A method for rapidly and accurately measuring platelet aggregation in which prior to counting, aliquot samples of whole blood are treated with anticoagulant, then an aggregating agent, shaken, and centrifuged. Method allows measurement of multiple samples simultaneously and the results obtained therefrom are statistically reproducible.

This invention relates to hematology and more particularly to an improved method for quickly and rapidly determining the platelet aggregation response from a sample of whole blood.

As practiced heretofore, platelet aggregation involved both lengthy and cumbersome procedures. Blood specimens were collected and anticoagulated. The anticoagulated blood was then centrifuged to provide a test sample of platelet-rich plasma. The plasma was then mixed with an aggregating reagent and a measurement of the change in optical density of the plasma sample was carried out to provide an index of the degree of aggregate formation.

Apparatus utilizing the prior art method is available and is known as the Bryston Aggregometer. It utilizes a magnetic stirring bar for the mixing operation; a heater to maintain the plasma at a pre-determined temperature during the aggregation test, and a light and photocell to measure the optical density of the plasma. In use, this instrument must be connected to a suitable recording apparatus, and a tracing of the aggregation curve is obtained following addition of the aggregating agent to the incubated plasma. A single determination of each plasma sample requires 5 to 15 minutes and the apparatus costs approximately $2,300 including the recorder.

On the basis of laboratory studies, we believe that to adequately assess the sensitivity of any given blood sample for platelet aggregation, multiple tests must be done using various concentrations of aggregating agent. Additionally, information in the literature and our own laboratory experience suggests that several agents are capable of producing platelet aggregation and that more than one mechanism of action is involved among the various aggregating agents. On the basis of the above considerations, it became increasingly desirable to make multiple measurements of the platelet aggregation response on aliquots from a given blood sample, utilizing a variety of agents as well as a variety of dose levels of each of the agents.

Use of the Bryston Aggregometer to obtain a full assessment of the aggregating characteristics in a given sample thus becomes impractical because it requires excessive blood volumes from the patient and takes much too long.

Other disadvantages of the methodology incorporating the Bryston Aggregometer arises from a basic defect in the system, from the physiological standpoint, because the platelets have to be isolated from the other blood cells in order to carry out the aggregation reaction; because it requires 1 to 1.5 mL of platelet-rich plasma to run each reaction and this amount of plasma requires 6 to 10 min. of whole blood (per single reaction); and because to obtain each plasma sample, the whole blood must be centrifuged for at least 10 minutes, during which time the failure to maintain precise physiological temperature will cause a change in the shape of the platelets, and therefore an abnormality. This is a reversible effect but to reverse it, the platelet-rich plasma must be reincubated for a short period of time at physiological temperature to restore the platelets to their normal disc shape before the aggregation reaction can be carried out, thereby requiring still more time.

The present invention is based upon the discovery of new and highly efficient methodology whereby both the time and the amount of blood needed to test inhibitory drugs is significantly reduced, the samples can be always maintained at physiological temperature, the test can be completed in the presence of normal cell population of whole blood since no isolation of platelets is needed until after aggregation has occurred. The invention thus presents as salient features the ability to use multiple samples of whole blood to effect an accurate, rapid, and statistically reproducible profile of platelet aggregatability.

Accordingly, it is a prime object of the present invention to provide a new and improved method of determining platelet aggregative response in a sample of whole blood whereby dose and reagent response profiles can be rapidly obtained while minimizing the blood volume required to obtain the measurement.

A further object of the present invention is to provide a new and improved method of measuring platelet aggregative response in a patient whereby whole blood samples can be used and a plurality of measurements simultaneously obtained.

These and still further objects as shall hereinafter appear are fulfilled by the present invention in a remarkably unexpected fashion as can be readily discerned from the following detailed description of a representative embodiment thereof.

In the practice of the present invention to measure platelet aggregation response from whole blood while using simultaneous multiple sample determinations, the following terms are used and are intended to carry the meanings indicated for them.

"Aggregating chemical" means a chemical capable of causing aggregation of blood platelets and is represented by the group consisting of adenosine diphosphate (A.D.P.), collagen suspension, epinephrine, serotonin, thrombin, latex particles, antigen-antibody complexes and the like.

"Anticoagulant" means a reagent capable of preventing or reducing the coagulation of blood and is represented by the group consisting of heparin, trisodium citrate, sodium oxalate, and like in vitro reagents.

The amount of anticoagulant used is species-dependent and it can be optimized using the normal skills of the art. For example, 0.05 mL of 3.8% trisodium citrate is appropriate for 0.5 mL of human whole blood, while 0.025 mL of 3.8% trisodium citrate is appropriate for 0.5 mL of rat whole blood.

In one practice of the present invention, determination of an aliquot sample of whole blood to which an anticoagulant has been added is obtained at a physiologic temperature by the following steps. An aggregating chemical is added to said sample and the sample is then agitated until aggregation is obtained. The aggregated sample is then centrifuged at approximately 500 r.p.m. for five minutes (radius equal to 8-10 inches) which allows the aggregates and red cells without sedimenting the individual platelets. Thereafter, the individual platelet concentration in the supernatant can be measured and the measurement obtained compared to a control which constitutes a sample of the same blood processed in the same manner but without the addition of an aggregating chemical. Suitable means for effecting such a measurement include Coulter Counters or the electronic counting
3,694,161

devices, hemocytometers, and turbidometric measuring devices.

As will be seen, the use in this invention of whole blood instead of plasma allows separate determinations of the aggregation response to be obtained on individual blood volumes of only 0.5 ml. Thus, a 7 ml blood sample can be utilized for fourteen separate determinations using the present invention so that one can utilize four dose levels of each of three aggregating agents, plus two control tubes, to obtain a complete picture of the aggregating response for a given sample of blood. Obviously, larger donor samples could be utilized for still further testing in the same way without exceeding the practical volume collection limit. Since the shaking operation and, following that, the centrifugation operation is expected to be carried on simultaneously for all tubes, the time requirement for multiple sample measurements is low and entirely within the practical range. Also, maintenance of the blood at 37° C. from the time of collection through aggregation obviates the need to reincubate at 37° C. to restore platelet shape and function.

Measurement of the aggregating response at a single point in time (following the addition of the aggregating agent to the blood sample) is adequate for our purposes and avoids continuous recording of the aggregating response. Obviously, elimination of the recorder achieves a further cost savings.

Following completion of the shaking operation, the samples may be centrifuged in a conventional International Model-K Centrifuge. Finally, a platelet-rich plasma samples are pipetted off, diluted appropriately, and platelet counts are measured with the Coulter Counter.

To aid in the understanding of the present invention, and not by way of limitation, reference is made to the following examples.

EXAMPLE I

To each of 12 test tubes, 0.05 mls. of anticoagulant solution (3.8% trisodium citrate) is added. The tubes are then placed in a 37° C. water bath. A 6 ml blood sample is taken from the subject's arm vein by suitable means, such as with a disposable syringe and needle. Aliquots of 0.5 ml of blood are added to each of the 12 tubes. Varying concentrations of an aqueous collagen suspension are added to 6 tubes (0, 2, 4, 8, 16 and 32 μg./tube). Simultaneously, varying concentrations of an aqueous solution of A.D.P. (0.05, 0.1, 2, 4 and 8 μg./tube) are added to the other 6 tubes. Such additions can be accomplished using a multi-channel pump or by other suitable means. The tubes are shaken for 5 minutes as by agitating a tube holder until the aggregating response is produced. Thereafter, the contents of the tubes are centrifuged at 500 r.p.m. for a five minute period and the plasma is drawn from each tube after centrifugation. Platelet counts are determined for each of the plasma samples using a Coulter Counter Model A or similar device. The resulting figures obtained provide a complete profile for the two different aggregating reagents over the concentrations shown.

EXAMPLE II

Replicate A.D.P. Induced platelet aggregation responses in a given subject

To show the reproducibility of platelet aggregation data in man using the methodology of this invention, the following study was performed.

Seven, 3 ml blood samples were taken from one healthy male volunteer at 45 minute intervals. Utilizing the procedure of Example I, platelet aggregation responses were obtained for each sample using A.D.P. as the aggregating agent at concentrations of 0, 0.5, 1, 2, 4, 8 μg./tube. Samples #1 through #3 were obtained from the subject during the morning hours. Samples #4 and #5 were taken after the subject had eaten lunch. Samples #6 and #7 were taken after the subject had exercised in an attempt to produce a stress situation. The resulting percent aggregation figures for all seven A.D.P. dose-response curves are shown below:

<table>
<thead>
<tr>
<th>Percent Aggregation</th>
<th>μg. A.D.P./tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample No.</td>
<td>0.5 1 2 4 8</td>
</tr>
<tr>
<td>1</td>
<td>15 44 55 79 85</td>
</tr>
<tr>
<td>2</td>
<td>13 38 69 81 93</td>
</tr>
<tr>
<td>3</td>
<td>35 30 65 79 91</td>
</tr>
<tr>
<td>4</td>
<td>11 27 65 81 92</td>
</tr>
<tr>
<td>5</td>
<td>8 32 63 83 92</td>
</tr>
<tr>
<td>6</td>
<td>8 27 62 88 94</td>
</tr>
<tr>
<td>7</td>
<td>7 37 65 79 96</td>
</tr>
</tbody>
</table>

From the data, it can be concluded that replicate platelet aggregation responses to A.D.P. are quite uniform; eating produces no change in platelet sensitivity to A.D.P.; and exercise, to the extent carried out, produces no change in platelet sensitivity to A.D.P.

EXAMPLE III

To further demonstrate the capabilities of the methodology of the present invention, the following experiment was performed:

Blood samples of 6 ml volume were taken from each of four healthy male volunteers. Utilizing the procedure of Example I, platelet aggregation responses were carried out with collagen at levels of 0, 2, 4, 8, 16 and 32 μg./tube and with A.D.P. at levels of 0, 0.5, 1, 2, 4 and 8 μg./tube.

Each subject then ingested one 5 grain aspirin tablet (a drug generally known to inhibit collagen-induced platelet aggregation in animals and man). Four hours later a second 6 ml blood sample was taken from each subject and tested in a manner identical to that of the earlier sample.

The results showed that prior to aspirin ingestion, there are differences between individuals for both collagen-induced and A.D.P.-induced platelet aggregation. Aspirin shifted all of the collagen dose-responses to the right, that is, reduced the aggregation for the respective dose levels. The post-aspirin collagen curves were quite similar for all subjects.

Aspirin showed little or no effect on the A.D.P.-induced aggregation.

EXAMPLE IV

Comparing conventional Bryston Aggregometer methodology, with the method of this invention provides an interesting contrast. If the aspirin experiment of Example III was conducted with the Bryston apparatus, 100 to 120 mls. of whole blood would be required from each subject. Only 12 mls. of blood were needed from each subject to obtain the same degree of quantitation using the present invention. Further, Bryston requires each aggregation determination to be carried out individually so that the time involved is vastly greater than that needed with the present invention which employs simultaneous measurements. Thus, determination requiring 30 minutes with this invention would require about 3 hours using the conventional Bryston approach.

Finally, the method of the invention in which the aggregation response is carried out in whole blood rather than plasma is considered “more physiological” than earlier techniques.

The ability of platelets to aggregate or clump together is thought to be of considerable importance physiologically, in terms of maintenance of normal hemostasis. Pathologically, the aggregation of platelets is thought to be a key etiologic factor in many types of thromboembolic disease.

While considerable progress has been made toward a more complete understanding of the causes and effects of platelet aggregation in animal studies, the rate of generation of new information from studies in man has been slow.
because of the absence of efficient methodology for studying the platelet aggregation response in man. It is believed that the subject matter hereof fills that void by bringing to the art new concepts for the study of platelet aggregation. Restated, the present invention involves multiple, simultaneous tests of aggregation responsiveness on aliquot of fresh, whole blood.

From the foregoing it becomes apparent that a new and improved method has been herein described which fulfills all of the aforestated objectives in a remarkably unexpected and unobvious fashion. More particularly, a method is described which reproducibly, accurately and efficiently measures the aggregation response of platelets to one or more aggregating agents, such as collagen, A.D.P., epinephrine, thrombin, antigen-antibody, etc., whereby quantitative differences in aggregation responses of platelets as they relate to variables such as age, sex, disease condition, effects of drugs, etc. may also be studied and it is believed that measures of the platelet aggregation response will be of great clinical importance in a variety of circumstances.

Accordingly, what is claimed is:

1. The method of measuring at physiologic temperature the platelet aggregation response in an aliquot sample of whole blood to which has been added an anticoagulant, comprising: adding an aggregating chemical to said sample of anticoagulated whole blood; agitating said sample at said temperature for a time sufficient to obtain aggregation; centrifuging said aggregated sample at a speed which will sediment the aggregates and red cells without sedimenting individual platelets; and thereafter measuring the individual platelet concentration in the supernatant for comparison with a like measurement obtained from a sample processed without the addition of an aggregating chemical.

2. The method of claim 1 in which said aggregating chemical is selected from the group consisting of adenosine diphosphate (A.D.P.), collagen suspension, epinephrine, serotonin, thrombin, latex particles and antigen-antibody complexes.

3. The method of claim 1 in which said anti-coagulant is selected from the group consisting of sodium oxalate, trisodium citrate and heparin.

4. The method of claim 1 wherein a plurality of samples are measured simultaneously.

5. The method of claim 4 wherein to each sample is added a different concentration of the same aggregating chemical.

6. The method of claim 4 wherein to each sample is added a different aggregating chemical.

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