A thrombocyte consumption test for the demonstration of autoantibody-like serum factors

GY. DÓBIÁS¹

From the Municipal Hospital, Budapest

SYNOPSIS The capacity of blood platelets to attach themselves aspecifically to antigen-antibody complexes in the presence of complement can be utilized for the demonstration of antibodies formed against homologous tissues.

A mixture of the serum-, antigen-complement, and of human thrombocytes is incubated, and the number of thrombocytes in the supernatant is ascertained and referred to a standard. Measurements less than 85% of the standard are regarded as positive.

Sera collected from patients with 'protracted' acute or chronic hepatitis and cirrhosis were found to be positive with the new method in 68.0% of the cases; the corresponding figures were 26.3% for 'non-protracted' acute hepatitis and 18.1% for diseases of the bile duct. No positive reaction occurred in other internal diseases studied or in healthy persons, but 40% of subjects with diverse infections gave positive results possibly because of autoantibody formation against tissues damaged by the infections. Comparative examinations suggest that the thrombocyte consumption test is more specific than the antihuman globulin consumption method.

In the presence of all components of complement (Siqueira and Nelson, 1961) blood platelets are aspecifically attached to the complex formed by corpuscular (Lamanna, 1957; Nelson and Nelson, 1959) and soluble antigens (Miescher and Cooper, 1960; Osler, Hawrisiak, Ovary, Siqueira, and Bier, 1957; Siqueira and Nelson, 1961) with their antibodies. This might explain why in states of allergy (Stavitsky, Stavitsky, and Ecker, 1949; Rocha e Silva, 1950; Storck, 1951; Storck, Hoigné, and Koller, 1951; Nilzén, 1953; Rice, 1955; Storck, and Hoigné, 1956; Korossy and Gózony, 1959; Bier and Siqueira, 1959) the thrombocyte count diminishes significantly after the administration of homologous antigen. Although it is only in recent years that investigators have gained some insight into these processes, Rieckenberg employed thrombocytes as a specific indicator system for the examination of *Leptospira* and *Trypanosoma* antibodies as far back as the year 1917. Hoigné and Storck elaborated in 1953 a thrombocyte-agglutination procedure for the diagnosis of drug sensitivity.

The present investigations were instituted to decide

if the decrease in the thrombocyte count after antigen-antibody reactions could be used to detect autoantibodies against homologous tissues and organs. The sera of patients suffering from various hepatic disorders were tested, and homogenized human liver was employed as antigen. We compared the results of examinations with the antihuman globulin consumption test carried out simultaneously.

MATERIALS AND METHODS

ANTIGEN The livers of young persons who had died accidentally were washed a few hours after death with 20 l. of ice-cold physiological saline through a cannula inserted into the portal vein, and then homogenized in a Waring blender. The liver brei was washed with ice-cold physiological saline until the last washing fluid gave a negative reaction for protein with a 20% solution of sulphosalicylic acid. The washed liver suspension was then distributed in 1 ml. ampoules and stored at -20°C . The content of each ampoule was suspended in 24 ml. of sterile veronal buffer² at pH 6.0–6.2 before use. The antigen suspension contained 5 to 10 mg./ml. of dry matter.

²Sodium chloride, 8.38 g., 5, 5-diethyl barbituric acid, 0.46 g., sodium 5,5-diethyl barbitol 0.30 g., sodium bicarbonate 0.25 g., magnesium sulphate, 0.012 g., and water to 200 ml.

¹Present address: Postgraduate Medical School, Budapest, XIII. Szabolcs-utca 33, Hungary.

Received for publication 17 September 1962

CLINICAL MATERIAL The patients were grouped clinically as follows: 44 cases of acute infective hepatitis, chronic hepatitis, or cirrhosis; 11 of cholecystitis; 15 of other internal diseases, comprising hyperthyroidism, generalized arteriosclerosis, heart injuries, hypertension; 15 of various infectious diseases comprising pneumococcal pneumonia, acute pharyngitis, influenza, acute tonsillitis, *Bact. coli* sepsis, and infectious mononucleosis. Fourteen healthy individuals were included in the trial. Blood was taken from the patients two to five times at weekly intervals; the 14 healthy individuals had only one bleeding. Two hundred and fifty samples were thus collected. The sera of patients were preserved sterile at -20°C . without the addition of any preservative.

STANDARD SERA Sera from five healthy persons were used as standards. The arithmetical mean of the thrombocyte count of these sera was used as the reference serum for values obtained from the test sera. It is advisable to retain large amounts of standard sera for continual reference throughout the tests.

THROMBOCYTE SUSPENSION Human blood platelets were used. Blood, drawn separately for each test from the cubital vein of healthy donors, was collected in a paraffin- or silicone-coated graduated centrifuge tube containing enough 3.8% sodium citrate to form a 1:5 mixture with the added blood. After centrifuging this mixture for eight minutes at 500 r.p.m., the thrombocyte count in the supernatant fluid was determined. A suspension containing 200,000 to 240,000 thrombocytes/c.mm. was used; if the value turned out to be higher, centrifugation was continued until the desired density was reached when the plasma was transferred to a paraffin-coated test tube, gently stirred, and used without delay. All superfluous mechanical operations which might damage the platelets, e.g., pouring or shaking, must be avoided. The whole procedure should be quick: no more than half an hour must elapse between the withdrawal of blood from the donor and its application.

REACTION Carefully cleaned test tubes (70×8 mm.), pretreated with sulphuric acid, were employed for the test. The ingredients were pipetted from accurately calibrated pipettes in the following order: 0.5 ml. of the serum to be tested (or 0.5 ml. of the standard sera), 0.1 ml. of the thrombocyte suspension (in the plasma containing fresh

thrombocytes complement is introduced to the system), and 0.4 ml. of antigen suspension. The test tubes were gently shaken and placed in a 37°C . water bath for 30 minutes. Shaking was repeated at 15 minutes. After incubation, the tubes were left at room temperature for 10 minutes, and then the number of thrombocytes per 0.01/c.mm. of the supernatant was determined. As the process is based on the linking of part of the thrombocytes added to the system, the method has been called the thrombocyte consumption test.

Not more than 20 sera were tested together so that the whole procedure could be finished within two hours from pipetting the ingredients.

THROMBOCYTE COUNT The chamber method of Hegedüs (Bálint and Hegedüs, 1959) was employed. A few drops of the supernatant were aspirated from the test tubes by paraffin-coated Pasteur pipettes and filled both fields of the Bürker chamber. The number of thrombocytes was determined in five rectangles per field, and the results added. The total represented the thrombocyte count per 0.01/c.mm. of the supernatant. (Where the distribution of thrombocytes is uneven, 10 rectangles per field are counted, and the result divided by 2.) The margin of error is negligible: if the tests are performed with precision, the extreme values of the standard sera will remain within the limits of $x \pm 5 \frac{x}{100}$ where x = the arithmetical mean of the number of thrombocytes in the standard sera.

ANTIHUMAN GLOBULIN CONSUMPTION TEST The consumption test was performed according to Steffen's original description (Steffen, 1954; 1955; Steffen and Schindler, 1955) with the antigen referred to above. The reaction was considered positive provided that the Coombs serum showed a twofold or even bigger decrease in titre.

RESULTS

The number of thrombocytes in the test sera, expressed as the percentage of the arithmetical mean of the thrombocyte values in the standards, is shown in Table I. In about a third of the test sera the number of thrombocytes was between 80 and 90% of the standard; in the remainder the results were below 80 and above 90% in descending sequence. The results

TABLE I
DECREASE IN THROMBOCYTE COUNT ACCORDING TO DISEASE

Diagnosis	Number of Serum Samples	Percentage of Arithmetical Mean of Thrombocyte Count in Standard Sera (arithmetic mean of standards = 100%)				
		<70%	70-79%	80-89%	90-99%	≥100%
'Protracted' acute hepatitis ¹ , chronic hepatitis, cirrhosis	113	8	27	44	18	16
'Non-protracted' acute hepatitis ²	59	2	6	17	15	19
Cholecystitis, obstructive jaundice	26	0	0	6	12	8
Healthy persons	14	0	0	0	7	7

¹Time in hospital more than 6 weeks; average, 57 days ²Time in hospital less than 6 weeks; average, 26 days

TABLE II

RESULTS OF ANTIHUMAN GLOBULIN AND THROMBOCYTE CONSUMPTION TESTS IN THE EXAMINED PATIENTS

Diagnosis	No. of Patients	Thrombocyte Consumption Test		Antihuman Globulin Consumption Test	
		Positive	Negative	Positive	Negative
'Protracted' acute hepatitis, chronic hepatitis, cirrhosis	25 ¹	17 (68.0%)	8 (32.0%)	19 (76.0%)	6 (24.0%)
'Non-protracted' acute hepatitis	19	4 (26.3%)	15 (73.7%)	9 (47.5%)	10 (52.5%)
Cholecystitis, obstructive jaundice	11	2 (18.1%)	9 (81.9%)	3 (27.2%)	8 (72.8%)
Various infections	15	6 (40.0%) ²	9 (60.0%)	9 (60.0%)	6 (40.0%)
Other internal diseases	15	0 (0.0%)	15 (100.0%)	3 (20.0%)	12 (80.0%)
Healthy persons	14	0 (0.0%)	14 (100.0%)	2 (14.2%)	12 (85.8%)

¹'Protracted' acute hepatitis 18, chronic hepatitis 4, cirrhosis 3²The six positive cases of diverse infections comprised pneumococcal pneumonia 2, infectious mononucleosis 1, influenza 2, *Bact. coli* sepsis 1.

TABLE III

EFFECT OF CORTICOSTEROID TREATMENT ON THROMBOCYTE CONSUMPTION TEST
IN 17 POSITIVE PATIENTS IN THE FIRST GROUP OF TABLE II

	Thrombocyte Consumption Test		Antihuman Globulin Test	
	Positive	Negative	Positive	Negative
During steroid treatment	7 (36.8%)	12 (63.2%)	7 (35.0%)	13 (65.0%)
Before and after steroid treatment	26 (63.4%)	15 (36.6%)	30 (73.1%)	11 (26.9%)

are different if the test sera are divided into categories according to clinical diagnosis. Most of the sera obtained from patients suffering from protracted acute or chronic hepatitis and cirrhosis contained fewer thrombocytes than 90% of the standard, but this value was above 90% in most cases in the sera of other patients and in the healthy persons. Table I shows that there is a correlation between the clinical picture and the decrease in the thrombocyte count.

The boundary between positively and negatively reacting test sera was found to be at 85% of the standard, and at this level the best correlation with the clinical state as well as with the results of the antihuman globulin consumption test carried out at the same time (Table II) was found. Test sera with a thrombocyte count below this value will be termed 'positive' and those above it will be called 'negative'. 'Negative' only applies to those patients whose sera had never given a positive reaction. Seventeen patients of the first group in Table II were 'positive' so serum from these patients was collected on 60 occasions, and positive reactions in 33 and negative in 27 instances were obtained. The reaction turned most frequently negative when the patient had received corticosteroids (Table III), a finding in accordance with the results of Vorländer (1954), Rissel, Steffen, and Wewalka (1957), Osztovcics, Marcsek, and Szász (1962). One or two days after starting administration of steroids the positive thrombocyte consumption test became negative, but a few days after ending the treatment it became positive again (Vorländer, 1954; Rissel *et al.*, 1957).

The most pronounced reduction in the thrombocyte count, *i.e.*, the most strongly positive case, was 48.2% of the standard. To study the reproducibility of the thrombocyte consumption test, 25 sera selected at random were analysed successively and the same results were obtained in 76.8%. Equivalent results seemed to be most frequent in sera which had been repeatedly frozen and thawed.

In some instances, thrombocytes showed fragmentation after the reaction. Since thrombocytes

TABLE IV

COMPARATIVE TESTS WITH LIVER AND HEART ANTIGEN

Serial No. of Cases	Diagnosis	Antigen	
		Liver	Heart
1	'Protracted' acute hepatitis	+	0
2	'Protracted' acute hepatitis	0	0
3	'Protracted' acute hepatitis	+	0
4	'Protracted' acute hepatitis	+	0
5	'Non-protracted' acute hepatitis	0	0
6	'Non-protracted' acute hepatitis	0	0
7	Obstructive jaundice	0	0
8	Obstructive jaundice	0	0
9	Cholecystitis	0	0
10	Carditis	0	+
11	Carditis	+	+
12	Carditis	0	0
13	Carditis	0	+
14	Carditis	0	0
15	Carditis	0	0
16	Carditis	0	+
17	Carditis	0	+

frequently appear as tiny particles under the microscope, the presence of dust or other granules in the preparation may easily give rise to errors; excessively turbid sera should not be used. Parallel analysis was carried out on the sera of adults suffering from various hepatic diseases and those of children suffering from carditis. Homogenized human liver and heart served as antigens. Results are summarized in Table IV.

The serum of one of the eight cardiac children reacted positively both with the heart and the liver antigen: the antihuman globulin consumption test was likewise positive in this case with both antigens.

DISCUSSION

These results justify the conclusion that the thrombocyte consumption test can be used to demonstrate autoantibody-like serum factors which react with homologous tissues. The reaction is based on the phenomenon of 'serological adhesion' (Lamanna, 1957; Nelson and Nelson, 1959) which is a two-phased process. First, the antigen-antibody-complement complex is formed, and to this, as a second step, the receptor substance covering the thrombocyte surface is linked. The hypothetical compound in the blood serum promoting the linking was named 'thrombocytobarin' by Kritschewsky and Tscherikower (1925). The precise mechanism of linking is not yet known. According to our observations, if we first added the serum containing the antibodies to the antigen, and only afterwards the complement and plasma containing thrombocytes were added to the system we obtained less satisfactory results.

The method under investigation has the advantage of simplicity. It does not require a serial cold washing of the mixture antigen-test serum, nor does it require an indicator system. This may be why no positive reaction occurred either in the sera of patients suffering from 'other' internal diseases or in those of the healthy individuals. On the other hand, 40% of the patients with infections did give positive reactions

which can be explained in two ways. First, in the various infections with a positive thrombocyte consumption test (Table II) the bacterial or virus infection or toxic impact may have injured the liver parenchyma sufficiently to promote the formation of autoantibodies against the hepatic tissue. Secondly, the high positivity might be attributable to the heterogeneous character of the antigen: liver homogenate contains not only hepatic cells but connective-tissue elements as well. Possibly the antibodies demonstrated are not liver but connective tissue specific. Further investigations of this hypothesis are in progress.

We are much indebted to Dr. Pál Baranyai, chief physician of the laboratory of the Heim Pál Hospital, and to Dr. Éva Kálmán for the blood serum of children with carditis and for the heart antigen.

REFERENCES

- Bier, O. G., and Siqueira, M. (1959). *Proc. Soc. exp. Biol. (N. Y.)*, **101**, 502.
- Bálint, P., and Hegedüs, A. (1959). *Klin. lab. diagn.* 159.
- Hoigné, R., and Storck, H. (1953). *Schweiz. med. Wschr.*, **83**, 718.
- Korossy, S., and Gózon, M. (1959). *Acta allerg. (Kbh.)*, **13**, 286.
- Lamanna, C. (1957). *Bact. Rev.*, **21**, 30.
- Kritschewsky, I. L., and Tscherikower R. S. (1925). *Z. Immun-Forsch.*, **42**, 131.
- Miescher, P., and Cooper, N. (1960). *Vox Sang. (Basel)*, **5**, 138.
- Nelson, R. A. Jr., and Nelson, D. S. (1959). *Yale J. Biol. Med.*, **31**, 201.
- Nilzén, A. (1953). *Acta derm.-venerol. (Stockh.)*, **33**, 456.
- Osler, A. G., Hawrisiak, M. M., Ovary, Z., Siqueira, M., and Bier, O. G. (1957). *J. exp. Med.*, **106**, 811.
- Osztovics, M., Marcsek, Z., and Szász, G. (1962). *Gyermekgyógyászat*, **13**, 97.
- Rice, C. E. (1955). *J. Immunol.*, **75**, 85.
- Rieckenberg, H. (1917). *Z. Immun-Forsch.*, **26**, 53.
- Rissel, E., Steffen, C., and Wewalka, F. (1957). *Wien. klin. Wschr.*, **69**, 885.
- Rocha e Silva, M. (1950). *Ann. N.Y. Acad. Sci.*, **50**, 1045.
- Siqueira, M., and Nelson, R. A. Jr. (1961). *J. Immunol.*, **86**, 516.
- Stavitsky, A. B., Stavitsky, R., and Ecker, E. E. (1949). *Ibid.*, **63**, 389.
- Steffen, C. (1954). *Wien. Z. inn. Med.*, **35**, 422.
- (1955). *Klin. Wschr.*, **33**, 134.
- , and Schindler, H. (1955). *Schweiz. Z. Path.*, **18**, 287.
- Storck, H., and Hoigné, R., (1956). *Dermatologica, (Basel)*, **112**, 405.
- , Koller, F. (1951) *Schweiz. med. Wschr.*, **81**, 195.
- (1951). *In C.r.I. Congr. internat. Allerg. Zürich, Karger, Basel*, 1952.
- Vorländer, K. O., (1954). *Verh. dtsh. Ges. inn. Med.*, **60**, 242.