Study of Leukopenias and Thrombocytopenias by the Direct Antiglobulin Consumption Test on Leukocytes and/or Platelets

By Jean Dausset, Jacques Colombani and Monique Colombani

The existence of leukopenia and thrombocytopenia of immunologic origin has been postulated for some years, but in vitro evidence has hitherto been lacking.

As the serous or indirect antiglobulin consumption test (indirect ACT) on leukocytes or platelets devised independently by Steffen1 and by Moulinier2 will not differentiate iso-antibodies developing after transfusion or pregnancy on the one hand from auto-antibodies on the other6, the cellular or direct antiglobulin consumption test (direct ACT) has been devised,3,4 by means of which it is possible to demonstrate the presence or absence of γ-globulin, on or in the patient's own platelets or leukocytes.

A known quantity of anti-human globulin serum is added to the washed leukocytes or platelets of the patient. After a suitable time the anti-human globulin serum is removed from the cells and its potency determined by titration against Rh positive red cells sensitized with incomplete anti-D serum, in parallel with an equal volume of the same reagent that has been in contact with normal platelets or normal leukocytes.

Material and Methods

Direct and indirect ACT was carried out in parallel on the platelets and leukocytes of 492 individuals, of whom 122 were examined at least twice. Twenty-four cases of diffuse lupus erythematosus (DLE), 111 patients with thrombocytopenia, 128 with leukopenia or leukothrombocytopenia, 101 miscellaneous non-cytopenic patients and 128 normal controls were examined. All cases of cytopenia seen over a two-year period were tested except (1) extreme cytopenias, where the direct test was technically impossible, and (2) some cases of secondary cytopenia, which occurs commonly in leukemic or malignant states.

Leukopenia was defined as a count of less than 4,000 leukocytes, granulocytopenia less than 2,000 granulocytes, and thrombocytopenia less than 140,000 platelets, all per cubic mm. Some patients show leukopenia or thrombocytopenia only during a definite stage of the disease, often only at the onset, and cases which were cytopenic less than two months before testing were included in the investigation. Numerous serologic investigations listed among the additional technics were also performed. Clinical and hematologic data were carefully analyzed in an attempt to evaluate the significance of the ACT.

Methods

All technics were carried out in siliconed glassware. All centrifuging was done at 4 C.

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Van Loghem et al.5 have shown that some auto-antibodies are still effective on papainized leukocytes, whereas iso-antibodies are not.
DIRECT ACT ON LEUKOCYTES AND PLATELETS

Direct ACT on Platelets and Leukocytes

1. Collection of blood: In the case of the normal controls, 45 ml. of blood were taken into 5 ml. of EDTA (disodium sequestrene) 5 per cent in saline. When a patient suffering from leukopenia or thrombocytopenia was to be examined, larger quantities of blood were usually needed.

2. Separation and washing of platelets: The blood was centrifuged for 10 minutes at 180 g. The plasma, rich in platelets, was removed and replaced by an equal quantity of 0.33 per cent EDTA in saline (platelet washing solution). The mixture was then centrifuged again, for 10 minutes at 180 g. (When the blood was from thrombocytopenic patients it was found better to centrifuge a number of times for short periods to avoid sedimentation of very large platelets, which are often found in such specimens.) The supernatant from the first centrifugation of the leukocyte suspension (see below) was added to 1/10 its volume of EDTA 5 per cent in saline and also mixed with the plasma rich in platelets. This platelet suspension was then washed six times with the platelet washing solution, the first centrifuging being carried out for 30 minutes at 1600 g, and subsequent ones for 15 minutes at 1600 g.

After the platelets had been washed five times, they were counted and adjusted so as to obtain a deposit containing 2 x 10^9 platelets after the sixth washing.

3. Separation and washing of leucocytes: The deposit obtained after the first centrifuging of the whole blood, containing red cells, white cells and many platelets, was suspended in three times its volume of a solution of gum acacia in saline.* The mixture was poured into tubes 14 cm. long and 1.6 cm. diameter. These were inclined at an angle of 45° and the red cells allowed to sediment for 20 minutes at room temperature. The tubes were then stood upright and sedimentation allowed to proceed for a further 10 minutes. The supernatant was then separated and this constituted the leucocyte suspension used. It should be noted that five times as many red cells as leucocytes remained in the suspension. After centrifuging the latter for 10 minutes at 180 g, the supernatant, rich in platelets, was separated and added to the platelet suspension as already described in section 2. The leucocytes and red cells forming the deposit were then washed six times with 1.2 per cent NaCl solution, each centrifuging being carried out for 10 minutes at 180 g. At the end of the fifth washing, the leucocytes and red cells were counted, and the amount of leucocytes adjusted in order to obtain a deposit containing 150 x 10^6 leucocytes after the sixth washing.

4. Separation and washing of red cells: Red cells were pipetted off the bottom of a tube of blood centrifuged for 15 minutes at 1600 g. They were practically free of leucocytes or platelets. Approximately the same number of red cells as was present in the leucocyte deposit were washed 10 times in 0.85 per cent saline, and served as a control for the leucocyte/red cell mixture.

5. Contact with the antiglobulin serum: An antiglobulin serum having a titer of at least 8,000 was used nine twofold dilutions below its end point. To each washed deposit of platelets, leukocyte/red cell mixture, and red cells, was added 0.15 ml. diluted antiglobulin serum. After the cells and serum had been incubated for 6 minutes at room temperature, the mixture was centrifuged, the supernatant quickly separated, and double diluted in saline.

*Ten Gm. of white gum acacia powder and 1 Gm. of anhydrous phosphate (Na2HPO4) were put in a mortar and 90 ml. of distilled water added slowly while the mixture was ground until homogeneous. The preparation was then autoclaved for 10 minutes at 116 C. at a pressure of 1 Kg. The container was kept at 4 C, until a precipitate of colloidal calcium phosphate had sedimented out over a period of approximately 24 hours. The supernatant was then removed and stored at 4 C. for a maximum of one week. Before use, 3 volumes of saline were added to 1 volume of the original solution; 4 volumes of this diluted solution were added to 1 volume of the packed cells from the first centrifugation of the whole blood (Greenwalt and Polka6).
6. Titration of the antiglobulin serum: This was performed by a standard indirect antiglobulin technic using group O Rh positive cells sensitized with an incomplete anti-D serum.

7. Interpretation: No or small decrease in the titer of the antiglobulin serum was obtained with the controls (normal platelets, normal leukocytes, and the red cells of the patient). A test was considered positive when the fall in titer was at least two dilutions. During this study only two different antiglobulins and anti-D were used.

Indirect ACT on Platelets and Leukocytes

1. Preparation of sera: These were inactivated by heating at 56 C. for 30 minutes* after which each specimen was added 1/10 volume of 2 per cent sequestrene.

2. Preparation of the leukocyte and platelet deposits: These were prepared in the same way and at the same concentration as those for the direct test, but were washed three times only.

3. Contact with the serum: Three-tenths ml. of serum was added to leukocyte and platelet deposits derived from the same whole blood, and the mixture incubated at 37 C. for one hour.

4. Washing of platelets and leukocytes: Leukocytes and platelets were then washed six times in their respective washing solutions.

All subsequent stages of the reaction and interpretation were identical to those in the direct test.

Other Technics

The following test methods were used: Direct antiglobulin platelet test (Dausset and Malinvaud7); Leucocyte agglutination (Dausset*); platelet agglutination at 37 C. (Dausset, Colin and Colombani9); platelet agglutination at 4 C. (Dausset and Malinvaud7); leukocyte, platelet or DNA precipitation (Seligmann10); Complement fixation tests with leukocyte and platelet extracts (Milgrom et al.11); Cr51-tagged platelet survival times (Najean, Larrieu and Bernard12). The method of fractionation of the serum was that of Dubert et al.13 The method of isolation of the leukocyte nuclei was from Nathan and Snapper.14 Leukocyte cytoplasm was precipitated by ethanol from the supernatant obtained after separation of the nuclei (Colomiani15).

Results

1. Results with the Direct Antiglobulin Consumption Test

In 52 cases, positive results were obtained both with platelets and with leukocytes, and of these 22 were suffering from DLE. Two tests were positive with leukocytes alone. Fifty-nine tests, of which 46 came from patients with idiopathic thrombocytopenic purpura (ITP), gave positive results only with platelets; 379 tests were negative both with platelets and with leukocytes.

After exclusion of the cases of DLE, a good correlation was found between the result of the direct ACT and the presence or absence of a cytopenia. Of the 183 thrombocytopenic patients, 42.2 per cent gave a positive result with platelets. Of the 128 leukopenic patients, 25.8 per cent gave a positive result with their leukocytes, whereas 98 per cent of the 299 individuals with neither leukopenia nor thrombocytopenia gave negative results both with leukocytes and with platelets. Nevertheless, no correlation could be established between the level of cytopenia on the one hand and the degree of antiglobulin consumption on the other. A severe cytopenia sometimes gave only a weakly positive

*We observed later that the inactivation of the sera increased the basic consumption.
Table 1.—Complete Results Obtained with the Direct Leukocyte and Platelet ACT

<table>
<thead>
<tr>
<th></th>
<th>Positive on leukocyte and platelet</th>
<th>Positive on leukocyte and negative on platelet</th>
<th>Negative on leukocyte and positive on platelet</th>
<th>Negative on leukocyte and platelet</th>
<th>Total number of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of cases</td>
<td>% total cases</td>
<td>Number of cases</td>
<td>% total cases</td>
<td>Number of cases</td>
</tr>
<tr>
<td>D.L.E.</td>
<td>32</td>
<td>91.6</td>
<td>0</td>
<td>1</td>
<td>4.15</td>
</tr>
<tr>
<td>Primary thrombocytopenia</td>
<td>0</td>
<td>0</td>
<td>46</td>
<td>49.4</td>
<td>47</td>
</tr>
<tr>
<td>Secondary thrombocytopenia</td>
<td>0</td>
<td>0</td>
<td>3*</td>
<td>16.6</td>
<td>15</td>
</tr>
<tr>
<td>Primary leukopenia and leukothrombocytopenia</td>
<td>21</td>
<td>48.7</td>
<td>1</td>
<td>2</td>
<td>4.1</td>
</tr>
<tr>
<td>Secondary leukopenia and leukothrombocytopenia</td>
<td>8</td>
<td>10</td>
<td>17</td>
<td>1.2</td>
<td>3</td>
</tr>
<tr>
<td>Miscellaneous non leukothrombocytopenic patients</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Normal controls</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>128</td>
</tr>
<tr>
<td>Total</td>
<td>52</td>
<td>2</td>
<td>59</td>
<td>379</td>
<td>492</td>
</tr>
</tbody>
</table>

*1 infectious, 1 malignant, 1 due to other known cause.
*2 toxic, 5 malignant, 1 due to another known cause.

consumption test, while a mild one might well give a much stronger result. It was also noticed that some patients who had clinically recovered, notably after splenectomy, still showed a positive reaction.

Correlation with hyperplasia or aplasia in the corresponding cell type of the marrow is much more difficult to establish. Most of the patients with thrombocytopenia giving a positive ACT had abnormal megakaryocytes, sometimes decreased in number and sometimes increased, with raised or lowered platelet production. Most of the patients with leukopenia giving a positive result showed a hypoplastic or even an aplastic marrow, but the criteria were not strict enough to permit of any definite conclusion.

If the cytopenic patients are further divided into those in whom the etiology of the condition was known, and those in whom it was unknown, six groups are formed (table 1 and fig. 1):

1. The 93 primary ITP gave positive results with platelets only in 49.4 per cent of cases.
2. The 18 secondary thrombocytopenias gave positive results in only 3 cases (16.6 per cent) with platelets only.
3. The 48 primary or idiopathic leukopenias or leukothrombocytopenias (ILT) gave a positive result on both leukocytes and platelets in 43.7 per cent of cases. One case was positive with leukocytes only and two with platelets only.
4. The 80 secondary leukopenias or leukothrombocytopenias gave a positive result with both leukocytes and platelets in 10 per cent of cases. One case gave a positive result on the leukocytes only and three on platelets only.
5. Among the 101 miscellaneous patients with neither leukopenia nor thrombocytopenia, only one gave a positive result with platelets and leukocytes, and 4 with both platelets and leukocytes.
Fig. 1.—Over-all results of the direct antiglobulin consumption test on leukocytes and platelets of 492 subjects. The columns represent the number of cases tested. On the left, the direct ACT on the patients’ leukocytes, and on the right, on the patients’ platelets. The shaded parts represent the positive results, and the unshaded parts, the negative results.

(6) None of the 128 normal controls gave positive results.

An attempt was made to subdivide the secondary cases according to the cause of the cytopenia into (a) toxic (where the administration of a known cytopenic drug was associated with a fall in the cell count); (b) infective; (c) malignant (where no antimetabolic or antimitotic drug was being used; and (d) other known causes of cytopenia, such as cirrhosis of the liver and Banti’s disease.

In secondary thrombocytopenia, of the three cases giving positive results on platelets, one was classified as infectious, one as malignant, and one was due to another known cause. Among the secondary leukopenias or leuko-thrombocytopenias, six were toxic in origin, five malignant, and one due to another known cause.

Among those patients who had no cytopenia, only one gave a positive result with both leukocytes and platelets. This patient had cirrhosis of the liver with multiple thrombosis.
Four patients in this group gave a positive ACT with platelets only, three having an atypical rheumatoid purpura and one a hemorrhagic syndrome without thrombocytopenia.

2. Results with the Indirect Antiglobulin Consumption Test

As has been shown by Van Loghem et al., a positive indirect ACT may be due either to iso- or to auto-antibodies against leukocytes or platelets. In the survey of our results of 492 subjects tested with this test, all cases of possible isoimmunization were eliminated.

The remaining cases showed a good correlation with the direct ACT (fig. 2). Eighty per cent of the cases with a direct ACT on platelets were associated with a positive indirect ACT. This percentage was 84.4 per cent in cases of ITP and 75 per cent in ILT. Seventy-eight per cent of the positive direct ACT on leukocytes were associated with a positive indirect ACT on the cells. In only four cases was a positive indirect ACT associated with a negative direct test. The close correlation between the two tests suggested that the substances fixed on the patient’s cells, and free in his serum, were identical.

Serologic characteristics of the free substances responsible for the indirect ACT in DLE, ITP and ILT: All three substances are heat stable at 56°C; they are not destroyed by heating for 10 minutes at 70°C; they are well preserved at -20°C; they cannot be absorbed by BaSO4; they react best at 37°C at a pH of between 6 and 8, they have all been found in the γ-globulin fraction. Their specificities have been studied by absorption/elution technics, using platelets, leukocyte nuclei and leukocyte cytoplasm as the antigens.

One ml. of serum was absorbed four times using 10 billion normal platelets each time and the nuclei or precipitated cytoplasm provided by 500 million normal leukocytes. Each absorption was carried out at 37°C for 1 hour. At the end of the fourth absorption, the serum was tested using the ACT, a negative finding showing in each case that all the substance had been removed. Elutions were performed by heating the antigens to 56°C for 30 minutes in 1 ml. of 2 per cent sodium chloride solution. The resulting eluates were then rendered isotonic by dilution with distilled water. The results as shown in table 4 demonstrate that:

(1) Sera from ITP contain a substance directed specifically against platelets.
(2) ILT sera contain two substances directed respectively and specifically against platelet and leukocyte cytoplasm,
(3) DLE sera contain three substances directed respectively and specifically against platelets, leukocyte nuclei and leukocyte cytoplasm.

Two experiments enabled us to differentiate the three antiplatelet substances and the two antileukocyte substances:

(a) It has been shown elsewhere (Dausset, Colombani and Colombani) that the antiglobulin component reacting with the antiplatelet substance of ITP is not identical with the component reacting with the other two antiplatelet substances. Thus the anti-platelet substance of ITP possesses a different serologic reactivity from that found in DLE or ILT sera.
(b) It has also been shown that the antileukocyte and antiplatelet substances present in the serum of patients suffering from DLE do not posses species specificity. They react with ox, pig, sheep and horse, as well as with
human, leukocytes and platelets. On the contrary, the antiplatelet substances present in ITP sera, and the antileukocyte and antiplatelet substance present in ILT, do not react with animal leukocytes or platelets (table 2).

3. Relationship with Other Serologic Tests

A. Direct tests

(1) The platelet antiglobulin test was performed systematically in parallel with the direct ACT. It was found positive in 7 cases of ITP, one case of hemorrhagic syndrome, one case of DLE, and two cases of atypical rheumatoid purpura. It was in all cases associated with a positive ACT (fig. 2).

(2) The L.E. test was performed in all cases of DLE and in most cases of leukopenia and of leukothrombocytopenia. It was positive in 17 cases of DLE and was always associated with a positive direct ACT. The latter appears more sensitive, remaining positive during remissions after the L.E. test has become negative. The L.E. test was never positive in ILT even when the direct ACT on the leukocytes was strongly positive (fig. 2).

B. Indirect tests

(1) Leukocyte and platelet agglutination tests were performed in all cases at 37°C, using at least three batches of leukocytes. Positive results were obtained in 38 cases, all of which were the result of isoimmunization by multiple transfusions and/or pregnancies, with the exception of one case of DLE in which a weak leukocyte agglutinin was found. There was no relationship between the presence of leukocyte agglutinins and the direct ACT. Some leukocyte agglutinins gave rise to a positive indirect ACT on leukocytes. Platelet agglutinins at 37°C were detected in 12 of these leukocyte agglutinating sera.

<table>
<thead>
<tr>
<th>Table 2.—Antiglobulin Consumption Test on Leukocyte Nucleus and Cytoplasm and on Platelet from Some Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibodies from</td>
</tr>
<tr>
<td>L.E.D.</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>I.T.P.</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>I.L.T.</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>Iso-antibody</td>
</tr>
<tr>
<td>9</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>Controls</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

N = Leukocyte nucleus.
C = Leukocyte cytoplasm.
P = Platelet.
The figures are the number of geometric dilutions lost by the antiglobulin titer.
Fig. 2.—Relationship between the direct antiglobulin consumption test on leukocytes and/or platelets and other direct or indirect immunologic tests. Each semicircle represents the results of the direct ACT. The spotted zone represents positive tests, and the white zone negative tests. The other test done simultaneously, when positive, is represented by the lined zone. The absence of this zone signifies a negative result of the other test.

(2) Platelet agglutinins, detected best at 4 C., were observed in 17 per cent of sera from cases of ITP with a positive direct ACT on platelets, as compared with only 4.3 per cent positive in those who gave a negative direct test. The significance of this finding is unknown.

(3) The complement fixation test on leukocytes, platelets, or DNA as well as leukocyte, platelet or DNA precipitin tests were found to be positive in those sera from cases of DLE which gave positive indirect ACT on leukocytes and/or platelets (Seligmann).[[85]](6)

(4) Ovary’s test (P. C. A.), performed with ITP sera giving a positive ACT, remains negative (Coombs,[[19]](6) Ovary).[[31]](6)

(5) The complement titer was normal in most cases, but the mean value in ITP with a negative ACT on platelets was lower than in cases where the test was positive.

4. Relationship Between the Direct Antiglobulin Consumption Test and Clinical Findings

The symptoms and signs which have been compared in the four groups of positive and negative thrombocytopenia, and positive and negative leuko-thrombocytopenia are listed in table 3. The differences observed will be discussed below.
Table 3.—Comparison of Some Characteristics in Cases Suffering from Idiopathic Thrombocytopenic Purpura and/or Idiopathic Leukothrombocytopenia with Positive or Negative Results Studied by the Direct Antiglobulin Consumption Test on Platelets and/or Leukocytes (% of Presence in Cases Studied)

<table>
<thead>
<tr>
<th></th>
<th>ITP 47 positive cases</th>
<th>ITP 47 negative cases</th>
<th>ILT 21 positive cases</th>
<th>ILT 3% negative cases</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age at the onset:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 years old</td>
<td>40.4</td>
<td>63.8</td>
<td>0</td>
<td>16.1</td>
</tr>
<tr>
<td><strong>History of infection</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>13.5</td>
<td>34.1</td>
<td>5.8</td>
<td>25.0</td>
</tr>
<tr>
<td><strong>Splenomegaly</strong></td>
<td>10.6</td>
<td>10.6</td>
<td>9.5</td>
<td>66.7</td>
</tr>
<tr>
<td><strong>Increased bleeding time</strong></td>
<td>82.9</td>
<td>61.0</td>
<td>58.3</td>
<td>84.2</td>
</tr>
<tr>
<td><strong>Non-platelet-producing megakaryocytes</strong></td>
<td>73.7</td>
<td>53.6</td>
<td>80.0</td>
<td>66.6</td>
</tr>
<tr>
<td><strong>Acute history (4 months)</strong></td>
<td>14.9</td>
<td>29.8</td>
<td>5.5</td>
<td>15.3</td>
</tr>
<tr>
<td><strong>Fatal</strong></td>
<td>10.6</td>
<td>2.1</td>
<td>33.3</td>
<td>31.3</td>
</tr>
</tbody>
</table>

Other characteristics have been studied, without any significant differences:

- **Etiology**
- **Symptoms**
- **Laboratory data**
- **Course and treatment**
  - **Sex**
  - **Pregnancies**
  - **Familial history**
  - **Intoxication**
  - **Hepatomegaly**
  - **Adenopathy**
  - **Sites of hemorrhagic syndrome**
  - **Anemia**
  - **Reticulocytes**
  - **Leukocytes**
  - **Marrow**
    - **Cellularity**
    - **Erythroblasts**
    - **Granulocytes**
    - **Lymphocytes**
    - **Megakaryocytes**
  - **Abnormal cells**
  - **Gamma globulins**
  - **Hemostasis**
  - **Recovery**
  - **Controlled more than 1 year**
  - **Not controlled**
  - **Transfusions (number)**
  - **Action of corticoids**
  - **Weak doses**
  - **High doses**
  - **Action of splenectomy**
  - **Immediate**
  - **Long term**

**Discussion**

The existence of leukopenia or thrombocytopenia due to immunologic causes has been generally accepted for several years. The direct ACT provides a method for the detection of an abnormal substance or a normal substance abnormally located on the blood cells. The test seems to give valid results, despite technical difficulties.

One of these difficulties is the basic consumption occurring after contact with normal cells both in the direct and indirect tests. This basic consumption leads to a decrease in the antiglobulin titer of not more than one doubling dilution, this being a constant finding with normal controls. By Outcherlony technic, the ratio of albumin/γ-globulin present in extracts of normal leukocytes and platelets was found similar to the serum ratio. In positive ACT leukocyte and platelet, the γ-globulins were increased (Seligmann)². From a technical point of view, two main methods are available for the ACT. Steffen¹ prefers to prepare five dilutions of his antiglobulin serum be-
forehand, and to test each against a separate volume of sensitized antigen. Moulinier,2 Van Loghem3 and Daussé prefer to dilute the antiglobulin serum after it has been in contact with one volume of sensitized antigen. The advantages and disadvantages of the two methods will be discussed elsewhere,14 but Steffen's technic requires too many cells for it to be used in the direct test.

The test requires the antigenic structure of the substance on the cells tested to have something in common with the anti-D used in the third stage of the reaction. Thus the substance detected, like anti-D, is probably a γ-globulin.

The γ-globulin fixed on or into the cells might be manufactured by the cell itself or it might come from the plasma. In either case, the γ-globulin could be an antibody, or a γ-globulin of no immunologic significance absorbed onto the cell. If it were an antibody, then it might have become non-specifically attached, or might be specifically attached to an antigen. The latter situation would correspond to that of an auto-antibody. Were the antibody merely absorbed on to the cell surface, then normal sera would be likely to be able to coat the cell and bring about such a reaction in the indirect test. It has been shown, however, that only pathologic sera can bring about this reaction, which shows a strict specificity for either platelets, or leukocytes, or both. Even where platelets and leukocytes are both sensitized, it has been shown that two substances are involved. This specificity, and the physicochemical nature of the substance, are in favour of the antibody hypothesis.

As in all other autoimmune diseases, the exact nature of the antigen involved remains unknown, and even its site is still not very definite. Our fractionation experiments involved only the nucleus and cytoplasm of leukocytes. It seems reasonable to assume that the cytoplasmic antigen and the platelet antigen are not situated on the surface of the cell. They may be hidden in a fold of the extracellular membrane or may indeed be intracellular. The following points favor these suggestions:

(1) Leukocyte or platelet autoagglutinins are very rarely observed.
(2) The antiglobulin mixed cell agglutination test is negative (Crombs19).
(3) The direct antiglobulin test is occasionally positive on platelets, but, in ITP at any rate, this occurs only when a strongly positive direct ACT can be obtained with these cells. Studies on specificity enabled a clear cut differentiation to be made between seven substances (table 5).

(1) An antiplatelet substance in ITP.
(2) An antiplatelet substance and an antileukocyte-cytoplasm substance in ILT.
(3) An antiplatelet substance, an antileukocyte-cytoplasm substance and an antileukocyte-nucleus substance in DLE.

None of these substances are identical. The three antiplatelet substances have been differentiated by the use of two different batches of antiglobulin serum, while those of DLE differ further by the absence of species specificity. The two antileukocyte-cytoplasm substances are differentiated by the absence of species specificity in DLE (table 5). The indirect ACT may also be positive as a result of the presence of antibodies developed after multiple isoimmunizations. Iso-antibodies can be differentiated from other antibodies by their agglutinating power.
Table 4.—Anticytoplasma, Antinucleus and Antiplatelet Antibodies Studied by the Indirect Antiglobulin Consumption Test

<table>
<thead>
<tr>
<th>Name</th>
<th>Trans-</th>
<th>Preg-</th>
<th>Direct ACT</th>
<th>Before absorption*</th>
<th>After absorption on</th>
<th>After absorption on</th>
<th>After absorption on</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>fusions</td>
<td>nacies</td>
<td>L</td>
<td>N C P</td>
<td>leukocyte nucleus</td>
<td>leukocyte cytoplasm</td>
<td>platelet</td>
</tr>
<tr>
<td>All.</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>8 7 8</td>
<td>0 6 8 3 1 0</td>
<td>7 0 8 0 4 0</td>
<td>8 7 0 0 0 3 8</td>
</tr>
<tr>
<td>Pag.</td>
<td>1</td>
<td>0</td>
<td>+</td>
<td>7 6 8</td>
<td>0 6 8 2 0 0</td>
<td>0 8 0 4 0 5 6 0 3 8</td>
<td></td>
</tr>
<tr>
<td>Sot.</td>
<td>0</td>
<td>0</td>
<td>+ (4)</td>
<td>6 6 8</td>
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</tbody>
</table>

L = Leukocytes, C = Leukocyte cytoplasm, N = Leukocyte nucleus, P = Platelet.

*The figures are the number of geometric dilutions lost by the antiglobulin titer (titrated against 0 Rh+ sensitized by incomplete anti-D) after contact with the antigens sensitized by the auto- or iso-antibody.
DIRECT ACT ON LEUKOCYTES AND PLATELETS

Table 5.—Differentiation Between the Antileukocyte and Antiplatelet Antibodies Detected by the Indirect Antiglobulin Consumption Test

<table>
<thead>
<tr>
<th>Antileukocyte antibodies</th>
<th>Action on animal leukocyte or platelet</th>
<th>Agglutinating power on leukocyte or platelet</th>
<th>Antibodies detected only by some antiglobulin sera</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLE</td>
<td>++</td>
<td>±</td>
<td>-</td>
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<tr>
<td>ILT</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Iso-antibody</td>
<td>-</td>
<td>+</td>
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</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antiplatelet</th>
<th>Action on animal leukocyte or platelet</th>
<th>Agglutinating power on leukocyte or platelet</th>
<th>Antibodies detected only by some antiglobulin sera</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLE</td>
<td>++</td>
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<tr>
<td>ILT</td>
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<td>ITP</td>
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<td>+</td>
</tr>
<tr>
<td>Iso-antibody</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

+ = presence, ± = doubtful presence, - = absence of characteristic.

The presence of platelet antibodies and of antibodies against leukocyte cytoplasm as distinct from antibodies against the leukocyte nucleus has been suggested by Seligmann21,22 and by Asherson.23

Clinical Discussion

Whatever may be the significance of the γ-globulin detected by the direct ACT on either platelets or leukocytes, the test has been found positive in three groups of patients:

1. In diffuse lupus erythematosus, the direct ACT is almost always positive on both platelets and on leukocytes, although it is unusual to find a lowering of the cell counts. This finding may possibly be explained as follows: Incubation of the cells in their own plasma was found both by Van Loghem et al.16 and by ourselves to enhance the direct leukocyte ACT, suggesting that the reaction took place better in vitro than in vivo. Cinematographic films by Robineaux24 showed that the antibodies had a specificity for cell nuclei, the cytoplasm being rejected and the nuclei alone phagocytized. Nevertheless, beside antinucleus antibodies (Miescher²⁰), the existence of anti-cytoplasmic antibodies has been proved. One must admit that an antibody against cytoplasm would not cause phagocytosis.

The 22 cases of DLE were followed for 2 to 3 years. Although their symptoms diminished markedly, the ACT nevertheless remained positive. More cases must be tested before we can be dogmatic about the sensitivity of the test, but it seems to be at least equal to the other tests available.

2. The existence of a serologic factor in idiopathic thrombocytopenic purpura was demonstrated in 1951 by the in vivo experiment of Harrington et al.25 Since that time, many in vitro tests have been devised, but none have showed a good correlation with the clinical data. Harrington et al.26 claimed to detect warm platelet agglutinins in 74 per cent of his adult cases. Tullis,27 using his own lysis-agglutination test, found 57 per cent positive in ITP and 66 per cent in “hypersplenism.” Steffen,28,29 using the indirect ACT, and Van der Wiel,30 using a similar technic, obtained respectively 66 per cent and 56 per cent positive reactions in ITP. All of these results were obtained using the patient’s
serum, but the platelets were from other individuals and the possibility of isoimmunization could not always be excluded.

In our hands the direct platelet ACT provided evidence that sensitization had probably occurred in vivo in 30 per cent of patients suffering from ITP, as well as 16 per cent of cases of secondary thrombocytopenia. The indirect ACT was positive in 85 per cent of those who gave a direct positive result.

A careful study was made of the two groups of ITP, the one giving a positive result and the other negative, with the direct ACT. Apart from the fact that a previous history of infection was more common and that the course of the disease was sometimes shorter in those cases in which the ACT was negative, no obvious difference could be found in the history, the clinical picture, or hematologic findings. Children were more numerous and the course of the disease shorter in the negative group. Corticosteroid therapy seemed to make no difference to the strength of the antibody among those who were positive, while these drugs seemed to have the same effect on patients, whether they belonged to the positive or negative groups. The test became negative in 38 per cent of cases following splenectomy (fig. 3). Some patients made a complete clinical recovery although the test remained positive. The immediate effect and long term result of splenectomy were studied in the two groups, but it is too early to conclude whether splenectomy is of more benefit in one group than in the other. Homologous or autologous Cr$^{31}$-labeled platelets, when transfused into the patient, survived for periods that bore no correlation to the presence or absence of a detectable antibody.12

3. The existence of a serologic cause for leukopenia or leukothrombocyto-
penia has not been as well established in the past as is the case with ITP. Nevertheless, transfusion of leukopenic blood was shown by Koszewski\textsuperscript{11} to cause a leukopenia in the recipient. Antileukocyte substances have been shown to be present in serum by Rejholec (76 per cent),\textsuperscript{12} Steffen,\textsuperscript{20} Van Loghem et al.,\textsuperscript{16} using the indirect ACT. Using the lysis-agglutination test, Tullis\textsuperscript{15} observed 61 per cent of positives in idiopathic cases and 83 per cent in "hyper-splenism."

By the direct leukocyte and platelet ACT it appeared that about 50 per cent of ILT and 15 per cent of secondary leukothrombocytopenias were sensitized in vivo to their own platelets and/or leukocytes. The indirect leukocyte and platelet ACT were positive in 79 per cent of the cases with a direct positive test, showing that the abnormal substances were usually free in the serum as well as fixed to the cells. Other tests, such as the L.E., and the complement fixation test in the presence of platelet or leukocyte extract, were negative, and also leukocyte agglutination, where there was no possibility of previous isoimmunization.

Cases of ILT were divided into two groups according to the finding of a positive or negative result with the direct ACT, and again a careful analysis of the history, clinical picture and hematologic findings was made. In the latter category, a lower mean age and a high frequency of infection was found. It is too early to classify ILT as a distinct entity, although it must be pointed out that both the serology and the clinical picture of DLE and ILT differ widely. Whereas the direct ACT is positive in both cases, the indirect ACT behaves differently. In DLE there is an antinuclear antibody, absent in ILT, while the antibodies of the former fail to show any species specificity.

It is impossible to be sure whether the substances detected by the leukocyte or platelet ACT are pathogenic or not. The role of the L.E. factor as a pathogenic substance is itself a matter of controversy. The demonstration of anticytoplasmic antibodies in DLE is rather difficult to reconcile with the normal leukocyte count in most of our cases. The same problem arises with ITP and ILT, but with these conditions it is important to notice that a positive finding is always accompanied by a depletion of the corresponding series. No correlation was, however, found between the intensity of the antiglobulin consumption and the cell count. Indeed, some cases of apparently cured ITP continue to show a strong direct platelet ACT.

In ILT, while the low white cell and platelet counts might be attributed to antileukocyte and antplatelet substances, the cause of the anemia remains obscure, for it is unusual to detect red cell antibodies in such cases. It might be supposed that the antileukocyte substances react with an antigen on a red cell precursor in the bone marrow during red cell maturation. However, other data must be accumulated before an answer can be given of the pathogenic role of the substances here detected.

The direct leukocyte and platelet ACT test is a new tool. It has some theoretical importance in that it enables the subdivision of thrombocytopenia and leukothrombocytopenia into two groups, according to whether or not \(\gamma\)-globulin can be found on the leukocytes or platelets. This abnormal, or abnormally located, \(\gamma\)-globulin could be an auto-antibody. Positive results are strikingly
more frequent in patients suffering from idiopathic forms of the conditions,
the findings thus resembling those in autoimmune hemolytic anemias, which
are also divided into idiopathic and secondary forms. It must again be em-
phasized that the test is positive only when the \( \gamma \)-globulin on the leukocytes
or platelets has some part of its structure in common with the anti-D used in
the final stage of the ACT. It is not impossible that by using another revealing
system, some of the negative results would become positive.

From a practical point of view, the direct test is time consuming and re-
quires a large quantity of blood to be taken from the patient. It need not be
performed on patients when the indirect ACT test is clearly positive, without
any history of isoimmunization. It must be admitted, however, that such pa-
tients, especially among cases of pancytopenia, are uncommon. The test
should be performed where there is a previous history of transfusion and the
indirect test is positive, in order to differentiate between iso- and auto-anti-
bodies. It is indicated in those patients having a negative indirect ACT, as the
latter was negative in 15 per cent of cases who had a positive direct ACT. In
our opinion the ACT is the first available biological method for the demonstra-
tion of intracellular antibodies, for no other test has as yet been developed for
the demonstration of antibody fixed within the substance of a cell.

**Summary**

A new test, the direct antoglobulin consumption test (direct ACT), has been
devised to be performed on the leukocytes and platelets of patients. It was
performed in parallel with the indirect antoglobulin consumption test (indirect
ACT), and other serologic tests on 492 individuals comprising 24 cases of dif-
fuse lupus erythematosus, 93 primary thrombocytopenias, 18 secondary
thrombocytopenias, 48 primary leukopenias or leukothrombocytopenias, 80
secondary leukopenias, 101 non-leukothrombocytopenic patients and 128 nor-
mal subjects.

A good correlation was obtained between a positive or negative result and
the presence or absence of a cytopenia in the corresponding cell series. Out
of 183 thrombocytopenic patients, 42.2 per cent gave a positive result with platelets, and out of 128 leukopenic patients, 25.8 per cent gave a positive re-
sult on leukocytes, whereas of the 299 patients with normal leukocyte and platelet counts, 98 per cent gave negative results. The test was found positive
in three categories of patients:

1) In **diffuse lupus erythematosus**, tests on both leukocytes and platelets
were almost uniformly positive. The indirect ACT permits a distinction
to be made between three substances in the serum of these patients.
These are antiplatelet, antileukocyte-cytoplasm and antileukocyte-nuclear
substances.

2) The **thrombocytopenic patients** were subdivided into two groups, namely
primary and secondary thrombocytopenia:

(a) Out of 93 cases of idiopathic thrombocytopenic purpura, about
50 per cent gave a positive result with platelets. Neither the
history nor the clinical picture suggested any differentiation be-
tween those who gave negative results, apart from the fact that
a history of infection was more frequent among the negative cases. Corticosteroid therapy did not affect the result of the test, but following splenectomy, 38 per cent of the cases become negative.

(b) Of 18 patients with a secondary thrombocytopenia, three cases (16 per cent) gave a positive result.

(3) The leukopenic and leukothrombocytopenic patients were also subdivided into two groups:

(a) Out of 48 primary or idiopathic cases, some 50 per cent gave a positive direct ACT either on leukocytes and/or on platelets. By using the indirect ACT it was possible to distinguish two substances in the serum, one being antiplatelet and the other an anti-leukocyte cytoplasmic substance.

(b) The 80 cases of secondary leukopenia or leukothrombocytopenia gave a positive result in 15 per cent of cases with leukocytes and/or with platelets.

Of the 101 non-leukothrombocytopenic patients, only five were found to give positive tests.

All the 128 normal subjects gave negative results.

The direct ACT provides direct evidence of the presence of a γ-globulin, probably an auto-antibody, on the leukocytes and/or the platelets of some 99 per cent of cases of diffuse lupus erythematosus, of about 50 per cent of cases of idiopathic thrombocytopenia and idiopathic leukothrombocytopenia, and in about 15 per cent of cases of secondary thrombocytopenia and leukothrombocytopenia. No marked difference was found in the history, clinical picture, or hematological findings between patients giving positive and negative results.

**Summario in Interlingua**

Esseva elaborate un nove test, le directe test del consumption de antiglobulina (directe TCA), a effectuar in le leucocytos e plachettas del paciente. Illo esseva effectuate in parallela con le indirecte test del consumption de antiglobulina (indirecte TCA) e altere probas serologic in 492 individuos. Iste includeva 24 patientes con diffuse lupus erythematosus, 93 con thrombocytopenias primari, 18 con thrombocytopenias secundari, 48 con leucopenias primari o leucothrombocytopenias, 80 con leucopenias secundari, e 101 non-leucothrombocytopenicos si ben como 128 subjectos normal.

Un bon correlation esseva trovate inter positive o negative resultatos del test e le presentia o absentia de cytopenias in le correspondente series cellular. In le gruppo de 183 patientes thrombocytopenic, 42,2 pro cento manifestava un resultato positive con le uso de plachettas, e in le gruppo de 128 patientes leucopenic, 25,8 pro cento manifestava un resultato positive con le uso de leucocytos, durante que in le gruppo de 299 patientes con normal numerationes leucocytic e plachettal, 98 pro cento produceva resultatos negative. Le test se mostrava positive in tres categorias de patientes:

1. In diffuse lupus erythematosus, tests in leucocytos etiam in plachettas esseva quasi uniformemente positive. Le indirecte TCA permitte le differentiation
de tres substantias in le seros de iste patientes. Iste substantias es un substantia anti plachettas, un substantia anti cytoplasma leucocytic, e un substantia anti nucleo leucocytic.

2. Le patientes thrombocytopenic esseva subdividite in duo gruppos secondo que lor condition esseva primari o secundari.

a) In le gruppo de 93 patientes con idiopathic purpura thrombocytopenic, circa 50 pro cento manifestava positive resultatos con le uso de plachettas. Ni le antecedentes ni le presente aspecto clinic suggereva un differentiation inter le resultatos positive e negative, a parte le facto que un anamnese de infeccion esseva plus frequente inter le casos negative. Therapia a corticostereides non afficeva le resultato del test. Tamen, post splenectomia, 38 pro cento del casos previemente positive deveniva negative.

b) Inter le 18 patientes con un thrombocytopenia secundari, tres casos (16 pro cento) produceva un resultado positive.

3. Le patientes leucopenic e leucothrombocytopenic esseva similemente subdividite in duo gruppus:

a) In le gruppo del 48 casos primari o idiopathic, circa 50 pro cento maniifestava un positive directe TCA con leucocytos e/o con plachettas. Per le uso del indirecte TCA il esseva possibile distinguere duo substantias in le sero. Le un esseva un substantia anti plachetta, le altere un substantia anti cytoplasma leucocytic.

b) In le gruppo del 80 casos de secundari leucopenia o leucothrombocytopenia, 15 pro cento manifestava un resultado positive con leucocytos e/o con plachettas.

Inter le 101 patientes non leucothrombocytopenic, solmente cinque esseva trovate con tests positive.

Omne le 128 subjectos normal manifestava resultatos negative.

Le directe TCA provide directe evidentia pro le presentia de un globulina gamma, probablemente un auto-anticorpore, super le leucocytos e/o piachettas de 99 pro cento, plus o minus, del casos de diffuse lupus erythematose, de circa 50 pro cento del casos de thrombocytopenia idiopathic e leucithrombocytopenia idiopathic, e de circa 15 pro cento del casos de secundari thrombocytopenia e secundari leucothrombocytopenia. Nulle marcate differentia esseva trovate in le anamnese, le aspecto clinic, o le constatationes hematologic inter patientes con resultatos positive e illes con resultatos negative.

ACKNOWLEDGMENTS

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Study of Leukopenias and Thrombocytopenias by the Direct Antiglobulin Consumption Test on Leukocytes and/or Platelets

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