Detection of Leukocyte Antibodies by the Complement Consumption Test

By V. CHUDOMEL, Z. JEZKOVA AND J. LIBANSKY

LEUKOCYTE ANTIBODIES can at present be demonstrated by various methods, the best known of which is the agglutination and antihuman-globulin consumption test.\textsuperscript{1-10,12-14,17-21,27} Several of these methods suffer from various deficiencies, and either lack sensitivity or are difficult to perform. It is therefore justified to search for new methods of investigation. This paper presents a new method by which leukocyte antibodies can be demonstrated by the decrease in the amount of complement \((C')\) in the serum examined after its contact with the leukocyte antigen.

METHODS

It can be assumed that the antigen-antibody reaction involves a certain amount of complement consumption. If complement consumption can be ascertained it means that the antigen-antibody reaction has taken place and that the serum contains leukocyte antibodies. The decrease in complement in this serum after its contact with the antigen can be determined by comparison with the amount of complement of the same serum to which no antigen has been added. Since leukocyte antibodies are weak as a rule and thus difficult to demonstrate, and since complement consumption is very small, it is important to find a method by which even this minute amount can be detected. For this purpose the complement contained in the serum itself is used for the reaction. Active serum is required, contrary to the usual complement methods, in which inactivated serum with an addition of guinea pig complement is used. It is of no consequence that the amount of complement differs in different individuals and may even vary in a single individual, since the evaluation of both positive and negative results is not dependent on the absolute amount of complement but on the difference of the amount of \(C'\) in the serum of the patient with and without the antigen.

The procedure described is divided into three parts: (1) the separation of leukocytes, (2) the antigen-antibody reaction, that is, the influence of the examined serum on the added leukocytes (fixation phase), and (3) the titration of complement (hemolytic phase, fig. 1).

1. Separation of leukocytes.—At the beginning this is done in a way similar to that described by Dausset\textsuperscript{7} but using dextran instead of polyvinylpyrrolidon. Two hundred and fifty ml. of group O blood suffices for about 8 examinations. After defibrination the blood is mixed with high molecular dextran (molecular weight about 200,000) in the ratio of 1:0.5. After sedimentation of the erythrocytes (45 min. at 37 C.) the supernatant liquid containing leukocytes and an admixture of erythrocytes is pipetted into conic test tubes of ca. 10 ml. and centrifuged for 10 minutes at 2,500 to 3,000 revolutions per minute. In this way a sediment of leukocytes with an admixture of erythrocytes is obtained. The sediment of the leukocytes is then washed with saline. After further centrifugation—10 minutes at 2,500 to 3,000 revolutions per minute—a sediment of leukocytes is obtained which after thorough removal of saline may be used as antigen for further reactions.

2. The fixation phase.—1.5 ml. of the active serum examined are put into each of two...
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Fig. 1.—The scheme of the detection of leukocyte antibodies by the complement consumption test. The active serum examined (3 ml.) is divided into two equal parts. To one part, antigen (sediment of leukocytes) is added and thoroughly mixed—upper row (A). To the other part no antigen is added—lower row (B). The second phase: incubation of both sera in the thermostat, at 37 C. for one hour (reaction antibody-antigen in the upper row [A]). Last phase: titration of C' of both sera and comparison between the titer of C' of serum A and serum B.

Test tubes. The serum in the first tube (A) is mixed with sedimented leukocytes (10 millions of leukocytes are added to 1 1/2 ml. of serum); the serum in the second tube (B) is left without leukocytes. The serum containing leukocytes (A) is then thoroughly mixed and left, together with B, for one hour in a thermostat at 37 C. During this time the serum containing leukocytes should be shaken several times in order to mix thoroughly. After one hour both sera are taken out of the thermostat, the leukocytes are thoroughly centrifuged and the supernatant serum poured into previously marked test tubes. In the presence of leukocyte antibodies consumption of C' occurs in the serum to which leukocytes were added as opposed to that serum without the addition of leukocytes and in which, therefore, no complement consumption takes place (fig. 1).

3. **Titration of C'.**—The examined serum which had been in contact with leukocytes (A, upper row) and that without leukocytes (B, lower row) are diluted with saline in the ratio of 1:20 and thoroughly mixed. 0.05 ml. of serum is pipetted into the first tube and 0.05 ml. more into each of the following tubes to a total of 0.95 ml. Saline is added to each tube to bring the amount up to 1 ml. 0.25 ml. of the hemolytic system (sensitized sheep erythrocytes) is then added and all test tubes placed for 15 minutes into a water bath of 37 C. (fig. 2). For the preparation of the hemolytic system five units of amboceptor are used. After 15 minutes the degree of hemolysis of sheep erythrocytes is read in both sera. The rack with the tubes is then left for another 15 minutes at room temperature, after which follows the final reading. Complete hemolysis is marked by —, partial hemolysis by ± to + +, no hemolysis by + + +. For example: while titrating C' in the lower row (B) the first complete hemolysis of sheep erythrocytes occurs in a certain tube. Since the amount of serum in this tube is known, how many units of C' it contains can be calculated. The same happens in a certain tube in the upper row (A). If no complement consumption took place during the antigen-antibody reaction, the first complete hemolysis can be observed in the same tube, both in the upper and the lower row. If, on the other hand, the consumption of C' takes place, the first complete hemolysis in the upper row occurs in the test tube with a higher amount of C', so that there is a difference between
The sedimentated leukocytes which are used as an antigen should be as dry as possible, i.e., without traces of saline, so as to avoid a dilution of the
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Table: Serum of a patient with acquired haemolytic anaemia.

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Table: Serum of a patient with SLE and acquired haemolytic anaemia.

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Figure 5 (top).—Result of the complement consumption test against erythrocytes of a group O donor in two cases of acquired haemolytic anemia. The result shows that, under the conditions of the experiment, no complement consumption occurred during the antigen-antibody reaction.

Figure 6 (bottom).—The serum of a patient with malignant reticulosis and that of a patient with acute leukemia were examined the same day by two methods: 1.) both sera were examined by the method described (active serum); 2.) the sera were inactivated and human complement added. The results show that in the first case (malignant reticulosis) antibodies could not be detected after inactivation of the serum, whereas in the second case, after inactivation of the serum, a marked decrease in the titer of antibodies against leukocytes occurred.

Serum and the complement in the upper row (A) which could influence the results.

2. Since the preparation of sedimented leukocytes involves the risk of an admixture of a certain amount of erythrocytes it could be argued that the presence of erythrocytes has no influence on the results if antibodies against erythrocytes are present in the serum examined. However, in several cases studied no complement consumption could be detected during the reaction between red cells and isoagglutinins. We further examined a few sera from patients with acquired hemolytic anemia, and no complement consumption could be proved by the manner described (fig. 5). Further studies concerning the consumption of C' in the antigen-antibody reaction of red cells by this method are in progress.

3. The fixation phase requires a precise relation between the amount of antigen and the antibodies. It is important that there should always be enough antigen for the fixation of all antibodies to obtain the maximum reaction.
4. Only active serum and the complement of the examined serum itself should be used. If the serum is inactivated the titer of leukocyte antibodies can be lowered considerably, as has been demonstrated by our experiments (fig. 6). Mushell et al. have shown that the titer of antibodies against Salmonella typhi decreased fourfold through inactivation. Since the titer of leukocyte antibodies is very low as a rule, a further decrease caused by inactivation of the serum is not desirable. We are, therefore, working only with active serum and are using the complement of the same examined serum. We also assume that the reaction might be made more sensitive if human complement were used instead of guinea pig complement, as Mushell has shown when demonstrating antibodies against Salmonella typhi.

5. In the fixation phase, during the contact of the antibody with the antigen, the serum is not diluted, so as to prevent dilution of the antibodies and to enhance the antigen-antibody reaction. On the other hand, while titrating C', the serum is diluted; the ratio between the diluted serum and the amount of hemolytic system used is of great significance. This ratio was arranged so as to allow the reaction to take place only in the center of the row so that in case of strong antibodies a sufficient interval could be maintained between the tubes with the first complete hemolysis in both A and B. The reaction is arranged in such a manner that the first complete hemolysis in the lower row (without the antigen) occurs usually between the fifth and eighth tube (fig. 7). Greater differences in the amount of C' in the serum, both in the positive and negative sense, may shift the interval in either direction. If the amount of C' in the serum is abnormally small the serum should be less diluted (e.g., 1:10) and 0.25 ml. of hemolytic system used, or if diluted as usual (1:20) a smaller amount of hemolytic system is needed. (0.125 ml.).

In routine testing by our method only a short series, i.e., the first 10 tubes, was used for the first examination. If the complement consumption was high in the upper row or if the complement of the patient was too low and complete hemolysis did not occur even in the tenth tube of the lower row, the titration of C' of the same serum had to be repeated in a long series—19 test tubes. According to the amount of complement the ratio between dilution of the serum while titrating C' and the hemolytic system can be adjusted. One to 2 normal sera are always tested simultaneously. The time required for the testing of 10 sera by the complement consumption test is about 6 to 7 hours.

The Evaluation of the Reaction

By the method described above leukocyte antibodies can be demonstrated by means of complement consumption in the antigen-antibody reaction. The result of the reaction is given by the difference between the first completely hemolyzed tube in the upper and lower rows. A difference of two tubes is regarded as suspect, and one of three tubes as a proof of the presence of antibodies against the used antigen. This difference can easily be determined by macroscopic inspection. Photocolorimetric reading is not required. An exact quantitative evaluation of the antibodies and a mutual comparison of
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**Fig. 7 (top).**—The figure shows how the result of complement consumption changes with different dilutions of serum, everything else being equal.

**Fig. 8 (bottom).**—The results of leukocyte antibody examinations of 463 various sera by means of the complement consumption test.

different sera cannot be made only from the difference in the number of tubes between the first hemolytic tube in the upper and the lower rows because of the various amounts of C' in the examined sera. Only sera with the same content of C' can be mutually compared. It is also not possible to evaluate and compare quantitatively the result simply by giving the difference in the number of test tubes between the first hemolytic tube in the upper and lower rows because even if the difference is the same, complement consumption while titrating C' at the beginning of the row differs from that at the end of the row as a result of the gradual dilution of C' in the single tubes. If the titer of the antibodies is to be stated more exactly, this difference can be expressed in units of C' or in percentage of complement consumption.

The first test tube with a 100 per cent hemolysis in the upper and lower rows contains just the amount of C' necessary for the complete hemolysis of the sensitized sheep erythrocytes. The amount of complement in the first tube with a 100 per cent hemolysis in both rows is thus the same. It can, therefore, be estimated from the difference between the completely hemolyzed tubes in the upper and lower rows how many units of C'
were used for the antigen-antibody reaction. If the definition of one international unit of complement is known (the amount of complement that causes complete hemolysis of sensitized sheep erythrocytes at 37 C. in 30 minutes) the calculation can be made according to the formula, in which

$$x = \left( \frac{m - n}{mn} \right) D.H.;$$

$x$ is number of units of $C'$ in 1 ml. of the examined serum, $m$ the amount of serum in the first 100 per cent hemolyzed tube in the upper row (A, with added antigen), $n$ the amount of serum in the first 100 per cent hemolyzed tube in the lower row (B, without the antigen), $D$, dilution of the examined serum, and $H$ the amount of the hemolytic system. An additional advantage of this method is that it makes it possible to estimate at the same time the entire amount of $C'$ in 1 ml. of the examined undiluted serum according to the formula

$$\frac{1}{n} \cdot D.H.$$

However, for practical use it is sufficient to ascertain only the significant difference between the first tube with a 100 per cent hemolysis in the upper and lower row instead of estimating the amount of complement used for the antigen-antibody reaction.

**RESULTS**

We examined 463 sera, of which 280 sera were obtained from normal, healthy individuals, 105 sera from patients with various internal diseases and 78 sera from patients with blood disorders. In normal sera positive results were obtained in 3.9 per cent, from patients with internal diseases in 7.6 per cent. In disorders of the blood the result was positive in 23 per cent (18 cases out of 78 sera examined). (figs. 8 and 9). The positive sera in disorders of the blood were examined repeatedly, and the results were almost always identical. Only in some cases of long duration was there a change, e.g., in a patient with Felty's syndrome whose serum was strongly positive several times and became negative after splenectomy. The result was positive in one of 11 cases of pancytopenia, in one of seven cases of myelofibrosis, in three of 10 cases of leukopenia, in two of six of splenic pancytopenia, in two cases of Felty's syndrome, in 5 of 12 cases of acute leukemia, in one of five of chronic myeloid leukemia, in two of six of malignant reticulosis; there was one positive result in a patient with lupus erythemathodes and marked pancytopenia (fig. 9). Figure 10 shows in more detail the positive results in our patients with disorders of the blood.

Patients suffering from disorders of the blood were examined repeatedly during the course of their illnesses. The positive finding of leukocyte antibodies was also evaluated with regard to transfusions and other forms of therapy. The analysis of our results will be given in a later paper.

**DISCUSSION**

By comparing the positive results in normal sera, in the sera of patients with internal diseases, and in those with blood disorders, it has been shown
that the maximum positive results occur in patients with disorders of the blood, especially with acute leukemia in which the results were positive in five out of 12 examined sera (41 per cent). Eighteen cases with disorders of the blood and positive findings of leukocyte antibodies by the complement consumption test were also simultaneously tested by the agglutination method, with positive results in only five cases. A comparison of both methods showed that the complement consumption test gave positive results more often than did the agglutination test; however, more cases should be compared to render the opinion definite. The method seems to be sufficiently sensitive and reliable, if all procedures are strictly observed. In our opinion probably both complete and incomplete antibodies can be demonstrated by our method. It is, however, possible that antibodies which do not need complement for interaction with antigen are not detected by our method.

The method also proved of value for the demonstration of thrombocyte antibodies. The principle is the same but instead of leukocyte antigen, thrombocyte antigen—the sediment of thrombocytes—was used. Details will be given in a later paper. We also believe that this method can be used for the demonstration of various tissue antibodies. Work on this subject is in progress. We know that complement can be inactivated not only by antigen-antibody reaction, but also by human plasmin. It is therefore possible that the positive result of our reaction—that is, consumption of C—does not always indicate the presence of antibodies only, but also of fibrinolysis, especially in patients with blood disorders. A more detailed study of this problem is desirable.
A new method for the demonstration of leukocyte antibodies by the complement consumption test is described. For this test active serum and the complement of the examined serum itself is used. In the presence of antibodies against the antigen used, complement consumption takes place during the antigen-antibody reaction. This consumption can be estimated while titrating C' by the difference between the first completely hemolyzed test tube in the lower row (serum to which no antigen had been added), and the test tube with complete hemolysis in the upper row (serum with added antigen).

Two hundred-eighty normal sera, 105 sera of patients suffering from various internal diseases and 78 sera from patients with disorders of the blood were examined. The complement consumption test gave positive results more often than did the leukoagglutination method.

The method described was also successful in the demonstration of thrombocyte antibodies. We believe that it is equally suited for the detection of various tissue antibodies.
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Summario in Interlingua

Es describite un nove methodo pro le demonstrazione de anticorpore anti-leucocytic per medio del test de consumption de complemento. In iste test, sero active e le complemento del sero examinare mesme es usate. In le presentia de anticorpore contra le antigeno usate, consumption de complemento occurre durante le reaction de antigeno e anticorpore. Le consumption pote esser estimate in le titration de C' per le differentia inter le prime completamente hemolysate tubo de proba in le fila inferior (sero a que nulle antigeno ha essite addite) e le completamente hemolysate tubo de proba in le fila superior (sero a que antigeno ha essite addite).

Esseva examinate 280 seros normal, 105 seros de patientes con varie morbos interne, e 78 seros ab patientes con disordines hematologic. Le test de consumption de complemento producereva resultatos positive plus frequentemente que le test de leuco-agglutination.

Le metodo describite esseva etiam successose in le demonstration de anticorpore anti-thrombocytic. Nos crede que illo es equalmente usabile in le detection de varie anticorpores histic.

REFERENCES

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