Leukocyte Antibodies in Human Sera and in Immune Rabbit Sera

By Roy L. Walford, E. Taylor Peterson and Patricia Doyle

This report details the results of a serologic study of anti-human-leukocyte antibodies occurring in human sera and in the sera of specifically immunized animals. The sera of three patients shown to contain anti-human-leukocyte antibody and the sera of rabbits immunized with extracts of human leukocytes were investigated by a number of methods. It was found that leukocyte antibodies in human sera may be manifested as leukoagglutinins, as agglutinins for antigen-coated un-tanned sheep erythrocytes, as inhibitors of phagocytosis by leukocytes, and as inhibitors of the ameboid motility of leukocytes. Leukocyte antibodies in immune rabbit sera may be manifested as leukoagglutinins, leukoprecipitins, and as agglutinins for antigen-coated tanned sheep erythrocytes.

Methodology as employed in this study will be given in considerable detail, and commented upon at some length where indicated. This is because problems of basic technic still constitute the main obstacle to advancement in that branch of immunohematology which is concerned with antigen-antibody reactions of the white blood cell.

Interest in leukocyte antibodies may stem from several fields of inquiry. The role of these antibodies in leukopenias of both the drug-induced and idiopathic varieties, their association with blood dyscrasias, and the role of multiple transfusions in their development raise questions of fundamental hematologic importance. The possible relationship of these antibodies to the so-called "homograft" reaction is of considerable interest, and certain of the hematologic problems just enumerated may perhaps be fruitfully viewed by considering the development of leukocyte antibodies to be a variety of homograft reaction. On the other hand, the peculiar immunologic nature of the homograft reaction has itself resisted serologic study for many years, and it is hoped that the devising of improved in-vitro methods for examining homospecific antigen-antibody reactions of nucleated cells may also aid in clarifying certain problems in the homograft field.

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CASE MATERIAL

A survey of 36 patients with various hematologic diseases (Table 1) disclosed 3 whose sera contained leukocyte antibodies. As the sera of these 3 patients were extensively employed in the studies to be reported, a synoptic presentation of each case history is pertinent at this point.

Case 1. (E. R.)—The patient was a forty-one year old white male painter with a 2 to 4 year history of fatigue, anemia, and a mild bleeding tendency. There was a history of occupational exposure to benzol for 9 years. Laboratory data within the past year fell within the following ranges: hemoglobin 8 to 10.5 Gm. per 100 ml.; white blood cell count 2,700 to 3,600 per cu. mm. with differential count of 44% neutrophils, 1% eosinophils, 48% lymphocytes, and 7% monocytes; platelets 18,000 to 38,000 per cu. mm. A reticulocytosis up to 7% and an occasionally elevated urinary urobilinogen was interpreted as equivocal evidence of a hemolytic tendency. Bone marrow aspiration and biopsy appeared normocellular but with decreased numbers of megakaryocytes and with moderate myeloid immaturity. The patient had received multiple transfusions in the past several years. Diagnosis: chronic pancytopenia, probably due to benzol poisoning.

Case 2. (V. J.)—The patient was a forty-eight year old white female with a two month history of weakness, fever, pallor, and bleeding of the gums. The admission hemoglobin was 12.4 Gm. per 100 ml. The white blood cell count was 600 with 4% neutrophils, 92% lymphocytes, and 4% monocytes. There was a marked thrombocytopenia. Bone marrow aspiration showed 64% monocytes in various stages of maturation. Leukoagglutinins were first observed after 5 transfusions had been given—not previously tested. The patient expired 3½ months after onset, with severe generalized hemorrhages. Autopsy and clinical diagnosis: acute monocytic leukemia, Schilling type.

Case 3. (I. G.)—The patient was a seventy-two year old female with a two year history of severe anemia responsive only to transfusions. The hemoglobin was 6 to 7 Gm. per 100 ml. The white blood cell count was 2,650 to 4,650 per cu. mm. with differential count varying from normal to 42% neutrophils, 1% eosinophils, 1% basophils, 47% lymphocytes, and 8% monocytes. The platelets were adequate in number but qualitatively abnormal, with large bizarre forms in the peripheral blood. The reticulocyte count was 0.6%. Bone marrow aspiration appeared hypercellular with both myeloid and erythroid immaturity. The patient had received multiple transfusions, about 2 every 5 weeks, for a prolonged period. Diagnosis: refractory anemia, neutropenia, and thrombocytopenia, etiology undetermined.

METHODS AND RESULTS

Preparation of leukocyte suspensions

Suspensions of white blood cells to be employed in leukoagglutinin tests were prepared from the blood of normal individuals by the methods outlined below. White blood cells to be used in preparing lyophilized cell mass for immunization, precipitin tests, and other procedures, were obtained from individuals with neutrophilic leukocytosis due either to infection or chronic myeloid leukemia. Except where otherwise specified, nonsiliconized glassware was employed throughout the studies reported in this paper.

Method 1 was a modification of Dausset’s technic.11 To 10 ml. of defibrinated blood in a 50 ml. test tube were added 2 ml. of a 6 per cent solution of dextran (average mol. wt. 200,000*) in 0.85% NaCl. The tube was stoppered with parafilm, inverted three times, and allowed to stand at room temperature until most of the red blood cells had settled out, as determined by visual inspection. The time required for settling varied with the sample but ranged from 15 to 45 minutes. The supernatant leukocyte-rich plasma was transferred to a clean test tube and a white blood cell count was performed. If the suspension was to be employed in the leukoagglutinin test, and the concentration of leuko-

* Commercial Solvents Corp., Terre Haute, Indiana.
cytes was less than 10 to 20,000 per cu. mm., the test tube was centrifuged for 5 minutes at 120 G's and an appropriate portion of the uppermost supernatant was discarded. Discarding the top one third layer of supernatant generally brought the concentration within the required range.

Method 2. To 10 ml. of defibrinated blood in a 50 ml. test tube were added 10 ml. of the dextran solution described above. Sedimentation with this amount of dextran was quite rapid. The supernatant leukocyte-rich plasma was drawn off in 10 to 15 minutes and centrifuged for 7 minutes at 500 G's. The leukocytes were thereby thrown to the bottom of the tube as a white button, with a thin layer of red blood cells lying on top. Six ml. of distilled water were then rapidly blown into the tube, all cells resuspended by agitation, and in exactly 25 seconds 2 ml. of a 3.4 per cent NaCl solution buffered with M/15 phosphate to a pH of 7.2 were added to the tube. The tube was centrifuged at about 100 G's for 5 minutes and the white blood cells resuspended in 0.85% buffered NaCl. The process of selective hemolysis with distilled water was repeated if any red blood cells remained intact. Finally, any visible clumps of leukocytes were broken up by gentle manipulation with a Pasteur pipette.

Fifty to sixty per cent of the white blood cells originally present in defibrinated normal blood were recovered by Method 1. This approximates the figures given by Skoog and Beck when they used siliconized glassware, heparinized blood, and a dextran to blood ratio of 1 to 4, but it is somewhat less than the yield obtained by Wasastjerna who employed 3.5 per cent polyvinyl pyrrolidone instead of dextran. Twenty to thirty per cent of the white blood cells originally present in the donor sample were sometimes lost by defibrination, as was also noted by Wasastjerna. Apparently they became enmeshed in the fibrin clot. We found that this degree of loss could be sharply reduced by using a minimum number of glass beads in the process of defibrination. Three or four beads sufficed for 10 to 20 ml. of blood. The leukocyte yield from normal blood with Method 2 was 70 to 80 per cent. The degree of nonspecific clumping of white blood cells in the final suspension was no greater than with Method 1. There was a total absence of contaminating red blood cells or platelets in the suspensions. Exposure of the leukocytes to the distilled water for a brief period did not alter their morphology on Wright-stained smears. Attempts to use saponin instead of distilled water as a selective hemolytic agent were unsatisfactory as saponin rendered the white blood cells excessively sticky. Damage to white blood cells by saponin was also noted by Wasastjerna.

When utmost morphologic and functional integrity of the leukocytes was desired, the technic of Lapin and Horonick was found to give superior results. This will be treated under the amoeboid motility studies. We found that the separation of leukocytes from whole blood by the technic of floatation upon serum albumin caused excessive nonspecific clumping of the white blood cells. While this procedure was found suitable for preparing white cell mass for immunization or for precipitin tests, we did not find it suitable for preparing leukocyte suspensions to be used in leukoagglutination or ameboid-motility studies.

Various additives have been suggested for preserving the integrity of leukocytes in suspension. The effect of additives on the suspension stability of white blood cells was investigated for periods up to 6 hours following the separation of the white blood cells from whole blood. Within this time interval the incorporation of sodium acetate into all solutions in a final concentration of 0.2 per cent had no effect upon suspension stability. Desoxyribonuclease was found to
mildly inhibit nonspecific clumping of suspended leukocytes, presumably through dissolution of nucleoprotein clots. A final suspension of 0.005 mg. per ml. was employed. The surface-active agent Triton-WR, used in platelet separation by Minor and Burnett, was found to exert a definite inhibitory effect upon nonspecific clumping of leukocytes when used in a final concentration of 0.1 per cent by volume. When additives were employed, they were incorporated into all solutions: the dextran of the initial separation, the solutions used for hemolysis and washing of the leukocytes (Method 2), and in the final suspension. Only Triton-WR exerted an effect of sufficient degree to recommend routine use in the separation of leukocytes from whole blood.

Preparation of anti-human-leukocyte immune rabbit serum

Technic 1. Three ml. of packed white blood cell mass prepared by Method 2 were emulsified in 4.5 ml. of Falba. To this was added 7.5 mg. of dried heat-killed Mycobacterium phlei suspended in 7.5 ml. of sterile mineral oil. One ml. of this final antigen-adjuvant mixture was injected subcutaneously into each of 3 rabbits. The remainder was stored in the frozen state. Two more subcutaneous injections were given each rabbit at weekly intervals, using freshly thawed antigen-adjuvant mixture. Three weeks after the last injection 1 ml. of a heavy saline suspension of freshly isolated white blood cells was injected intravenously. The rabbits were exsanguinated one week later. Serum was separated from the clot, pooled, and stored in the frozen state.

Technic 2. This was a modification of the multiple injection technic described by Thompson. White blood cells separated by Method 2 were lyophilized and 100 mg. of this material were shaken with 1 ml. of sterile saline and the suspension was emulsified in 1 ml. of Falba. Three mg. of dried heat-killed M. phlei suspended in 2 ml. of Bayol F were added to the emulsified preparation. Each rabbit was injected with 1 ml. of this preparation in each gluteal region. Three times weekly for three weeks thereafter each rabbit received intradermal, intravenous, intraperitoneal, subcutaneous, and intramuscular injections of 1 ml. quantities of saline-suspended lyophilized white blood cells. The rabbits were bled two weeks after the last injection.

The leukoagglutinin titer of the immune serum prepared by Technic 1 was 1 to 200. Potency was retained after storage in the frozen state for as long as one year. The leukoagglutinin titer from Technic 2 was 1 to 500. A zone reaction was observed with low dilutions, agglutination being maximal at a dilution of 1 to 16 to 1 to 32. All immune rabbit sera were adsorbed with normal sheep or human red blood cells before use, as indicated for the various experiments described below.

Leukoagglutinin test

Serum was either tested immediately or preserved with one tenth volume of 1 to 1,000 sodium azide and frozen. It was inactivated at 56 C. for 30 minutes and adsorbed with packed red blood cells from the same donor whose white blood cells were to be used in the test. The serum was set up undiluted and in dilutions of 1 to 4 and 1 to 16 in 0.85 per cent NaCl. A leukocyte suspension of 10,000 to 20,000 cells per cu. mm. was prepared by either Method 1 or 2. One tenth ml. of the leukocyte suspension was added to 0.1 ml. of each dilution of test serum in a 10 x 75 mm. test tube. The tubes were incubated in a 37 C. water bath for 1 hour. Each tube was then vigorously tapped against a hard surface 20 times. This important step served to break up nonspecific clumping without in-

* McNerney Chemical Corp., Los Angeles, Calif.
† Penola Inc., Esso Oil Co., Philadelphia, Penn.
fluencing true agglutination. One to two drops of the suspension were then coverslipped on clean glass slides and examined microscopically. Agglutination was graded as negative to four plus (fig. 1). When using Method 1 for the separation of cells, a drop of 1 percent acetic acid was added to each tube before the final agitation in order to hemolyze contaminating red blood cells.

The leukoagglutinin test proved to be a convenient and reliable method for detecting anti-leukocyte antibodies in both human and animal sera providing that certain controls were rigidly maintained and certain criteria adhered to. These were as follows:

(a) A strongly positive control, e.g., high-titered immune rabbit serum, was always included in each test run.

(b) At least 2 negative serum controls were always included.

(c) To be considered positive, a test had to reveal not only definite clumps of agglutinated leukocytes but also a paucity of leukocytes between the clumps. This relation had to be seen in all low-power microscopic fields and not just focally.

(d) A test was not considered positive unless it was consistently reproducible.

Failure to use appropriate controls and sufficiently rigid criteria in judging leukoagglutination reactions has led to considerable confusion in the past. Examples of negative, two-plus, and four-plus reactions are shown in figure 1. The main difficulty has been with false positive reactions. These may be due to contaminated serum, the phenomenon of "leukergy", the presence of foreign material, agglutination of leukocytes that have participated in erythrophagocytosis, or to unknown causes. Butler's evidence that the increased incidence of leukocyte clumping in ABO-incompatible situations is not due to the presence of a true leukoagglutinin is an illuminating example. Doan observed that about 5 per cent of human donors possess leukocytes which show spontaneous loss of amoeboid motility when exposed to the serum of any other individual. It is possible that a similar situation may exist with regard to leukoagglutina-

![Fig. 1.—Leukoagglutinin test. Examples of negative (A), two-plus (B), and four-plus (C) reactions employing the same leukocyte suspension. A slight degree of nonspecific clumping is illustrated in the control (A). A true positive reaction is indicated by strong agglutination in the test serum in the presence of a negative or weakly reacting control, by a paucity of non-agglutinated leukocytes between the clumped cells in all low-power fields, and reproducibility.](image-url)
tion, and that the fortuitous use of leukocytes from such a donor might cause false positive reactions.

Ninety per cent of tests set up by the procedure we have outlined above gave clearcut results. False positive reactions occurred in the remaining 10 per cent as indicated by clumping in normal human serum controls, by highly irregular clumping in the test serum, or by lack of reproducibility. In every instance of false positive reaction, repetition of the test with a leukocyte suspension from another donor gave clearcut negative results. No false negative reactions occurred insofar as we are aware.

**Table 1.**—Survey of 96 patients with hematologic disease for the presence of leukocyte antibodies, by means of leukoagglutinin, leukoprecipitin, and tanned erythrocyte methods. Thirty-one of the patients had received multiple transfusions. Positive leucoagglutination reactions were observed in 1 case of chronic pancytopenia, 1 case of refractory anemia with neutropenia and thrombocytopenia, and 1 case of acute monocytic leukemia. No leukoprecipitins or agglutinins for antigen-coated tanned erythrocytes were found in the human sera.

<table>
<thead>
<tr>
<th>Disease</th>
<th>No. patients</th>
<th>Number examined with each of 3 tests</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Leukoagglutinin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>tins</td>
</tr>
<tr>
<td>Acute and subacute acquired hemolytic anemia</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Chronic pancytopenia</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Refractory anemia, neutropenia, and thrombocytopenia</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Dissem. L. E.</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Idiopathic neutropenia in newborn</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Infectious mono.</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Miliary the with splenic involvement</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Banti’s syndrome</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Hodgkin’s disease</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Leukemia, acute, undifferentiated</td>
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<td>5</td>
</tr>
<tr>
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<td>2</td>
<td>2</td>
</tr>
<tr>
<td>monocytic</td>
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<td>1</td>
</tr>
<tr>
<td>Leukemia, chronic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>myeloid</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>lymphatic</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

**Table 2.**—Leukoagglutinin test. The sera of three patients shown to contain leukocyte antibody were tested against suspensions of leukocytes from different donors.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Number of suspensions</th>
<th>Number with pos. reaction</th>
<th>Average titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1. (E. R.)</td>
<td>18</td>
<td>16</td>
<td>1 to 16</td>
</tr>
<tr>
<td>Case 2. (V. J.)</td>
<td>12</td>
<td>11</td>
<td>1 to 8</td>
</tr>
<tr>
<td>Case 3. (I. G.)</td>
<td>5</td>
<td>5</td>
<td>1 to 2</td>
</tr>
<tr>
<td>Immune rabbit serum</td>
<td>18</td>
<td>18</td>
<td>1 to 500</td>
</tr>
<tr>
<td>(pos. control)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
In the three human cases showing definite positive reactions the sera retained its potency after preservation in the frozen state for at least 6 months and unfrozen in the ice-box for 10 days. The antibody could be specifically adsorbed by isolated white blood cells. No difference between the leukoagglutinating activity of inactivated and non-inactivated sera was noted, although Dausset has reported contrary results in some instances and therefore suggested that a labile inhibitory factor may exist in some sera. The addition of guinea-pig complement to the inactivated sera had no effect upon the reaction. Dausset et al. reported that the use of siliconized glassware tended to inhibit leukoagglutination. Our experience agrees with this finding. Because of the potentiating effect of added dextran upon agglutination of sensitized red blood cells, the addition of 0.1 ml. of a 5 per cent dextran solution to the final serum-leukocyte incubation mixture was investigated. A mild potentiating effect was noted, but this was of insufficient degree to warrant including dextran into the routine procedure.

Thirty six patients surveyed during the course of this study are categorized in table 1. All of these patients had hematologic disease in which one might suspect the presence of leukocyte antibodies. Thirty one had received multiple transfusions. Table 2 lists the reactions of the three positive sera with leukocyte suspensions from various individuals. Most but not all of these leukocyte suspensions were from group O donors. As shown in table 2, the patients' sera reacted against most but not quite all suspensions of normal leukocytes. It should be pointed out, moreover, that the suspensions giving a negative reaction with the serum of Case 1 (table 2) gave a positive reaction with the serum of Case 2, and vice versa. A considerable variation of the degree of agglutination of the various leukocyte suspensions exposed to the same patient serum was also noted. Similar results have been reported by Dausset et al. The serum of Case 1 (E. H.) gave a negative leukoagglutinin test when set up against a suspension of his own leukocytes. Studies for anti-leukocyte auto-antibodies could not be performed in the other two cases.

Leukoprecipitin tests

(1) Tube method: Test sera were inactivated at 56°C for 30 minutes and adsorbed with equal volumes of washed packed human red blood cells of blood group AB. Lyophilized leukocyte mass was finely ground in a tissue grinder with a small amount of saline buffered to a pH of 7.2. Saline was then added to aliquots of the homogenate until the concentration of leukocyte mass had been adjusted to 0.002, 0.02, 0.2, and 5 mg per ml. respectively. Each aliquot was then filtered through Whatman #3 filter paper under positive pressure, and carefully layered over undiluted test sera in precipitin tubes. The tubes were incubated for 2 hours at 37 C., left overnight in the refrigerator, and read against a black background. A positive reaction was indicated by the presence of a white flocculation zone at the interphase.

(2) Agar-plate precipitin test: Agar plates were prepared using 2 per cent Difco agar containing 0.1 Gm. of merthiolate and 0.02 Gm. of methyl orange per liter, according to the method of Wilson and Pringle. One central and four peripheral basins were molded into the agar during hardening (fig. 2). Filtrate was obtained after homogenizing 10 mg. of lyophilized white cell mass in 1 ml. of buffered saline. The central basin was charged with a mixture consisting of equal parts of this filtrate plus 2 per cent agar. The 4 peripheral basins were charged respectively with immune rabbit serum, normal rabbit serum, and the sera of Cases 1 and 2, all diluted with an equal volume of agar. The plates were
kept at room temperature in a closed container over water. All basins were recharged at 10 day intervals with the respective solutions throughout 6 weeks of observation.

No definite positive reactions were observed with any of the patients’ sera with the tube method. Positive reactions were consistently obtained, however, using immune rabbit sera. The optimal antigen concentration lay between 0.2 and 0.02 mg. of leukocyte cell mass per ml. of saline. The tube precipitin reactions using sera of Cases 1, 2, and 3, the sera of 3 other patients, and the sera of immunized rabbits were also compared before and after treatment of the sera with alkali, according to the method of Stertberger et al. for demonstrating so-called “non-precipitating” antibody. Pre-treatment with alkali moderately enhanced the degree of flocculation encountered with the rabbit sera but had no effect in the other instances.

Micro Kjeldahl analysis of the lyophilized White cell mass obtained by our technic showed approximately 0.48 mg. nitrogen per 5 mg. of cell mass. However, analysis of the filtered saline extract obtained after homogenizing 5 mg. of cell mass in 1 ml. of saline revealed only 0.1 mg. of nitrogen per ml. It appeared therefore that about 80 per cent of the total nitrogenous component of the lysate had failed to go into solution. Pirofsky, using similar extraction technics, found the protein content of his leukocyte extracts to be “minute.” Seligman and his associates demonstrated positive leukoprecipitin tests in pathologic human sera, particularly acute leukemia, and stated that an antigen concentration of at least 1.5 mg. of protein per ml. may be required for an optimum reaction. These authors obtained homogenized leukocyte extract by means of sonic oscillation. It is therefore possible that inadequate extraction technic might ex-

Fig. 2.—Leukoprecipitin test in agar-medium. Central basin contains leukocyte extract. (A) is immune rabbit serum; (B), normal rabbit serum; (C), serum of Case 1; (D), serum of Case 2. The two lines of precipitation between (A) and the central basin indicate the presence of at least 2 reacting antigen-antibody systems between the immune rabbit serum and the leukocyte extract.
plain some of our negative reactions with patients' sera, and further work is in
progress to clarify this situation. The lack of correspondence between the leuko-
agglutinin and leukoprecipitin tests of cases 1, 2, and 3 is in itself not surprising,
for Dausset was unable to demonstrate leukoagglutinins in sera in which Selig-
man had found leukoprecipitins. Leukoprecipitins have been found mainly in
acute leukemia, whereas leukoagglutinins are associated with a much broader
spectrum of hematologic disease. It may be that leukoagglutinins and leukopre-
cipitins are directed towards different antigenic components of the cell.

Results of precipitation in an agar medium are shown in figure 2. Two distinct
lines of precipitation formed between the basins of immune rabbit serum and of
antigen after three weeks, indicating the presence of at least two antigen-antibody
systems. No precipitation was noted between the basins of patient sera and
antigen.

Agglutination of antigen-coated tanned erythrocytes

Sheep erythrocytes less than 1 week old were washed three times or until clear,
and made up to a 2% suspension in 0.85% saline buffered to a pH of 7.2 with M/15 phosphate.
An equal volume of this cell suspension was added to a freshly prepared saline solution
of 1/20,000 tannic acid. This mixture was incubated exactly 10 minutes at 37°C, centri-
fuged lightly, washed once in buffered saline, and made up to a 2% suspension in
saline. Two ml. of this 2% suspension of tanned sheep erythrocytes was centrifuged
and the supernatant discarded. Ten to 41 mg. of lyophilized white cell mass was homo-
genized in 2 ml. of saline buffered to a pH of 6.4. The homogenate was Seitz-filtered. The
filtrate was then added to the button of tanned sheep erythrocytes and the erythrocytes
were resuspended. A control tube was introduced at this point, consisting of a similar
button of packed tanned sheep erythrocytes resuspended in saline at a pH of 6.4. Both
tubes were allowed to stand exactly 15 minutes at room temperature, then washed twice
with a 1 to 250 saline dilution of normal rabbit serum, and resuspended to a 5% concentration
in 1 to 250 normal rabbit serum. Serial fourfold dilutions of test sera from
patients and from normal and immunized rabbits were set up in saline, after complete
adsorption of the sera with untreated sheep erythrocytes. To 0.4 ml. of each dilution of
the various sera in a series of 10 x 75 mm. test tubes were added 0.1 ml. of the 5% suspension
of antigen-coated tanned sheep erythrocytes. In a parallel series, uncoated
tanned erythrocytes from the control tube were added to the sera. All tubes were kept at
room temperature for one half hour with frequent shaking, then overnight in the refrig-
erator. The cell pattern was then read as a hemagglutination test.

Strongly positive reactions were noted with immune rabbit sera and negative
reactions with normal rabbit and all human sera examined. The titer of the im-

Fig. 3.—Hemagglutination by immune rabbit serum of tanned sheep erythrocytes
coated with leukocyte antigenic material. Upper row is test; lower row, control. Titers
from left to right are: undiluted, 1 to 2, 1 to 4, 1 to 16, 1 to 32. All test sera show a strong
positive reaction.
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mune rabbit sera was about 1 to 128. A representative pattern is shown in figure 3. Reactions employing human and rabbit red blood cells as the antigen-carrier were comparable to those using sheep cells.

Agglutination of antigen-coated un-tanned sheep erythrocytes

This technic is modified from that of Middlebrook and Dubos for demonstrating anti-tuberculin antibodies, and depends upon the removal of all proteins from the cell-sensitizing fraction by means of prolonged extraction with phenol.

The final sensitizing fraction contains complex polysaccharide material.

Leukocytes were frozen and thawed 8 times, then lyophilized. One hundred twenty mg. of the lyophilized cell mass were added to 2.5 ml. of 88 per cent phenol and stirred at 35 C. for 20 hours at low speed. After centrifugation at 15,000 rpm for 15 minutes, the supernatant was discarded. The sediment was resuspended in 2.5 ml. of fresh phenol, stirred another 20 hours, and recentrifuged. The final sediment was washed for one half hour in fresh phenol, and again centrifuged. The sediment was now washed 3 times with 2.5 ml. aliquots of cold acetone to remove the phenol, and allowed to dry on a watch glass at room temperature. The dried material was suspended in a 5 ml. quantity of a solution composed of 0.55% NaCl, 0.50% Na2HPO4, and 20% methanol in distilled water, and stirred for 20 hours at 37 C. After centrifugation at 5,000 r.p.m. the precipitate, if any, was discarded. The supernatant was adjusted to a pH of 6.0 with 1/10 N. NaOH, dialyzed against running tap water for 6 hours, then against 3 changes of distilled water for a total of 2 days, and finally centrifuged at 5,000 r.p.m. The supernatant was placed in a dialysis bag and evaporated to about 2 ml. volume by being placed in front of an electric fan. It was finally dialyzed for 3 days against saline buffered to a pH of 7.0. This preparation contained the sensitizing fraction.

One tenth ml. of washed packed sheep cells were added to the 2 ml. of final extract, incubated therein for 2 hours at 37 C., and washed 3 times in saline buffered to a pH of 7.0. The coated cells were then made up to a 0.5 per cent suspension. The test sera were inactivated and adsorbed completely with washed sheep erythrocytes. Duplicate rows of 10 x 75 mm. test tubes were set up for each serum specimen. The specimens were tested undiluted, and in dilutions of 1 to 4 and 1 to 16. To a 0.4 ml. quantity of each serum dilution in the first row were added 0.4 ml. of the suspension of polysaccharide-coated sheep cells. In a similar manner, untreated control cells were added to corresponding test tubes in the second row. The test tubes were incubated for 2 hours at 37 C., shaken vigorously, left overnight in the ice-box, and read as a hemagglutination test.

This test was set up using sera of Cases 1, 2, and 3, normal rabbit serum, immune rabbit serum, and normal human sera from group O donors. Positive reactions were obtained with the serum of Case 2 to a dilution of 1 to 4. Negative reactions were obtained in all other instances.

Effect of leukocyte antibody from human sera upon phagocytic action of human leukocytes

Three hundredths ml. of the test sera diluted in donor plasma (table 3) were added to 0.3 ml. of whole defibrinated blood and the mixture incubated for 1 hour at 37°C. A few drops of a slightly opaque suspension of heat-killed staphylococcus aureus was then added to each tube. The tubes were incubated another one half hour. Wright-stained smears were made of each specimen and 100 neutrophils were examined microscopically. The per cent of neutrophils containing phagocytized cocci was recorded for each slide. It was also desirable to determine in so far as possible the average number of cocci per phagocytic cell. The phagocytic cells were therefore divided into 2 groups. The first group consisted of those cells containing from 1 to 10 cocci, and in this group the actual number of cocci in
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**Table 3.**—Effect of leukocyte antibody from human sera upon phagocytosis of staphylococci by human neutrophils. All dilutions were made in donor plasma. The number of cocci per phagocytic cell was less in the exposed cells than in control cells.

<table>
<thead>
<tr>
<th>Test serum</th>
<th>Dilution</th>
<th>Percent cells with 1 or multiple cocci</th>
<th>Percent cells with over 10 cocci</th>
<th>Total no. cocci per total no. cells containing from 1 to 10 cocci</th>
<th>Average no. cocci per cell in this group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal human</td>
<td>Undiluted</td>
<td>75</td>
<td>14</td>
<td>285/61</td>
<td>4.7</td>
</tr>
<tr>
<td>Normal human</td>
<td>1 to 4</td>
<td>70</td>
<td>17</td>
<td>342/62</td>
<td>5.5</td>
</tr>
<tr>
<td>Donor plasma</td>
<td>Undiluted</td>
<td>73</td>
<td>20</td>
<td>231/53</td>
<td>4.4</td>
</tr>
<tr>
<td>Case 1</td>
<td>Undiluted</td>
<td>74</td>
<td>3</td>
<td>272/71</td>
<td>2.8</td>
</tr>
<tr>
<td>Case 1</td>
<td>1 to 4</td>
<td>81</td>
<td>14</td>
<td>388/67</td>
<td>5.7</td>
</tr>
<tr>
<td>Case 2</td>
<td>Undiluted</td>
<td>71</td>
<td>6</td>
<td>261/65</td>
<td>4.0</td>
</tr>
<tr>
<td>Case 2</td>
<td>1 to 4</td>
<td>88</td>
<td>14</td>
<td>322/74</td>
<td>4.4</td>
</tr>
</tbody>
</table>

Each cell was recorded. The second group consisted of those cells containing more than 10 phagocytized cocci. The actual number of cocci in each cell of this group could not be accurately determined by visual inspection because of close crowding of the cocci.

In Table 3 are shown results for cases 1 and 2 as well as control sera. While the overall per cent of neutrophils containing phagocytized cocci was not appreciably affected by prior incubation in the test sera, the number of cocci per phagocytic cell was less in the exposed cells than in the control cells. This effect was noted only with undiluted sera.

**Effect of leukocyte antibody from human sera upon the amoeboid motility of leukocytes**

A suspension of human white blood cells was prepared according to the method of Lapin and Horonick.* Scrupulously cleansed, siliconized, oven-dried glassware was employed throughout. Normal leukocytes in a concentration of 10 to 20,000 per cu. mm. were suspended in an equal volume of donor plasma, in normal human sera, and in sera of Cases 1, 2, and 3 that had been diluted with donor plasma (Table 4). Four one hundredths ml. of the final suspension in each instance was deposited on a siliconized slide. The slide was carefully coverslipped, all air bubbles expressed, and the edges sealed with a mixture of paraffin and petrolatum (melting point 45 C.). The slides were placed in a 37 C. incubator and examined with a phase contrast microscope after 3 hours and 18 hours. The per cent

Table 4.—Per cent of total leukocytes from group O blood that showed active amoeboid motility after 3 and 18 hours incubation in donor plasma, normal human sera, and sera of Cases 1, 2, and 3. Results with leukocyte suspension from 2 separate donors are shown. A significant decrease in the per cent of motile leukocytes after 18 hours incubation in the patient's sera, but not after 3 hours, is apparent with both leukocyte suspensions.

<table>
<thead>
<tr>
<th>Test serum or plasma</th>
<th>Dilution</th>
<th>Percent motile at 3 hours</th>
<th>Percent motile at 18 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Donor #1</td>
<td>Donor #2</td>
</tr>
<tr>
<td>Donor plasma</td>
<td>Undiluted</td>
<td>61</td>
<td>81</td>
</tr>
<tr>
<td>Normal human serum</td>
<td>Undiluted</td>
<td>77</td>
<td>91</td>
</tr>
<tr>
<td>Normal human serum</td>
<td>1:4</td>
<td></td>
<td>81</td>
</tr>
<tr>
<td>Normal human serum</td>
<td>Undiluted</td>
<td>73</td>
<td>—</td>
</tr>
<tr>
<td>Case 1</td>
<td>1:4</td>
<td>72</td>
<td>68</td>
</tr>
<tr>
<td>Case 2</td>
<td>1:4</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td>Case 3</td>
<td>1:4</td>
<td>76</td>
<td>72</td>
</tr>
</tbody>
</table>
of leukocytes showing amoeboi motility was determined by careful scrutiny of 100 cells on each slide (fig. 4). The amoeboi motility counts were confined to non-agglutinated individual cells or to only weakly agglutinated (2 to 3 cell) clumps. Agglutination of the leukocytes in the patients' sera was much inhibited although not entirely abolished by use of siliconized glassware.

In control sera no significant difference in the per cent of motile leukocytes between 3 and 18 hours was noted (table 4). This agrees with the data of Lapin and Horonick. As compared to these control values, the sera of cases 1, 2, and 3 demonstrated a delayed cytotoxic effect upon the leukocytes. The per cent of motile leukocytes in these sera after three hours of incubation was similar to that of the controls. However, after eighteen hours of incubation in the antileukocytic
sena the number of cells demonstrating amoeboid motility had greatly diminished (table 4). Cytologic detail of control cells, and of cells exposed to anti-leukocyte antibody, for 18 hours, are shown in Figure 4 as viewed by phase contrast. Wright-stained smears of the preparations revealed severe degenerative and lytic changes in those leukocytes exposed to the antisera for 18 hours, and no change beyond mild cytoplasmic vacuolization in the leukocytes suspended in normal serum or in plasma for a similar period. The serum of Case 1 (E. R.) had no inhibitory effect upon the motility of his own leukocytes, while at the same time demonstrating a marked effect upon donor leukocytes. It was not possible to run similar auto-antibody studies on cases 2 and 3.

**Discussion**

Leukocyte antibodies are rarely demonstrated in human sera by current methods, but in selected cases of hematologic diseases can probably be demonstrated in 5 to 10 per cent. Dausset found leukoagglutinins in 8 to 9 per cent of 700 pathologic sera, but does not state on what bases these sera were selected. In the present investigation 36 patients with hematologic disease were surveyed in order to obtain positive sera for experimentation, and of that number 3 (8.3 per cent) showed demonstrable leukocyte antibodies.

The leukoagglutinin test is the simplest and most reliable method available at present for the detection of leukocyte antibodies. Satisfying and clearcut reactions are obtainable providing that certain controls are always included in each test series and rigid criteria are adhered to before a reaction is regarded as positive. Some slight damage to leukocytes may be required for actual agglutination by homologous antibody. This is suggested by the moderate inhibitory effect of siliconized glassware upon the leukoagglutination reaction. Nevertheless, it is probable that such antibody does attach to or act upon relatively intact leukocytes, as shown by a pronounced diminution of amoeboid motility of white blood cells incubated for a prolonged period in human anti-leukocytic sera.

More than one and doubtless multiple antigens are present in the human leukocyte, as indicated in this study by precipitin technics using immune rabbit sera against lysate of human white blood cells. Miescher and Fauconnet presented evidence interpreted as distinguishing nuclear from cytoplasmic antigenic leukocyte material. Employing immune rabbit sera, Seligmann and his associates demonstrated the existence of a variable number of antigens in human leukocytes. Our results are in reasonable accord with this finding. Contrary to the Seligmann group, however, we were unable to demonstrate anti-human-leukocyte antibodies in human sera (i.e., homologous antibody) by precipitin technics. This discrepancy might stem from differences in antigen-extraction procedures, or equally well from the simple lack of precipitin antibodies in our human material.

Leukocyte antigenic material can be adsorbed by tanned sheep red blood cells as shown by subsequent hemagglutination of these cells by adsorbed immune rabbit sera. Human anti-leukocytic sera, however, failed to cause hemagglutination of such cells under the conditions of our experiments. We have found no previously reported studies employing this general technic with heterologous leukocyte antibody. Previous attempts have been made to demonstrate hemagglutina-
tion of antigen-coated tanned erythrocytes with homologous leukocyte antibody, but without success. Since it is clear from our results with immune rabbit sera that antigenic material was actually adsorbed onto the erythrocyte surfaces, the failure of homologous antibody (sera of Cases 1, 2, and 3) to react therewith might be due to one or more of the following causes: insufficient amount of antigenic material was taken up by the cell surfaces; the titer of homologous antibody was too low; the homologous antibody had a narrower range of specificity than heterologous antibody and the leukocyte antigenic material was sufficiently altered by the experimental procedure to prohibit antigen-antibody union. The last possibility seems to us the most likely.

One of the three human anti-leukocytic sera reacted with un-tanned sheep red blood cells which had been coated with polysaccharide extracted from human leukocytes. Sera of the other two test cases, the immune rabbit sera, and normal human sera did not so react. This again illustrates the antigenic diversity of the leukocyte. The concept that in some instances acquired homologous antibody may be directed against the complex polysaccharide moiety of nucleated cells may be important, inasmuch as we appear to be dealing in these experiments with the biological basis of individuality.

Mattoth et al. could not demonstrate any inhibition of phagocytosis of starch particles by leukocytes previously exposed to a patient’s serum that gave a positive leukoagglutinin test. By in vivo experiments Matsuno presented evidence that heterologous leukocyte antibody may actually stimulate phagocytosis by leukocytes. He used heat-killed tubercle bacilli. In our experiments comparing control leukocytes to those exposed to leukocyte antibody we found no difference between the overall per cent of cells engaging in phagocytosis. However, the average number of cocci taken up by each cell did seem diminished by exposure to the antibody.

Demonstration of a delayed cytotoxic effect of homologous leukocyte antibody upon leukocytes, as measured by our ameboid motility studies, is of considerable theoretical interest. Suggestive evidence of such an effect was obtained by Francke who estimated cytolysis by doing white blood cell counts at various time intervals on normal blood incubated with sera from a number of cases of chronic pancytopenia. His results could not be confirmed by Butzengeiger and Gartz. Braunstein16 failed to observe cell-lysis by leukoagglutinating sera, although time intervals of exposure were not stated by him. These conflicting results might be explained by the plausible assumption that among the patients studied by Francke some possessed leukocyte antibodies in their sera, and that none of those studied by Butzengeiger and Gartz happened to possess such antibodies. This interpretation is in accord with current knowledge of the distribution of leukocyte antibodies. Braunstein16 perhaps did not follow his cell preparations for a sufficient period of time to observe cytolytic changes.

In studies on the nature of immunity to homologous grafted skin, Billingham and Sparrow showed that isolated epidermal rabbit cells would not survive transplantation back to their original donor animal after the cells had been exposed for 22 hours to the serum of a previously grafted “immune” rabbit. Exposure for $3^{1/2}$ hours, however, had no effect upon the graft survival. Their
experiments were adequately controlled. Their results bear some analogy to our own studies of the delayed effect of homologous antibody on amoeboid motility.

It is not our purpose in this report to speculate at length regarding the clinical significance of leukocyte antibodies; however, a few words of comment may be allowed. Dausset's summarizing article⁹ as well as the Colloque⁷ should be consulted for a detailed survey of clinical information. The following facts require answer in any theory about the development and significance of leukocyte antibodies in man: (1) Nearly all cases have hematologic disease, (2) The great majority of cases show neutropenia, (3) The great majority have received multiple transfusions prior to the demonstration of leukocyte antibodies in their sera. While it would be tempting to attribute the neutropenia to auto-antibodies, we know of only a few cases (excepting those associated with drug-sensitization⁸) in which auto-antibodies were actually demonstrated, although one may accept the L. E. factor as being a special type of auto- or pan-antibody.¹⁴, ²⁸ The observation that erythrocyte and platelet auto-antibodies may exist concomitantly with leukocyte antibodies that do not appear to react with the patient's own white blood cells, but only against donor cells, has been recorded.², ²⁹, ⁴² It has been inferred from this concomitance that the leukocyte antibody involved must be of a peculiar type—one that is really an auto- or pan-antibody, but that appears to be an iso-antibody. This strikes us as being too much in the way of an antibody-masquerade to be accepted on present data. In a few cases of neutropenia studied by Dausset before and after multiple transfusions, leukocyte antibodies were found only after the transfusions. It is true that leukocyte antibodies have been found in untransfused patients and therefore postulated to be auto-antibodies. Such a postulate is an oversimplification, however, in view of the analogous fact that iso-antibodies to erythrocyte factors other than ABO can be occasionally found in untransfused patients. The idea that transfusions widen the specificity of a pre-existing leukocyte auto-antibody is open to the criticism that these supposed auto-antibodies simply do not appear to react with the patient's own white blood cells under experimental conditions. Indeed, all that can be said on present data with respect to the genesis of leukocyte antibodies in man is that they tend to develop in patients with hematologic disease, usually not until multiple transfusions have been given, that many of these patients have an associated neutropenia, and that the relation of the neutropenia to the general process is wholly unknown.

A possible analogy between leukocyte antibodies and the homograft reaction has been suggested elsewhere.⁴⁹ The transfusion of white blood cells can legitimately be regarded as a type of homograft. Leukocytes, skin, and doubtless other organs, share common antigens within the individual. Skin homografting may itself lead to the development of leukocyte antibodies.¹ Prior injection of white blood cells from a particular donor into a recipient leads to accelerated breakdown of skin that is subsequently homografted from the same donor to the same recipient (i.e., it leads to a type of "second-set phenomenon"—in which there is accelerated breakdown of a tissue graft in an animal who has received and rejected a previous graft from the same donor). Medawar²³ has shown that on a quantitative basis the intradermal route is 18 times more effective than the
intravenous route in eliciting this "second-set phenomenon" with injected white blood cells. If one admits of the comparison, it might be suggested that both multiple transfusions and an underlying hyperactive reticulo-endothelial system such as occurs in many hematologic diseases are required for the development of leukocyte antibodies, largely because the intravenous pathway is a poor route of immunization with homologous antigenic material. Further study of this analogous relationship might be fruitful.

SUMMARY

A study of leukocyte antibodies is presented using (1) the sera of rabbits immunized with human leukocytes, and (2) the sera of three patients screened for the presence of such antibodies from among 36 patients with hematologic disease, 31 of whom (including the 3 studied in detail) had received multiple transfusions. The following techniques are described and were employed: Leukoagglutination, leukoprecipitation including tube and agar-plate methods, agglutination of antigen-coated tanned and untanned sheep erythrocytes, the effect of antisera upon phagocytosis of heat-killed staphylococci by leukocytes, and upon ameboid motility of leukocytes.

The leukoagglutinin test gives reliable clearcut results providing that appropriate controls are included and certain criteria adhered to, in order to facilitate the recognition of clumping due to other factors than true antigen-antibody union.

No leukoprecipitins were detected in human sera with the techniques used in this study. Immune rabbit sera, on the other hand, gave two reaction-lines in agar media, when set up against leukocyte extract.

Immune rabbit sera reacted strongly with antigen-coated tanned sheep red blood cells. Human sera did not so react. One of the three selected human sera reacted with antigen-coated untanned erythrocytes, suggesting the presence of a polysaccharide antigen extractable from human leukocytes and capable of stimulating antibody formation in the human. Immune rabbit sera, and other human sera, did not react in this test.

A suggestive but perhaps not a conclusive effect upon phagocytosis of bacteria by leukocytes exposed to human leukocyte antibody for 1 hour could be demonstrated.

By means of ameboid motility studies, a cytotoxic effect of the human antisera upon human leukocytes could be demonstrated after 18 hours of incubation, but not after 3 hours. This was interpreted as evidence of a delayed reaction.

Certain cardinal points from a clinical and theoretical standpoint with regard to the genesis of leukocyte antibodies in man are briefly reviewed. A possible analogy between leukocyte antibody formation and the homograft reaction is discussed. It is suggested that the rarity of leukocyte iso-antibody formation following transfusion is related to the fact that the intravenous pathway may be a poor route of immunization for these antigens.

SUMMARIO IN INTERLINGUA

Es presentate un studio de anticorpos a leucocitos. Esseva usate (1) le seros de conilios immunisate per medio de leucocitos human e (2) le seros de tres
patients human, seligite per tests pro le presentia de tal anticorpores ab un serie total de 36 subjectos con morbo hematologic. Trenta-un del 36—incluse le tres seligite pro le presente studio—habeva recipite multiple transfusiones. Le techniques empleate es describite. Illos eseva leucoagglutination, leucoprecipitation (a tubo e a disco de agar), agglutination de tannisate e non-tannisate erythrocytos ovin a revestimento de antigeno, e le effectos de antiseros super le phagocytose de thermo-occiditestaphylococcos per leucocytos e super le motilitate ameboide de leucocytos.

Le test a leucoagglutinina produce clar e precise resultatos, providite que omne le requirite mesuras de controlo es observate e que certe criterios es prendite in consideration a fin de facilitar le recognition de formas de coagulo que es causete per factores altere que le union de antigeno e anticorpore.

In seros human, nulle leucoprecipitinas esseva trovate per medio del techniques usate in iste studio. Del altere latere, seros immunisate de conilio—quando confrontate con extracto leucocytic in un medio de agar—exhibiva duo lineas de reaction.

Seros immunisate de conilio reageva fortemente con tannisate erythrocytos ovin a revestimento de antigeno. Seros human non reageva assi. Un del tres seligite seros human reageva con non-tannisate erythrocytos a revestimento de antigeno. Isto pareva indicar le presentia de un antigeno polysaccharidic que es extrahibile ab leucocytos human e que es capace a stimular le formation de anticorpore in humanos. Seros immunisate de conilio e altere seros human non reageva in iste test.

Esseva possibile demonstrar un suggestive ben que fors an conclusive efecto super le phagocytose de bacterios per leucocytos post lor exposition, durante 1 hora, a anticorpore a leucocytos human.

Per medio de studies de motilitate ameboide, il esseva possibile demonstrar un efecto cytotoxic exercite per antiseros human super le leucocytos human. Iste efecto esseva notate post 18 horas de incubation sed non post 3 horas, e le conclusion esseva derive que il se tracta hic de un reaction retardate.

Es presentate un breve revista de certe punctos cardinal ab le puncto de vista clinie e theoric con respecto al genese de anticorpores contra leucocytos in humanos. Es discutite le possibile analogia del formation de anticorpore a leucocytos con reactiones post homograffage. Es signalate le possibilitate que le raritate del formation de iso-anticorpore a leucocytos post transfusiones de sanguine es relationate al facto que le via intravenose es un inefficace canal de immunisation pro iste antigenos.

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Leukocyte Antibodies in Human Sera and in Immune Rabbit Sera
ROY L. WALFORD, E. TAYLOR PETERSON and PATRICIA DOYLE

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