Determination of Leukoagglutinin Specificity by In Vivo and In Vitro Studies*

ALI A. HOSSAINI AND HENRY E. WILSON

Departments of Clinical Pathology and Medicine, The Ohio State University, Columbus

Antibodies of various specificities may be found in patients receiving multiple blood transfusions. Investigations in the past decade have established that some of these patients may develop antibodies specifically directed against leukocyte antigens and not against those of the red cells (Bridges, Boyd, and Nelson, 1962; Dausset, 1954; Felbo and Jensen, 1962; Killman, 1958; Payne, 1947; van Loghem et al, 1958; van Rood, Leeuwen, and Eernisse, 1959; van Leeuwen, 1963). The transfusion of patients whose sera contain these antibodies, known as leukoagglutinins, frequently results in febrile transfusion reactions. In the past, most febrile transfusion reactions were attributed to the presence of pyrogens in the blood-collecting apparatus. However, with the use of disposable equipment, this source of reactions has been virtually eliminated, and most authors agree that leukocyte-leukoagglutinin incompatibility is a major cause of such febrile reactions today (Bridges, Boyd, and Nelson, 1962; Brittingham, and Chaplin, 1957; Dausset, 1954; Hossaini; Killman, 1958; Payne, 1957; van Loghem et al, 1958; Wilson, Rheins, Naegeli, and George, 1959). This study was undertaken to determine in vivo and in vitro the specificity of leukoagglutinins, and to establish the nature of the pyrogenic reaction.

* Recent work on this project has been supported by grant #T-362 from the American Cancer Society, and by the American Cancer Society institutional grant (#64-2).

Materials and Methods

Normal sera were obtained from donors in the blood bank of the Ohio State University Hospital. All pathologic sera were obtained from patients on the hematology service of the department of medicine.

Leukocyte Suspensions

Blood was collected from normal donors in the blood bank in 15 ml amounts in siliconized tubes containing 0.3 ml of 5% disodium versenate (EDTA). After thorough mixing of blood and anticoagulant, 2 ml of 6% dextran were added, and the contents of the tube were mixed again by inversion. The blood was divided equally and pipetted into three 22 x 1.5 cm test tubes. After 90 minutes of sedimentation, the supernatant plasma, rich in leukocytes and platelets, was transferred to a 15 ml graduated centrifuge tube. The tube was spun in a centrifuge at 800 rpm for 15 minutes. The supernatant fluid was removed and, by the same centrifugation technique, the cells in the sediment were washed twice with saline-serum-anticoagulant (SSA) solution. The latter solution was prepared by mixing 2 ml of inactivated normal blood group AB serum with 0.5 ml of 5% EDTA, and then diluting it to 200 ml with physiological saline. After the second washing, the supernatant was removed as completely as possible, and the button of cells was homogeneously suspended in a fresh 0.5 ml aliquot of SSA solution. A white cell count was made, and additional SSA was added to produce a cell density of 8 to 10,000 per mm^3.

Red Cells

The red cell portion remaining after sedimentation was washed in saline and was used for the absorption of erythrocyte isoagglutinins. All erythrocyte isoagglutinins were removed from test sera by absorption with washed red cells of the leukocyte donor before leukoagglutination tests were performed.

Leukoagglutination Test

The leukoagglutination tests were performed by placing 0.2 ml of a 1:8 dilution of the absorbed sera in physiological saline in 10 x 75 ml tubes, followed by one drop of bromelin solution (Dade Reagents, Inc., Miami, Florida) or 0.1 ml of the SSA suspended leukocytes. The

tubes were shaken, left to stand for 15 minutes at room temperature, and then were spun for 30 seconds in a serofuge (Clay-Adams). After tapping the tubes gently to disperse the cells in the sediment, the solutions were examined for agglutination by holding the tubes in the direct beam of a light source over a concave magnifying mirror.

A known positive control and a negative control were included in each series of tests. Each serum was tested against two cell suspensions prepared from randomly selected donors.

Induction of Febrile Transfusion Reaction

The following sera were used:

1. Serum from a Group A, Rho (D) positive blood patient with aplastic anemia, whose serum had shown strong leukoagglutinating activity.
2. Serum from an apparently normal individual (negative control) of the same blood group and Rho (D) as that of the patient.

Five ml of both the control and the leukoagglutinin-positive sera were diluted to 30 ml using injectable physiological saline followed by Seitz-filtration.

Basal white cell and differential, red cell, reticulocyte, and platelet counts were obtained by finger stick; temperature, pulse rate, and blood pressure were recorded on a male volunteer (AAH). Two hours after obtaining the basal data, a slow intravenous transfusion of 250 ml of physiological saline was begun. Twenty-five minutes later the control Seitz-filtered serum was injected into the saline bottle and infusion continued. At half-hourly intervals for six hours, counts were taken, and temperature, pulse, and pressure were recorded.

Under similar environmental, temporal and nutritional conditions, the same volunteer was injected one week later with an equal amount of the identically processed positive serum, and similar observations were recorded as above.

Results

In all, 395 sera obtained from 375 normal individuals and patients with various pathological conditions were tested for leukoagglutinins. When a patient gave a negative test and subsequently became positive, the reactions were recorded as two specimens; this accounts for the difference in numbers. There were 20 such cases. Many of the pathological groups had received multiple transfusions. Results of the sera tested for leukoagglutinins and the number of transfusions received by each patient prior to the first testing of the serum are shown in Table 1. Table 2 lists the diagnoses of patients whose sera were tested for leukoagglutinins before they received any blood transfusions. None of the seven leukoagglutinin-positive sera was from a woman who had ever been pregnant.

Cytologic studies of agglutinated cells revealed participation of both the granulocytes and mononuclear cells in the clumps. Clumping resulted in complete loss of the ameboid movement of cells in the positive tubes, whereas cells in the negative sera remained mobile, though sluggish.

Transfused patients were classified by clinical diagnosis. Those showing transfusion reactions, leukoagglutinins, or both, are presented in Table 3.

TABLE 1

Distribution of 395 Sera Tested for L.A.*

Number of blood units	Number tested	No L.A.	L.A. demonstrated
From patients never transfused prior to testing	247	240†	7
From patients receiving less than 8 units	63	48	15
From patients receiving more than 8 but less than 20 units	59	42	17
From patients receiving more than 20 but less than 50 units	16	9	7
From patients receiving more than 50 units	10	3	7
Total	395	342	53

* L.A.: Leukoagglutinins

† Some of these patients were subsequently transfused and re-tested.

TABLE 2

Diseases Found in Patients Showing Negative and Positive Leukoagglutinis Prior to Receiving any Transfusion

Disease	Number tested	Number positive
Rheumatoid arthritis	9	4
Chronic pyelonephritis	4	1
Acute monoblastic leukemia	18	1
Healthy	76	1
Miscellaneous*	133	0
Total	240	7

* Diseases included:

1. Other acute leukemias
2. Chronic leukemias
3. Lymphomas
4. Hemolytic anemias
5. Refractory anemias
6. Lupus erythematosus disseminatus
7. Coagulation defects
8. Various forms of non-hematologic diseases

TABLE 3

Correlation between Disease, Transfusion Reaction and L.A. Production

Disease Group	Number Showing				
	Reaction only	Reaction and L.A.	L.A. only	Both negative	Total
Acute leukemias	3	7	6	22	38
Chronic leukemias	4	10	4	19	37
Lymphomas	4	4	1	15	24
Hemolytic anemias	4	2	0	6	12
Refractory anemias	4	4	0	7	15
Thrombocytopenic purpuras	0	2	0	0	2
Coagulation defects	1	0	0	4	5
Non-hematologic diseases*	2	5	1	5	13
Insufficient data					2
Total	22	34	12	78	148

* Six untransfused patients showed leukoagglutinins (L.A.). (See Table 1)

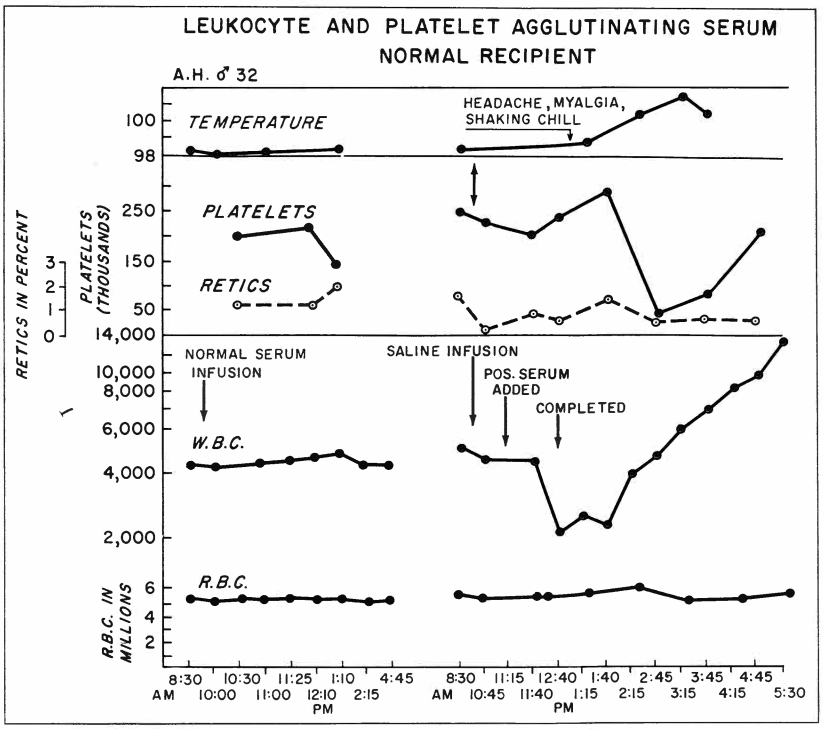

Fig. 1.

Figure 1 shows white cell, red cell, platelet, and reticulocyte counts, temperature, and symptoms experienced during the infusion of both the control and positive sera. No change occurred in these parameters following the infusion of the control serum. On the contrary, the following alterations occurred after the infusion of the positive serum. One hour after the start of the infusion of serum, there was a marked leukopenia, lasting for 90 minutes, followed by a gradual return to the basal level, and a subsequent leukocytosis over the next three hours. Only slight changes in the reticulocyte and red cell counts occurred, but a significant thrombocytopenia was observed. Platelet levels remained at the basal value for two hours, but 30 minutes later the count showed a precipitous drop. The thrombocytopenia persisted for two hours, and then the count returned to normal levels. No further counts were made.

Fifteen minutes before the completion of the infusion (40 minutes after starting the serum infusion), the subject developed a frontal headache, which increased in intensity over a period of an hour. Within 15 minutes after the onset of the headache, a shaking chill, a mild tachypnea (28 breaths per minute), and a 2–3° rise in temperature (which reached its peak within three hours) developed.

Table 4 shows the basal red cell, reticulocyte, platelet, white cell, and differential counts and the values obtained over the six hour study period following the injection of the positive serum. Since detailed listing of the hourly values following the injection of the control serum did not contribute additional information, only the recorded range of values were included in this table. The values obtained with regard to the red cell, reticulocyte, platelet, and total white cell counts have been mentioned already in Figure 1. Differential counts obtained after the infusion of the control serum varied slightly from basal values. However, there were marked absolute neutrophenia, eosinopenia, lymphopenia and monocytopenia. The neutrophenia continued for over three hours and was followed by a neutrophilic leukocytosis. Values for the remaining white cell elements remained below normal levels over the six-hour period. However, the monocytes showed signs of reappearance five hours after infusion.

TABLE 4

Changes Induced in a Human Volunteer by Injection of Leukoagglutinin Positive Control Sera

Cells		Control Serum	Test Serum						
		Range of values over 6 hours	Time in hours after injection						
			0	1	2	3	4	5	6
RBC's in millions		5.2–5.3	5.8	5.5	5.5	6.1	5.1	5.1	5.2
Reticulocytes %		1.2–2.0	1.6	0.2	0.8	0.4	1.4	0.4	0.6
Platelets in 1000's		190–208	214	184	204	36	74	194	—
WBC	Total	4150–5000	5000	1950	2300	4500	6500	8650	12950
Polys.	Total	3050–3071	3300	1287	1656	4095	6175	8391	12600
	%	61–74	66	66	72	91	95	97	95
Eos.	Total	0–250	0	0	0	0	0	0	0
	%	0–5	0	0	0	0	0	0	0
Baso.	Total	0–0	0	0	0	0	0	0	0
	%	0–0	0	0	0	0	0	0	0
Lympho.	Total	747–900	1300	546	552	360	325	173	88
	%	18–27	26	28	24	8	5	2	3
Mono.	Total	125–150	400	117	92	45	0	86	259
	%	2–3	8	6	4	1	0	1	2

Discussion

This study confirms the work of other investigators (Bridges, Boyd, and Nelson, 1962; Dausset, 1954; Felbo and Jensen, 1962; Killman, 1958; van Rood, Leeuwen, and Eernisse, 1959; van Rood and von Leeuwen, 1963; Wilson, Rheins, Naegeli, and George, 1959) that the mechanism of the leukoagglutination phenomenon is immunologic and is specific for leukocytes. There is a significantly higher frequency of occurrence of leukoagglutinins in patients receiving multiple transfusions as compared to patients who were not transfused. Table 1 shows that only seven out of 247 sera from the latter group gave a positive test. In contrast, 46 out of 148 sera from transfused patients were positive.

Twelve of the leukoagglutinin-positive patients had been tested before any transfusion. All their sera were negative then, but became positive after one or more blood transfusions. Yet other patients, who had been tested at different stages of transfusion therapy, were found to become leukoagglutinin-positive as the number of transfusions was increased. Table 1 shows the direct relationship between the number of leukoagglutinin-positive patients and the number of units of blood they had received. Among the 46 leukocyte agglutinating sera from transfused patients, only three were known to possess a concomitant red cell antibody. Therefore, the increase in the number of leukoagglutinin-positive patients with increasing transfusions may have been caused by further stimulation by the antigen or antigens of transfused leukocytes.

Absence of reaction following the injection of the control serum indicates that all pyrogenic agents had been eliminated from the serum. Variations in differentials, in blood counts and temperatures, in pulses and pressures during the negative control experiment were within the range of physiological variations and experimental error.

Excluding the unlikely possibility of sensitization by the negative control serum, it may be assumed that the reaction to the positive serum was due to the specific effect of the injected leukoagglutinins on circulating leukocytes. The possibility of a pyrogenic reaction must be considered, however, as marked leukopenia is not characteristic of this type of transfusion reaction.

The marked leukopenia in the absence of a change in the red cell

count indicates that the reaction was not caused by erythrocyte antibodies. The simultaneous onset of neutropenia, eosinopenia, lymphopenia and monocytopenia is additional evidence suggesting that the antibody is directed against granular and mononuclear leukocytes. This reinforces the findings by microscopic studies that both cell types participate in the clumps of cells seen in positive leukoagglutinin tests. The thrombocytopenia was shown to be associated with the presence of platelet antibodies in the injected serum.

The injection of a serum known to contain leukoagglutinins into a normal individual resulted in the development of a febrile reaction and a mild leukopenia. In an earlier report, one of us (AAH) explained this on the basis of passive transfer of leukoagglutinins.

Using the continuous-flow electrophoresis cell, Wilson, Rheins, and Naegeli (1959) fractionated seven negative sera and ten sera from six leukoagglutinin-positive patients. Results showed a rise in activity of the γ-globulin fraction only in the positive sera. Persistence of leukoagglutinins in positive sera after absorption indicates that the red cells lacked the corresponding antigens.

There was an absence of a marked reduction in the severity of febrile transfusion reactions when packed red cells with a leukocyte count of less than 1000 per mm^3 were given to three leukoagglutinin-positive patients who had experienced multiple febrile transfusion reactions. The mild reactions after receiving the processed blood might have been due to the almost inevitable contamination of packed, washed, red cell preparations by a few unremoved leukocytes.

Summary

Results of testing sera from normal individuals and pathological patients, using a bromelin technique for the detection of leukoagglutinins, are reported. These results, supported further by an in vivo experiment, suggested that the phenomenon of leukoagglutination is immunologic in nature and that leukoagglutinins may be the cause of some febrile transfusion reactions. These are specifically directed against leukocytes, and do not involve the red cell series. Furthermore, the antigen (or antigens) is probably present in both the granulocytes and the mononuclear leukocytes. Anti-platelet antibodies could be presumed to be the cause of thrombocytopenia.

References

BRIDGES, J. M., D. A. BOYD, AND M. G. NELSON. Leucocyte antibodies and transfusion reactions. *Lancet* 2: 223–226, 1962.

BRITTINGHAM, T. E., AND H. CHAPLIN, JR. Febrile transfusion reactions caused by sensitivity to donor leukocytes and platelets. *J. Amer. Med. Assoc.* 165: 819–825, 1957.

DAUSSET, J. Leukoagglutinins. IV. Leukoagglutinins and blood transfusion. *Vox Sang.* 4: 190, 1954.

FELBO, M., AND K. G. JENSEN. Death in childbirth following transfusion of leukocyte-incompatible blood. *Acta Haemat.* 27: 113–119, 1962.

HARRINGTON, W. J., C. C. SPRAGUE, V. MINNICH, C. V. MOORE, R. C. AULVIN, AND R. DUBACH. Immunologic mechanisms in idiopathic and neonatal thrombocytopenic purpura. *Ann. Int. Med.* 38: 433–469, 1953.

HOSSAINI, A. A critical review and a new approach to the serological detection of leukoagglutinins. Dissertation for Ph.D. degree, Ohio State University, Columbus, 1960.

KILLMAN, S. A. A study of antigens of human leukocytes. *Vox Sang.* Ser. 2, 3: 409–425, 1958.

KILLMAN, S. A. Febrile transfusion reactions in patients with leukocyte agglutinins. *Dan. Med. Bull.* 5: 178–183, 1958.

KISSMEYER-NIELSEN, F. Demonstration of a thrombocytopenic and a leukopenic factor in the blood of patients with thrombocytopenia and leukopenia. *Acta Haemat.* 9: 337–345, 1953.

LALEZARI, P., AND G. E. BERNARD. Identification of a specific leukocyte antigen: another presumed example of 5^b. *Transfusion* 5: 135–142, 1965.

PAYNE, R. Leukocyte agglutinins in human sera. Correlation between blood transfusions and their development. *A.M.A. Arch. Int. Med.* 99: 587–606, 1957.

PAYNE, R. The association of febrile transfusion reactions with leukoagglutinins. *Vox Sang.* Ser. 2, 2: 233–241, 1957.

VAN LOGHEM, J. J., JR., M. VAN DER HART, W. HIJMANS, AND H. R. E. SCHUIT. The incidence and significance of complete and incomplete white cell antibodies with special reference to the use of the Coombs consumption test. *Vox Sang.* Ser. 2, 3: 203–223, 1958.

VAN ROOD, J. J., AND A. VAN LEEUWEN. Leukocyte grouping. A method and its application. *J. Clin. Invest.* 42: 1382–1390, 1963.

VAN ROOD, J. J., A. VAN LEEUWEN, AND J. G. EERNISSE. Leukocyte antibodies in sera of pregnant women. *Vox Sang.* Ser. 2, 4: 427–444, 1959.

WILSON, H. E., M. S. RHEINS, S. A. NAEGELI, AND W. GEORGE. Leukocyte agglutinins. I. Method of detection and clinical observations. *J. Lab. Clin. Med.* 53: 854–862, 1959.