Cell Antigens and Cell Specialization. II. Demonstration of Some Red Cell Antigens of Human Normoblasts

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In a previous communication, it was reported that A, B and H antigen receptors are present on human normoblasts. These receptors were shown to be present on the surface of normoblasts in all stages of development. These findings suggested that cell antigens appear early in cell formation.

In the present study, we report the results of the determination of a large number of red cell isoantigens on human normoblasts. This paper also reports preliminary results of the detection of in vivo coating of normoblasts and reticulocytes by antibodies in cases of autoimmune hemolytic anemia and of erythroblastosis fetalis.

Materials

Preparation of nucleated cell suspension of fresh bone marrow: Thirty bone marrow samples (1–3 ml.) were obtained by sternal aspiration from adult patients with high erythrocyte sedimentation rates (more than 25 mm. in 60 minutes). The individuals from both sexes ranged between 41 and 76 years of age. All bone marrow samples were collected in Versenate Disodium (1 mg. per ml. of bone marrow). The particles were removed and the remaining material was allowed to sediment for 1½ to 2 hours in Wintrobe hematocrit tubes at 25 C. At the end of this period, the marrow fat and remaining bone marrow particles were removed, and the marrow plasma rich in nucleated cells was separated. The cells were then washed three times with isotonic saline containing 1 mg. of Versenate Disodium (EDTA-saline) per ml. and centrifuged at 600 G. for 2 minutes. Following this, the supernatant was removed completely and the suspension restored approximately to its original volume in EDTA-saline solution. Only those specimens free of microscopic agglutinates and containing 15 or more normoblasts, and approximately 30 erythrocytes and/or reticulocytes per 100 nucleated cells were used in the present study.

Preparation of nucleated cell suspension of bone marrow from immune hemolytic anemia patients: Approximately 2 ml. of bone marrow was aspirated from the tibia of a newborn infant with anti-Rh<sub>e</sub> erythroblastosis and from the iliac crest of a 13-year-old patient with autoimmune hemolytic anemia. (Cases 1 and 2) The nucleated cell suspensions were prepared in identical manner to that described above.

Preparation of reticulocyte rich cell suspension: Venous blood (5 ml.) was obtained from one patient with anti-Rh<sub>e</sub> erythroblastosis. (Case 2) The blood was collected in Versenate Disodium (1 mg. per ml. of blood). The blood was allowed to sediment for 3 hours in 4 Wintrobe hematocrit tubes at 25 C. At the end of this period, the supernatant was centrifuged at 600 G. for 10 minutes. The red cell button was then washed 3 times in EDTA-saline. After this, four per cent red cell suspensions in EDTA-saline were prepared.

Antisera: The AB serum was prepared from a non-secretor AB healthy donor. Anti-A, anti-B, non-agglutinating anti-D, anti-C, anti-E, anti-c, anti-M, anti-N and anti-Lu<sub>b</sub> and

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anti-human globulin were obtained commercially. The anti-e, anti-K, anti-Fya, anti-S, anti-s, anti-Tja, and anti-P antibodies were supplied by Mrs. Jane Swanson from the War Memorial Blood Bank of Minneapolis. The complete anti-D, the anti-k, anti-Fyb, anti-Leb, anti-Jk\(a\), anti-Jk\(b\) and anti-I\(i\) (from a patient with acquired autoimmune hemolytic anemia), were obtained from patients at the University of Minnesota Hospitals. All sera complement were inactivated by the addition of 1 mg. of Versenate Disodium per ml. of serum.

**Case Histories**

Case 1—This is a 13-year-old boy who came to the hospital on August 8, 1963 with a recent history of sore throat and fever and a past history of cleft palate, bilateral hearing loss, idiopathic thrombocytopenic purpura at age 3, and hemolytic crises in 1959 and 1960. Ten days after admission, the hemoglobin dropped from 12.4 to 5.6 Gm. per cent in 48 hours, and remained between 4.3-5.5 Gm. per cent during the third, fourth and fifth weeks after admission. From August 19 to August 28 the reticulocyte count was always below 1 per cent and reached a low of 0.2 per cent on August 23. The bone marrow aspirated August 23 showed some features consistent with hemolysis (increase of iron storage and moderate erythrophagocytosis), but also showed normal cellularity and a normal percentage of normoblasts. A portion of the bone marrow specimen was processed as described under Methods. Immunohematologic studies performed on the same day revealed a 3+ direct antiglobulin test performed with a broad spectrum antiglobulin serum. This reaction was negative when using an anti-human non-gamma globulin serum, and gave a 3+ reaction with anti-human gamma globulin serum. The serum of the patient was shown to contain an autologous cold agglutinin possessing a wide range of thermal reactivity giving a positive indirect Coombs test. A sucrose gradient centrifugation study of the patient's serum showed that all the antibody activity was present in the 7S globulin fraction and that it reacted at 4, 25 and 37 C. as an agglutinating antibody, and it reacted more strongly at 37 C. when using the antiglobulin reaction. The specificity of the antibody was tested against a large number of group O erythrocytes, including ii cells, pp cells, and Bombay erythrocytes. The antibody was capable of producing agglutination of all cells tested. In view of the hematologic findings, the diagnosis of "hypoplastic" crisis of autoimmune hemolytic anemia was made. Treatment with steroids was started and the patient showed improvement of his anemia. On October 5, his reticulocyte count was 26.6 per cent. Two days later, his hemoglobin was 7.0 Gm. per cent, the reticulocyte count was 8.2 per cent, the direct and indirect Coombs tests were negative and he was discharged from the hospital.

Case 2—The patient was a newborn weighing 2490 Gm., admitted to the University Hospital because of marked jaundice at 14 hours of age. On admission, the patient had a total bilirubin of 12.2 mg. per cent, a hemoglobin of 11.7 Gm. per cent and a white cell count of 20,050 per mm.\(^3\) with 32 normoblasts per 100 white cells. The reticulocyte count was 23 per cent. A bone marrow study showed moderate hyperplasia with 35 per cent normoblasts in the differential count. A portion of the bone marrow specimen was processed as described under Methods. The newborn erythrocytes were typed as group A, Rh positive, and the direct Coombs test gave a 4+ reaction. The eluate obtained from the infant's erythrocytes contained anti-Rho specificity. The mother's erythrocytes were typed as group A, Rh negative, and the indirect Coombs test gave a 4+ reaction. The maternal anti-Rh

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*Ortho Foundation, Raritan, N. J.
†Reported with the kind permission of Dr. William Krivit.
‡Hyland Laboratories, Los Angeles, Calif.
§Performed by Dr. Ralph C. Williams of the Dept. of Internal Medicine of the University of Minnesota.
¶Reported with the kind permission of Dr. John Anderson.
titer was found to be 1:256. Because the total bilirubin level rose over 17 mg. per cent, exchange transfusions on two occasions were necessary before discharging the patient.

**METHODS**

The agglutination methods used varied according to the different testing antisera. The slide direct agglutination method was used with the antisera containing an agglutinating antibody reacting at 25 C. and with an avidity of 120 seconds or less [anti-Rho(D), anti-Tja, anti-M, anti-N, and anti-I]. The other isoantigens were tested using the tube method: One 0.1 ml. drop of nucleated cell suspension of fresh human bone marrow was placed in a 10 x 75 mm. tube. All tubes were centrifuged at 600 G. for 2 minutes and all the saline supernatant removed by capillary pipette. Amounts of 0.15 ml. of testing serum was added to each experimental tube. The cells and antiserum were mixed thoroughly and incubated at room temperature (25 C.) for 5 minutes or at 37 C. for 60 minutes. When albumin agglutinins were used [anti-(c) and anti-s], the cells were completely resuspended in an equal volume of 22 per cent albumin and then 0.15 ml. of testing serum was added followed by incubation at 37 C. for 60 minutes. Many of the isoantigens tested were detectable by the antiglobulin reaction (table 1). With this technic, the sensitized cells were washed three times with EDTA-saline and incubated at 25 C. for 5 minutes with human antiglobulin serum, followed by three washings with EDTA-saline.

Each bone marrow sample served for testing at least 6 different sera selected according to the erythrocyte typing so at least two of the antigens tested gave negative reactions serving as controls. Each experiment was accompanied additionally with a negative control consisting of mixture of nucleated cells and AB serum. The control of the antibodies requiring the antiglobulin reaction consisted of the addition of AB serum in the place of the testing serum followed by the antiglobulin reaction. Whenever using the tube typing method after the incubation period or after the addition of antiglobulin serum, all cell mixtures were centrifuged at 600 G. for one minute and then the cells were gently resuspended; one drop of the suspension was then placed on a slide under a coverglass and any excess fluid was removed by gentle pressure applied over the coverslide with a piece of blotter paper. The preparations were sealed with Kroening wax and examined under phase microscopy. Positive reactions were read as presence of normoblasts agglutinated with erythrocytes and reticulocytes. Each one of the positive reactions was examined for differential counts of nucleated cells present free or in agglutinates. These results were compared with the definitive counts of the control samples as previously described. The strength of a positive reaction was classified according to the total number of normoblasts and erythrocytes agglutinated as follows: When at least 75 per cent of the normoblasts were agglutinated (4) at least 50 per cent of the normoblasts (3), at least 25 per cent of the normoblasts (2), less than 25 per cent of the normoblasts (1), a negative reaction (0), was read as lack of agglutination of any cell types.

**Testing of coated normoblasts and reticulocytes in a case of erythroblastosis fetalis and of normoblasts in a case of autoimmune hemolytic anemia:** One 0.1 ml. of EDTA-saline washed marrow nucleated cells (from a patient with erythroblastosis fetalis, and a patient of autoimmune hemolytic anemia) were placed in 10 x 75 mm. tubes and added to 0.1 ml. of antihuman globulin serum. The procedure was identical to that described above, with the modification that in each test the negative controls consisted of AB serum with the nucleated cell suspension, and in a second tube, the nucleated cell suspension was mixed with absorbed antihuman gamma globulin.* The antiglobulin reaction of the reticulocyte cell suspension was performed in a way similar to that used for the bone marrow. However, the antiglobulin serum and the absorbed antiglobulin serum used were prepared by addition of equal amounts of 1 per cent brilliant cresyl blue. The mixture of cells and anti-
globulin-brilliant cresyl blue was incubated at 25 C. for 10 minutes and then centrifuged at 350 C. for one minute. The cells were then resuspended completely to produce small agglutinates and air-dried smears were made. The preparations were then counter-stained with Wright-Giemsa and examined for the presence of reticulocytes.

**RESULTS**

Nineteen out of 30 nucleated cell suspensions were suitable for study. There were ten group O, seven group A, and two group B individuals. Four of the nineteen individuals were Rh0 negative and fifteen were Rh0 positive. Classified according to their Rh phenotypes, the marrow samples were classified as follows: Seven Rh0 (D), rh'(C), hr'(c) hr''(e) positive and rh''(E) negative, three Rh0, rh', hr'' positive and rh' and hr' negative; two Rh0, rh', rh'', hr' and hr'' positive, and three Rh0, rh'', hr', hr'' positive and rh' negative. Each individual was tested for at least 6 isoantigens, care being taken in many cases of testing the antigens present in the homozygous state so that the serum for the allele antigen would serve as a negative control. For example, if the individual was homozygous for the rh'(C) antigen, the nucleated cell suspension was tested with both the anti-rh'(C) and the anti-hr'(c) antisera. Each bone marrow tested was always studied with a control of AB serum, and in those cases requiring the antiglobulin reaction, the nucleated cell suspension was mixed with AB serum and then tested with anti-human globulin.

Table 1 summarizes the results of the agglutination reactions of nucleated cell suspensions of different bone marrows with different iso-antibodies. It shows that normoblasts give agglutination reactions similar to those of the isoantigens present in the erythrocytes. The reactions were considered specific on grounds of negative results with AB serum and with sera known to give negative results with the erythrocytes obtained from the same individual from which the bone marrow was obtained.

Because the genotypes pp, kk, ii and Lu^aLu^a are rare, the negative controls for the reactions of Tj^a, K, I and Lu^a antigens consisted only of negative reactions obtained with AB serum and other antisera studied.

The differential counts of normoblasts, erythrocytes and white cells present as part of the agglutinates or as free cells showed that the positive reactions are specific for the corresponding normoblasts and erythrocytes and not for the white cells. The white cells counted in the agglutinates varied between 2 to 8 per cent of nucleated cells. However, only the reactions with the anti-Tj^a serum produced agglutination of a large number of white cells.

Table 1 also shows the results of the strength of the agglutination reactions. It can be seen that the strongest reactions were seen with the antisera containing agglutinating antibodies of avidity of less than 120 seconds (anti-Rh0, anti-Tj^a, anti-M, anti-N, and anti-I).

No attempts were made to study the normoblasts in different stages of development. However, the examination of the agglutinated cells disclosed early and late forms of the nucleated red cells of the bone marrow.

*Results of the antiglobulin reaction of normoblasts of erythroblastosis fetalis*
and autoimmune hemolytic anemia and of reticulocytes of erythroblastosis fetalis: The antiglobulin reaction of nucleated cell suspensions of human bone marrow was performed on 12 of the 19 samples of bone marrow studied after incubation of the sample with AB serum for 60 minutes at 37°C. These tests were negative and served as controls for the antiglobulin reactions of the autoimmune hemolytic anemia and erythroblastosis fetalis.

Table 2 shows the results of the differential counts (1,000 nucleated cells counted) of normoblasts, erythrocytes and white cells of bone marrow present free or as part of agglutinates as a result of the antiglobulin reaction in a patient with autoimmune hemolytic anemia (fig. 1) and a patient with erythroblastosis fetalis. It demonstrates that the antiglobulin reactions are specific on grounds of negative agglutination reactions with absorbed antiglobulin serum.

The antiglobulin reaction of reticulocytes of the patient with erythroblastosis fetalis gave a 3+ reaction (fig. 2). The antiglobulin reaction of reticulocytes was considered specific on grounds of negative agglutination reaction with absorbed anti-human globulin.

**DISCUSSION AND CONCLUSIONS**

In previous studies, we described the presence of A, B and H antigens on human normoblasts including the pronormoblasts, anti-anti-sera to specific antisera, it has been possible to demonstrate the iso-antigens of the following blood systems on normoblasts: Rh, MNSs, P, Lutheran, Kell, Lewis, Duffy, Kidd and I. Under the experimental conditions de-
Table 2.—Differential Counts of Free and Agglutinated Normoblasts, Erythrocytes and White Cells of Bone Marrow Obtained from (1) a Patient with Autoimmune Hemolytic Anemia and (2) a Patient with Erythroblastosis Fetalis Tested by the Direct Antiglobulin Method

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<th>Autoimmune Hemolytic Anemia</th>
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<td>Absorbed human antiglobulin</td>
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<td>% Agglutinated cells</td>
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<td>Erythrocytes</td>
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<td>Normoblasts</td>
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<td>White cells</td>
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scribed in this paper, the treatment of nucleated cell suspension of the bone marrows with specific antisera produced agglutination of normoblasts and red cells leaving free most of the other elements of the bone marrow. A small percentage of white cells was usually found in the agglutinates, although pure white cell preparations were found to be lacking in all antigens tested with the exception of Tja. It is possible that the white cells present in the agglutinates were related to "mechanical trapping."

The demonstration of isoantigens on normoblasts is in keeping with previous reports, and suggests that the appearance of cell antigens occurs early.

Fig. 1.—Direct antiglobulin positive agglutination reaction of normoblasts and erythrocytes of a patient with autoimmune hemolytic anemia. Phase contrast microscopy. X 500. Print = 2000 X.
in cell formation and independent of the maturation of the cell. Many of the isoa antigens were demonstrated by both agglutinating and non-agglutinating antibodies.

Severe reticulocytopenia, the result of bone marrow "aplasia" is now recognized to be an uncommon but important complication of autoimmune hemolytic anemia. Acute "aplastic" crises complicating autoimmune hemolytic anemia have been reported by others. Some workers have found by the antiglobulin test that nucleated erythrocytes can be coated by antibodies from patients with autoimmune hemolytic anemia. The two cases reported in this article illustrate the acute "aplastic" crises complicating autoimmune hemolytic anemia with reticulocytopenia and lack of normoblastic hyperplasia, and a severe case of erythroblastosis fetalis with normoblastic hyperplasia and reticulocytosis. In both cases, the normoblasts were shown to be coated by gamma globulin. In the erythroblastosis, where reticulocytes were sufficiently numerous to study, they were also shown to be coated. Some investigators have stressed the importance of the failure of erythrocyte production in the pathogenesis of the anemia of hemolytic disease of the newborn. It is thought that the bone marrow hypoplasia ("aregenerative anemia") may occur in severe erythroblastosis as a result of the direct suppressive effect on the erythropoietic centers by the offending agglutinins. Other investigators were not able to demonstrate a significant variation in the normoblastic activity of infants with erythroblastosis fetalis suffering from post-exchange transfusion anemia.10,11

It has been reported that blood samples rich in reticulocytes may give a "false" positive antiglobulin reaction12,13 which do not depend on the anti-gamma globulin specificity of the antihuman globulin serum.14 We have shown that the coating of reticulocytes and normoblasts in the two cases
reported is specific on grounds of negative results with absorbed antihuman globulin serum.

In conclusion, the findings in the patient with autoimmune hemolytic anemia support the concept that a normoblastic hypoplasia of the bone marrow may occur as cell destruction exceeds cell production, explaining the reticulocytopenia. On the other hand, the finding of gamma globulin coating reticulocytes from peripheral blood and normoblasts from the bone marrow of the infant with erythroblastosis fetalis suggests that anti-Rho antibodies can coat the immature erythrocytes in vivo. Bone marrow depression is rare in autoimmune hemolytic anemia and in erythroblastosis fetalis and might depend on multiple factors, including the physicochemical properties of the antibody, the damaging effect of normoblasts and reticulocytes by the offending antibodies, the severity of the hemolytic process and the ability of the individual to produce red cells. Studies on these questions are in progress at present in our laboratory.

**SUMMARY**

Evidence is presented for the presence of the antigens of nine blood group systems on normoblasts. The antigen receptors are detected on all stages of normoblasts. The in vivo demonstration of gamma globulin coating the reticulocytes and the normoblasts of a patient with erythroblastosis fetalis and the normoblasts of a patient with autoimmune hemolytic anemia are discussed in relation to the pathogenesis of the anemia in both conditions.

**SUMMARIO IN INTERLINGUA**

Es presentate constatationes in evidentia del presentia de antigenos de plure systemas de gruppos de sanguine in le normoblastos. Le receptores antigenic es detegite in omne stadios del normoblastos. Le demonstration in vivo de globulina gamma "revestiente" le reticulocytos e le normoblastos de un patiente con erythroblastosis fetal e le normoblastos de un patiente con autoimmun anemia hemolytic es descutite in relation con le pathogenese del anemia in le duo conditiones.

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**REFERENCES**


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