Immuno-Electronmicroscopic Studies of Surface Antigens of Blood Elements
I. Autoantibody on Erythrocytes in Acute Hemolytic Anemia

By GIORGIO TONIETTI, GIUSEPPE A. ANDRES, LIDIA ACCINNI, MARIA PURPURA AND KONRAD C. HSU

FERRITIN-LABELED ANTIBODY has been used for the localization, by electron microscopy, of antigens at the surface of cells. Lee and Feldman, Harris, and Haberman et al. investigated blood group antigens on the cell membranes of human erythrocytes with ferritin-labeled antibody to A, B or Rh antigen. Müller-Eberhard and his associates demonstrated the ultrastructural localization of various components of complement on sheep erythrocytes previously treated with antibodies and complement.

In autoimmune hemolytic anemia it has been assumed that an antigen, as yet unidentified, located on the surface of the red cells is responsible for evoking the production of the autoantibody which is then bound to the patient's own erythrocytes. The purpose of the experiments reported here was to examine the sites of localization of the autoantibody on the red cells of a patient with autoimmune hemolytic anemia, and further to study the sites of localization of the patient's autoantibody on the erythrocytes of other individuals of the same blood group. Ferritin-labeled antibodies to human immunoglobulins and to a fraction of complement were used in an attempt to tag the sites of the red cells where the patient's autoantibody had been bound.

METHODS AND MATERIAL

Source of Erythrocytes

The patient (L.S.), a seven year old male, was studied at the Centro Nazionale Transfusione Sangue, C.R.I., in March 1967. His history of anemia dated back to May 1964.

Antiglobulin Tests

Direct agglutination tests performed with the patient's erythrocytes and antiserum to human globulin and antiserum to human IgG were positive. Absorption of the antihuman...
Physical examination showed a deeply jaundiced boy. On palpation of the abdomen, the liver was felt 2 cm. below the right costal margin and the spleen 3 cm. below the left costal margin. The superficial lymph nodes were minimally but definitely enlarged. Examination of the blood revealed a red cell count of 1.8 million, a total white count of 3,600 and a platelet count of 180,000 per cu. mm. Bilirubin was 4.9 mg./100 ml. and the osmotic fragility of the red blood cells was normal. The blood group was O CCDee.

globulin with pure IgG rendered the serum incapable of agglutinating the erythrocytes of the patient, thus showing that the antibody coating the erythrocytes was of the IgG class.

The patient's serum and the antibody eluted from his erythrocytes were tested for reactivity with a panel of eleven samples of Group O blood. The latter were selected to allow detection of antibody for the following blood group factors: Rh (Cc, Cw D, E, e, F), V, MN, S, P, Tja, Lewis, Lutheran, Kell, k, Kp, Duffy, Kidd, Sutter and Xg. None of the test cells were agglutinated in saline at 4 C., room temperature, or 37 C. With the indirect Coombs' test, however, all cells in the panel were agglutinated. Likewise the serum and eluate agglutinated all cells which had been treated with papain or trypsin. The antibody concerned therefore could not be related specifically to any of the blood groups represented.

Conjugation of Antibody with Ferritin

Antibody to human IgG, IgM, and β1C were conjugated with ferritin according to a method described elsewhere. Conjugated antibodies to rat globulin and purified (unconjugated) ferritin were also prepared to be used as controls.

Preparation of Erythrocytes for Testing with Ferritin-Conjugated Antisera

Samples of blood were drawn from the patient and from normal subjects represented in the panel into equal volumes of acid citrate dextrose (ACD) solution. Samples for immunologic testing were prepared as follows: 1) Erythrocytes from the patient were washed three times in saline at room temperature, with alternate centrifugation and resuspension. A 10 per cent suspension of the washed erythrocytes was prepared; and 0.3 ml. samples were exposed to equal volumes of ferritin-conjugated antibody to human IgG, IgM, β1C and rat globulin and to purified ferritin. 2) Another sample of 0.3 ml. of washed erythrocytes obtained from the patient was preincubated with unlabeled antibody to human IgG for 15 minutes at room temperature, washed, and then exposed to the ferritin-conjugated antibody to human IgG. 3) Thrice-washed erythrocytes obtained from the panel were incubated at 37 C. for 1 hour with an equal volume of eluate prepared from red blood cells of the patient as described above. The red cells were then washed and five samples of 0.3 ml. each were incubated with equal volumes of the four ferritin-conjugated antibodies mentioned in 1), as well as with ferritin alone. 4) Another sample of erythrocytes obtained and treated as in 3) was incubated at 37 C. with the serum of the patient for 1 hour, washed, and then treated with the same four ferritin-conjugated antisera and with pure ferritin. 5) Finally, a sample of the normal red blood cells from the panel was washed and 0.3 ml. were directly incubated with equal volumes of ferritin-conjugated antibody to human IgG.

All the incubation with ferritin-conjugated antibodies was performed for 15 minutes at room temperature with gentle shaking. After three more washings in saline the red cells were fixed for 20 minutes in buffered osmium tetroxide. Pellets were obtained by centrifugation at 1,000 rpm. for 10 minutes. These were dehydrated in graded solutions of acetone and embedded in Araldite. Thin sections were cut in a Leitz ultramicrotome and observed in the electron microscope either unstained or after staining with lead hydroxide.

Results

The results obtained by electron microscopic study of erythrocytes treated with ferritin-labeled antibody to IgG, IgM and β1C and with the labelled antibody to rat globulin and purified ferritin are summarized in Table 1. When
Table 1

<table>
<thead>
<tr>
<th>Source of Erythrocytes</th>
<th>Incubated with</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
<td>Ferritin-conjugated antibody to human IgG</td>
<td>positive</td>
</tr>
<tr>
<td>&quot;&quot;</td>
<td>&quot;&quot; IgM</td>
<td>negative</td>
</tr>
<tr>
<td>&quot;&quot;</td>
<td>&quot;&quot; IgG</td>
<td>negative</td>
</tr>
<tr>
<td>&quot;&quot;</td>
<td>&quot;&quot; rat globulin</td>
<td>negative</td>
</tr>
<tr>
<td>&quot;&quot; Purified ferritin</td>
<td></td>
<td>negative</td>
</tr>
<tr>
<td>&quot;&quot; Unlabelled antibody to human IgG followed by ferritin-conjugated antibody to human IgG</td>
<td>negative</td>
<td></td>
</tr>
</tbody>
</table>

Normal subject + eluate from patient's erythrocytes

<table>
<thead>
<tr>
<th></th>
<th>Ferritin-conjugated antibody to human IgG</th>
<th>positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&quot;&quot; IgM</td>
<td>negative</td>
</tr>
<tr>
<td></td>
<td>&quot;&quot; IgG</td>
<td>negative</td>
</tr>
<tr>
<td></td>
<td>&quot;&quot; rat globulin</td>
<td>negative</td>
</tr>
<tr>
<td>Purified ferritin alone</td>
<td></td>
<td>negative</td>
</tr>
</tbody>
</table>

Normal subject + serum of the patient

<table>
<thead>
<tr>
<th></th>
<th>Ferritin-conjugated antibody to human IgG</th>
<th>positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&quot;&quot; IgM</td>
<td>negative</td>
</tr>
<tr>
<td></td>
<td>&quot;&quot; IgG</td>
<td>negative</td>
</tr>
<tr>
<td></td>
<td>&quot;&quot; rat globulin</td>
<td>negative</td>
</tr>
<tr>
<td>Purified ferritin alone</td>
<td></td>
<td>negative</td>
</tr>
</tbody>
</table>

Normal subject

|                                    | Ferritin-conjugated antibody to human IgG | negative |

* Based on electronmicroscopic observation.

the erythrocytes of the patient were exposed to ferritin-conjugated antibody to human IgG the antibody-carrying ferritin molecules appeared clustered on the surface of red blood cells at fairly even intervals (Fig. 1). In cross sections the clusters lined the margin of the cells. In tangential sections the clusters appeared on the erythrocytes' surface with a more irregular pattern. The relative thickness of these sections probably is responsible for the observation of ferritin clusters localized at different levels. Treatment of the erythrocytes of the patient with unlabeled antibody to human IgG, prior to immersion in ferritin-conjugated antibody to IgG, blocked the reaction.

When normal red cells from the panel were incubated with the eluate obtained from the erythrocytes of the patient or with his serum and then treated with ferritin-conjugated antibody to IgG, clusters of the ferritin-antibody complexes were visible on their surface. Ferritin particles in each such cluster appeared to be less numerous than they were on the erythrocytes of the patient treated with the same ferritin-conjugated antiserum (Fig. 2), but the mean distance between clusters appeared to be the same.

When the erythrocytes of the patient were tested with ferritin-conjugated antibodies to IgM, IgG, or rat globulin, or with purified ferritin, there was no localization (Fig. 3). There was also no localization of ferritin-conjugated antibody to IgM, IgG, or rat globulin on normal erythrocytes from the panel previously incubated with the eluate obtained from the red blood cells of the patient or with the serum of the patient. The ferritin-conjugated antibody to IgG did not localize on normal erythrocytes from the panel.
Fig. 1.—Electronmicrograph of patient's red blood cells treated with ferritin-conjugated antibody to human IgG. In cross section clusters of ferritin molecules are seen on surface of cells at fairly even intervals and in tangential section clusters appear with more irregular pattern. × 45,000 (original magnification).

DISCUSSION

Intense binding of ferritin-conjugated antibody to IgG was observed on the surface of erythrocytes from a patient with hemolytic anemia. Ferritin-conjugated antibody to IgM did not localize at the surface of the same cells. Elution of the antibody from erythrocytes of the patient and its binding with
Fig. 2.—Electronmicrograph of normal red cells which have been incubated with the eluate from patient's erythrocytes prior to treatment with ferritin-conjugated antibody to IgG. Clusters of ferritin particles are seen in similar distribution as illustrated in Figure 1. × 45,000 (original magnification).

erythrocytes from normal individuals represented in the panel has been confirmed at the electron microscopic level. As in the case of the patient's own red cells, only ferritin-labeled antibody to IgG was bound.

The pattern of localization of ferritin-conjugated antibody was the same on erythrocytes taken from the patient as it was on the erythrocytes of individuals of the same blood group represented in the panel exposed to the serum of the patient or to the eluate from his red cells. It is difficult to ascertain whether the clusters of ferritin on the red cells in each instance represented all the sites of the specific antigen. This seems probable, however, because of the uniform distribution of the ferritin in the three reactions.

The pattern of localization of the patient's IgG-antigen complex on the surface of the erythrocytes in each case was similar to that described by Lee and Feldman for Group A antigen studied with specific ferritin-labeled antibody. It has been proposed that "warm" autoantibodies may preferentially be directed against Rh antigens. However, the pattern of distribution of the ferritin-conjugated antibody in this patient did not resemble the distribution of the anti-Rh antibody, as described by Lee and Feldman. Autoantibodies against A and B antigens are reported to be rare. A correlation between the specificity of autoantibodies and their distribution on the surface of red blood cells requires further study.

Ferritin-conjugated antibody to β1C was not bound to the surface of the patient's red blood cells. "Warm," incomplete antibody requires complement for lysis, but may fix to red blood cells in the absence of the thermolabile com-
ponents of complement. If some cells had fixed complement, they may have been lysed and therefore have not been observed.

The use of the immunoferritin technic for tagging antigens on the surface of cells is particularly simple because no prefixation is necessary. This technic proved useful in localizing the antigenic sites on the red cells which bound the autoimmune antibody responsible for the patient's disease. It should also have application to problems concerned with the ultrastructural localization of antigens in other formed elements of the blood.

**SUMMARY**

Tagging, by means of the immunoferritin technic, of autoantibody on the erythrocytes of a patient with autoimmune hemolytic anemia, and on cells from a panel of blood of normal individuals of blood group O which have been incubated either with the patient's serum or eluate from his cells, is described. It was found that: 1) Ferritin-labeled antibody to human IgG was localized on the surface of the patient's erythrocytes at fairly even intervals. 2) Ferritin-labeled antibody to human IgM, β1C, rat globulin or pure ferritin alone was not bound to the patient's cells. 3) None of the ferritin-conjugates mentioned in 1) or 2) or pure ferritin was bound to red cells from normal individuals represented in the panel. 4) Only ferritin-conjugated antibody to human IgG was localized, in a similar pattern, on the surface of the normal red cells which had been incubated either with the patient's serum or the eluate from his cells, whereas none of the conjugates in 2) or pure ferritin was bound to these treated cells.
SUMMARIO IN INTERLINGUA

Es describite un technica de marcar, per medio del methodo a immunoferritina, le autoanticorpore super le erythrocytos de un patiente con autoimmun anemia hemolytic e super cellulas ab un batteria de specimens de sanguine ab subjectos normal del grupp sanguine O incubate con le sero del patiente o con eluato ab su cellulas. Le uso del technica resultava in le sequente constatationes: 1. Ferritino-marcate anticorpore anti IgG human esseva localisate al superficie del erythrocytos del patiente a intervallas satis uni-forme. 2. Ferritino-marcate anticorpore anti human IgM, β1C, globulina de ratto, o ferritina pur per se non esseva ligate al cellulas del paciente. 3. Nulle del conjugatos a ferritina mentionate sub (1) o (2) o ferritina pur esseva ligate a erythrocytos ab le subjectos normal representate in le grupp. 4. Solo ferritino-conjugate anticorpore anti human IgG esseva localisate, in un simile configuration, al superficie del erythrocytos normal que habeva esiste incubate con le sero del patiente o con le eluato ab su cellulas, durante que nulle del conjugatos mentionate in (2) o ferritina pur esseva ligate a iste tractate cellulas.

REFERENCES

8. Weiner, W.: Eluting red-cell antibod-
Immuno-Electronmicroscopic Studies of Surface Antigens of Blood Elements: I. Autoantibody on Erythrocytes in Acute Hemolytic Anemia

GIORGIO TONIETTI, GIUSEPPE A. ANDRES, LIDIA ACCINNI, MARIA PURPURA and KONRAD C. HSU

Updated information and services can be found at:
http://www.bloodjournal.org/content/33/2/179.full.html
Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml