In Vivo Localization of Heterologous Anti-Erythrocyte Antibodies in the Rat Bone Marrow and Their Effect on the Proliferative Capacity of Immature Erythroid Cells

By TAKEO KUROYANAGI, MASANOBU SAITO AND AKIRA KURISU

Patients with acquired auto-immune hemolytic anemia associated with a decreased red cell production have been reported by several authors. We have observed such cases among patients with acquired auto-immune hemolytic anemia due to incomplete antibodies of the warm variety. There have been many reports suggesting the sensitization and damages of erythroblasts in the bone marrow by anti-erythrocyte antibodies. Recently Yunis and Yunis have demonstrated A1, A2 and B receptor on orthochromatic and basophilic normoblasts. These results suggest that anti-erythrocyte antibodies not only sensitize circulating red cells but also exert some cytotoxic effects on nucleated red cells in the bone marrow.

However, little attention has been so far directed to the localization of anti-erythrocyte antibodies in the bone marrow and their effect on immature erythroid cells. By means of the I labeled antibody method, the in vivo localization of heterologous anti-erythrocyte antibodies was determined in the rat bone marrow. The effect of anti-erythrocyte antibodies on the proliferative capacity of nucleated red cells was studied in vitro by the incorporation of tritiated thymidine.

METHODS

1. Production of Anti-Erythrocyte Antibodies

Two ml. of 50 per cent suspension of rat erythrocytes in physiologic saline were injected intravenously into rabbits seven times at an interval of 3 days. Two weeks after the last injection rabbits were sacrificed to obtain anti-erythrocyte serum. Globulin was fractionated from the above serum by sodium sulfate method.

2. I Labeling of Antibody

The modified iodination method of Pressman and Eisen described by the authors in the previous paper was used in this study for the I labeling of antibody.

3. Assay for the in Vivo Localization

One ml. of the I labeled anti-erythrocyte antibody was injected into the tail vein of 5 normal rats. Twenty-four hours after the injection rats were weighed, heparinized and perfused completely with physiologic saline. Tissue samples weighing less than 1 Gm. were used for the determination of radioactivities. Usually the kidney and the spleen were counted as a whole, whereas the liver was counted from cut sections. Both femurs were...
weighed and counted as a whole, followed by the removal and weighing of the bone marrow. The weight of total bone marrow was calculated as three per cent of body weight according to Fairman and Corner.\textsuperscript{13}

4. \textit{Determination of Per Cent Localization}

A well-type scintillation counter with a sodium iodide crystal and 3 inches lead shield was used for the determination of radioactivities in tissues. The background was less than 170 cpm and counts were considered significant only if more than five times this background.

Per cent localization was calculated by dividing total counts in the tissues with total counts of injected $^{131}$I-labeled antibodies. Average values of per cent localization determined in 5 rats were considered to represent the in vivo localization of each antibody sample.

The total radioactivities localized in each organ is the sum of specific localization of radiolabeled antibodies and nonspecific accumulation of radiolabeled normal globulin. In order to determine the nonspecific uptake, the in vivo localization of $^{131}$I-labeled normal rabbit globulin was determined in 40 rats. The mean and 95 per cent confidence limits in these rats were: bone marrow, 0.79 $\pm$ 0.1 per cent; liver, 1.4 $\pm$ 0.7 per cent; and spleen, 0.14 $\pm$ 0.08 per cent, respectively. These mean localizations of radiolabeled normal globulin were considered to represent the background localization.

The normal globulin (diluted to 1 : 10 with physiologic saline) was heated at 70 C. for 60 minutes, cleared by centrifugation and then lyophilized. The heat-treated normal globulin was labeled with $^{131}$I by the above mentioned method. The $^{131}$I-labeled heat-treated normal globulin was injected into 20 rats intravenously in order to determine the per cent localization. The mean and 95 per cent confidence limits of per cent localization of heat-treated normal globulin were: bone marrow 0.42 $\pm$ 0.3 per cent, liver 1.5 $\pm$ 0.7 per cent, and spleen 0.2 $\pm$ 0.1 per cent, respectively. The mean per cent localization was considered to represent the background localization in the experiments in which the in vivo localization of the heat-treated anti-erythrocyte antibody was studied.

The specific localization (net localization) due to antibody was determined by calculating differences between the observed total per cent localization and the above background per cent localization.

5. \textit{Labeling Technic with Tritiated Thymidine}

Four and one-half ml. of the bone marrow cell suspension containing approximately $10^8$ cells per cu. mm. was added to 0.5 ml. of stock solution containing tritiated thymidine and Na$_2$-EDTA. The final concentration of tritiated thymidine was 0.5 $\mu$c. per ml.

After incubating the above mixture for 1 hour at 37 C., smears were made on glass slides from the cell concentrates, fixed in methylalcohol, and covered with films which were developed after a two-week exposure period in a cold and dark room, and then stained through the film with the Giemsa stain. A total of 100 cells was enumerated at each degree of maturation of erythroblasts and the per cent of labeled cells was calculated.

\section*{RESULTS}

1. \textit{In Vivo Localization of Heterologous Anti-Erythrocyte Antibodies in the Rat Bone Marrow}

The in vivo localization of three different samples of anti-erythrocyte antibodies in normal rats was determined. Their hemagglutinin and hemolysin titer averaged 1 : 2560 and 1 : 560, respectively.

As shown in table 1, the net localization in the bone marrow averaged 2.4 per cent, ranging from 1.5 to 3.3 per cent. The net localization in the liver and the spleen averaged 0.8 per cent and 0.3 per cent respectively.

When anti-erythrocyte antibodies were heated for 60 minutes at 70 C.
TABLE 1.—In Vivo Localization of Heterologous Anti-Erythrocyte Antibodies in the Rat Bone Marrow

<table>
<thead>
<tr>
<th>Antibody Lot No.</th>
<th>Bone Marrow</th>
<th>Liver</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total Bkgd Net</td>
<td>Total Bkgd Net</td>
<td>Total Bkgd Net</td>
</tr>
<tr>
<td>1</td>
<td>2.3 0.8 1.5</td>
<td>2.0 1.4 0.6</td>
<td>0.7 0.1 0.6</td>
</tr>
<tr>
<td>2</td>
<td>3.2 0.8 2.4</td>
<td>2.5 1.4 1.1</td>
<td>0.2 0.1 0.1</td>
</tr>
<tr>
<td>3</td>
<td>4.1 0.8 3.3</td>
<td>2.1 1.4 0.7</td>
<td>0.4 0.1 0.3</td>
</tr>
<tr>
<td>Mean</td>
<td>3.2 0.8 2.4</td>
<td>2.2 1.4 0.8</td>
<td>0.4 0.1 0.3</td>
</tr>
</tbody>
</table>

Total: Total per cent localization; Bkgd: Background per cent localization; Net: Net per cent localization. The mean per cent localization of I\(^{131}\) labeled normal rabbit globulin as determined in 40 rats.

their hemagglutinin and hemolysin titer reduced markedly. The presence of incomplete antibodies in these heat-treated anti-erythrocyte antibodies was confirmed by an indirect antiglobulin reaction using duck anti-rabbit globulin serum. These heat-treated anti-erythrocyte globulins were injected into normal rats intravenously. Their net localization in the bone marrow, liver and spleen averaged 1.7 per cent, 0.5 per cent and 0.3 per cent, respectively, indicating that the localizing antibody is thermostable (table 2).

2. Effect of Absorption of Anti-Erythrocyte Antibodies with Erythrocyte Membrane on Their In Vivo Localization

Normal rat red cells were hemolyzed with distilled water and sediments were collected by centrifugation. The anti-erythrocyte antibodies were mixed with these sediments and incubated for 1 hour at 37 C. and then at 4 C. overnight. The above mixture was centrifuged and sediments were discarded. The supernatants were collected and lyophilized. Then these supernatants were labeled with radioactive iodine. These I\(^{131}\) labeled antibodies were injected intravenously to determine their in vivo localization. As shown in table 3, the net localization in the bone marrow averaged 0.2 per cent, ranging from 0.1 to 0.3 per cent. The net localization in the liver and the spleen averaged 0.5 per cent and 0.1 per cent, respectively.

3. Plasma Clearance of Anti-Erythrocyte Antibodies

In order to determine the plasma clearance of heterologous anti-erythrocyte antibodies, the determination of hemolysins and hemagglutinins titer and the anti-globulin test using duck anti-rabbit globulin serum were carried out in rats receiving rabbit anti-erythrocyte serum at various intervals following the intravenous injection. The clearance of the localizing antibody from plasma was also studied.

(a) Hemolysin, hemagglutinin and anti-globulin test. The hemolysin and hemagglutinin titer in serum of rats receiving intravenous injection of anterythrocyte antibodies was determined at various intervals after injection. Hemolysins and hemagglutinins could not be demonstrated in sera collected at 6 hours and during the first week following injection. The indirect anti-globulin reaction using duck anti-rabbit globulin serum was also negative.
Table 2.—In Vivo Localization of Heat-Treated Heterologous Anti-Erythrocyte Antibodies in the Rat Bone Marrow

<table>
<thead>
<tr>
<th>Antibody Lot No.</th>
<th>Bone Marrow</th>
<th>Liver</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bkgd</td>
<td>Net</td>
<td>Bkgd</td>
</tr>
<tr>
<td>1</td>
<td>2.4</td>
<td>0.9</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>2.3</td>
<td>0.9</td>
<td>1.4</td>
</tr>
<tr>
<td>3</td>
<td>3.1</td>
<td>0.9</td>
<td>2.2</td>
</tr>
<tr>
<td>Mean</td>
<td>2.6</td>
<td>0.9</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Bkgd: The mean per cent localization of $^{131}$-labeled heat-treated normal rabbit globulin as determined in 20 rats.

Table 3.—Effect of Absorption of Anti-Erythrocyte Antibodies with Erythrocytes Membranes on Their in Vivo Localization

<table>
<thead>
<tr>
<th>Antibody Lot No.</th>
<th>Bone Marrow</th>
<th>Liver</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bkgd</td>
<td>Net</td>
<td>Bkgd</td>
</tr>
<tr>
<td>1</td>
<td>1.0</td>
<td>0.8</td>
<td>0.2</td>
</tr>
<tr>
<td>2</td>
<td>0.9</td>
<td>0.8</td>
<td>0.1</td>
</tr>
<tr>
<td>3</td>
<td>1.1</td>
<td>0.8</td>
<td>0.3</td>
</tr>
<tr>
<td>Mean</td>
<td>1.0</td>
<td>0.8</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Bkgd: The mean per cent localization of $^{131}$-labeled normal rabbit globulin as determined in 40 rats.

The direct anti-globulin test using duck anti-rabbit globulin serum was positive 6 hours after injection of antibodies and remained positive for 1 week.

(b) Absence of the localizing antibody in rats serum collected 30 minutes after injection of anti-erythrocyte antibodies. $^{131}$-labeled anti-erythrocyte antibodies were injected intravenously into normal rats which were sacrificed 30 minutes later to obtain serum. These sera contained a large amount of radioactivity. They were injected intravenously into normal rats, in order to determine the in vivo bone marrow localization. In these experiments the mean per cent localization of $^{131}$-labeled normal rat globulin as determined in 20 rats was considered to represent the background localization. The mean background localization of normal rat globulin was as follows: bone marrow, 0.5 ± 0.1 per cent, liver, 1.0 ± 0.2 per cent and spleen, 0.1 ± 0.08 per cent, respectively.

The net localization of the above serum averaged 0.2 per cent in bone marrow, 0.6 per cent in liver, and 0 per cent in spleen, respectively.

4. Effect of Anti-Erythrocyte Antibodies on the Proliferative Capacity of Immature Erythroid Cells

Effects of anti-erythrocyte antibodies on the proliferative capacity of immature erythroid cells in rat bone marrow were studied with in vitro incorporation of tritiated thymidine.

The percentage of labeled basophilic and polychromatic erythroblasts of the normal rat bone marrow averaged 58.6 per cent and 36.7 per cent, respectively.
**Table 4.—Absence of Localizing Antibody in Serum Collected 30 Minutes after Injection from Rats Receiving Heterologous Anti-Erythrocyte Antibodies**

<table>
<thead>
<tr>
<th>Antibody Lot No.</th>
<th>Bone Marrow Total</th>
<th>Bkgd</th>
<th>Net</th>
<th>Liver Total</th>
<th>Bkgd</th>
<th>Net</th>
<th>Spleen Total</th>
<th>Bkgd</th>
<th>Net</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.6</td>
<td>0.6</td>
<td>0</td>
<td>1.6</td>
<td>1.0</td>
<td>0.4</td>
<td>0.2</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>2</td>
<td>0.9</td>
<td>0.6</td>
<td>0.3</td>
<td>1.5</td>
<td>1.0</td>
<td>0.5</td>
<td>0.1</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>0.6</td>
<td>0.4</td>
<td>1.8</td>
<td>1.0</td>
<td>0.8</td>
<td>0.1</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td><strong>0.8</strong></td>
<td><strong>0.6</strong></td>
<td><strong>0.2</strong></td>
<td><strong>1.6</strong></td>
<td><strong>1.0</strong></td>
<td><strong>0.6</strong></td>
<td><strong>0.1</strong></td>
<td><strong>0.1</strong></td>
<td><strong>0.1</strong></td>
</tr>
</tbody>
</table>

Bkgd: The mean per cent localization of $^{131}$-labeled normal rat globulin as determined in 20 rats.

Five-tenths ml. of anti-erythrocyte serum was added to 4.0 ml. of the bone marrow cell suspension containing approximately $10^6$ cells per cu. mm. in Hank's solution and incubated for 1 hour at 37 C. Then 0.5 ml. of the solution containing tritiated thymidine and $Na_2$-EDTA was added to the above mixture and incubated for 1 hour at 37 C. Smears were made on glass slides from the cell concentrates of the mixture for the radioautography. The percentage of labeled basophilic and polychromatic erythroblasts averaged 48.7 per cent and 14.0 per cent, respectively.

The proliferative capacity of bone marrow cells obtained 24 hours after injection from rats receiving the injection of 1 ml. of anti-erythrocyte serum was studied with in vitro incorporation of tritiated thymidine. The percentage of labeled basophilic and polychromatic erythroblasts averaged 45.0 per cent and 14.3 per cent, respectively.

**Discussion**

An increasing body of evidence suggests that anti-erythrocyte antibodies may not only sensitize circulating erythrocytes, but also exert some effects on immature erythroid cells. However, until recently little attention has been directed to the in vivo bone marrow localization of antierythrocyte antibodies. This matter was therefore studied by the in vivo localization of heterologous erythrocyte antibodies in the rat bone marrow using $^{131}$-labeled antibody. The net localization of $^{131}$-labeled anti-erythrocyte antibodies in the bone marrow averaged 2.4 per cent, indicating their specific localization. When anti-erythrocyte antibodies were heated at 70 C. for 1 hour, hemolysin and hemagglutinin titer decreased markedly. The net localization of the heat-treated anti-erythrocyte antibodies in the bone marrow averaged 1.7 per cent, indicating that the localizing antibody was thermostable.

When anti-erythrocyte antibodies were absorbed thoroughly with red cell membranes, their net localization in the bone marrow decreased to 0.2 per cent, indicating the absence of the localizing antibody. These results demonstrate that anti-erythrocyte antibodies localize specifically in the bone marrow and the localizing antibody is produced against red cell membrane antigens.

Plasma clearance of hemolysins and hemagglutinins was determined in rats receiving the intravenous injection of anti-erythrocyte antibodies. No
Table 5.—Effect of Heterologous Anti-Erythrocyte Antibodies on Tritiated Thymidine Uptake of Rat Erythroblasts in Vitro

<table>
<thead>
<tr>
<th>Per Cent of Labeled Cells</th>
<th>Normal Rat Bone Marrow</th>
<th>Normal Rat Bone Marrow + Antibody</th>
<th>Bone Marrow of Rats Receiving Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basophilic erythroblasts</td>
<td>58.6 ± 4.5</td>
<td>48.7 ± 6.3</td>
<td>45.0 ± 5.7</td>
</tr>
<tr>
<td>Polychromatic erythroblasts</td>
<td>36.7 ± 5.2</td>
<td>14.0 ± 5.3</td>
<td>14.3 ± 5.2</td>
</tr>
</tbody>
</table>

Normal Rat Bone Marrow + Antibody: Anti-erythrocyte serum was added to the bone marrow cell suspension in Hank’s solution in vitro and the mixture was incubated for one hour at 37°C.

Bone Marrow of Rats Receiving Antibody: Normal rats were given the intravenous injections of 1 ml. of anti-erythrocyte serum. The bone marrows collected from rats 24 hours after injection were studied for the radioautography by tritiated thymidine.

Hemolysins and hemagglutinins were demonstrated in serum collected 6 hours after injection of antibodies. Neither was the incomplete anti-erythrocyte antibodies demonstrated by means of an indirect anti-globulin test. However, the direct anti-globulin test became positive within 6 hours and remained positive for 1 week. The sera collected from rats receiving 131I-labeled anti-erythrocyte antibodies 30 minutes after injection showed a high radioactivity. When these sera were injected into normal rats, their net localization in the bone marrow averaged 0 per cent, indicating the absence of localizing antibody.

These results indicate that hemolysins and hemagglutinins in anti-erythrocyte antibodies are rapidly cleared from plasma as they enter circulating blood. The incomplete antibodies became attached to circulating erythrocytes within 6 hours. Red cell sensitization persisted for 1 week. The localizing antibody in anti-erythrocyte antibodies localized in the bone marrow within 30 minutes, leaving no activity in plasma.

Pisciotta,7 Rossi,8 Yunis and Yunis10 demonstrated the presence of common antigens in erythroblasts and erythrocytes. Thus it seemed likely that anti-erythrocyte antibodies may exert some cytotoxic effects on erythroblasts. Therefore, the effect of anti-erythrocyte antibodies on the proliferative capacity of immature erythroid cells was determined with tritiated thymidine uptake in vitro.

The percentage of basophilic and polychromatic erythroblasts of normal rats averaged 59 per cent and 37 per cent, respectively. The addition of anti-erythrocyte serum to the bone marrow cell suspension in vitro resulted in a decrease of the percentage of labeled basophilic and polychromatic erythroblasts, averaging 49 per cent, respectively. The bone marrow erythroid tissue of rats receiving anti-erythrocyte antibodies was studied with in vitro incorporation of tritiated thymidine. The percentage of labeled basophilic and polychromatic erythroblasts decreased markedly, averaging 45 per cent and 14 per cent, respectively.

Thymidine is incorporated solely into newly formed DNA, and tritium gives the superb radioautograph with least background. Thus with the introduction of tritiated thymidine,14-16 a highly specific and efficient DNA label has become available. The proportion of labeled cells in a population may indicate the length of DNA synthesis period to that of total generation cycle. There-
fore, the decreased percentage of labeled cells in a cell population would indicate the prolongation of the length of total generation cycle based on an assumption that DNA synthesis period were the same for all cells in that population.

Thus, it is evident that anti-erythrocyte antibodies exert an inhibitory effect on the proliferative capacity of immature erythroid cells.

**Summary**

The in vivo localization of heterologous anti-erythrocyte antibodies in the rat bone marrow was determined by the I\(^{131}\)-labeled antibody technic. I\(^{131}\)-labeled anti-erythrocyte antibodies localized specifically in the bone marrow indicating the presence of localizing antibody. Both the localizing antibody and the incomplete antibody were thermostable, whereas hemolysins and hemagglutinins were thermolabile. Following an intravenous injection of anti-erythrocyte antibodies in rats, hemolysins and hemagglutinins were cleared rapidly from the plasma. The incomplete antibodies became attached to circulating red cells within 6 hours and red cell sensitization persisted for 1 week. The localizing antibody localized in the bone marrow within 30 minutes, leaving no activity in plasma.

The anti-erythrocyte antibodies markedly reduced the uptake of tritiated thymidine by erythroblasts in vitro, demonstrating their inhibitory effect on the proliferative capacity of erythroblasts.

**Summary in Interlingua**

Le localisation in vivo de heterologe anticorpore anti erythrocytos in le medulla ossee del ratto esseva determinate per medio del technica a anticorpore marcate con I\(^{131}\). Assi marcate anticorpore anti erythrocytos se localisava specificament in le medulla ossee, lo que indicava le presentia de anticorpore localisatori. Le anticorpore localisatori e etiam le anticorpore incomplete esseva thermostabile, sed le hemolysinas e le hemagglutininas in le systema esseva thermolabile. Post le injection intravenose de anticorpore anti erythrocytos in rattos, le hemolysinas e hemagglutininas esseva rapidemente removite ab le plasma. Le anticorpore incomplete se attachava a erythrocytos in le circulation intra 6 horas, e le sensibilisation del erythrocytos persisteva durante un septimana. Le anticorpore localisatori se localisava in le medulla ossee intra 30 minutas, de maniera que nulle activitate remaneva in le plasma.

Le anticorpore anti erythrocytos reduceva marcatemente e acceptation de tritiate thymidina per erythroblastos in vitro, lo que demonstra su effecto inhibitori super le capacitate proliferatori del erythroblastos.

**References**

4. Moeschlin, S., Siegenthaler, W., and


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