Evidence for the in Vivo Localization of Heterologous Anti-Leukocyte and Anti-Bone Marrow Antibodies in the Rat Bone Marrow

By TAKEO KUROYANAGI, AKIRA KURISU AND HAJIME SUGIYAMA

THE PRESENCE of leukoagglutinins has been well known in several diseases, suggesting their possible roles in the mechanism of immunoleukopenia in man.1-2 It is generally believed that leukoagglutinins sensitize circulating leukocytes intravascularly. However, little is yet known on the effect of leukoagglutinins on immature granulocytic cells in the bone marrow.

It is well established that heterologous anti-leukocyte antibodies sensitize circulating leukocytes and induce their rapid destruction in experimental animals.21-30 However, their cytotoxic effect on immature granulocytic cells of the bone marrow has been denied by some authors, while others have accepted it as a possibility.21,24,31 Therefore, the exact effect of heterologous anti-leukocyte antibodies on the bone marrow still remains to be determined.

The lack of a simple, reproducible, and reliable method has prevented an attempt to clarify the localization of heterologous anti-leukocyte antibodies in bone marrow until recently.

Several experiments were therefore carried out to determine the localization of heterologous anti-leukocyte and anti-bone marrow antibodies in the rat bone marrow and to evaluate their cytotoxic effects on bone marrow cells.

METHODS

1. Production of Antibodies

(a) Production of heterologous anti-leukocyte antibodies: Six hours after intraperitoneal infusion of 20 to 30 ml. of 5 per cent glucose solution into albino rats of Wistar strain, ascitic fluid was collected. Almost 90 per cent of the cells in ascitic fluid were granulocytic leukocytes. After centrifugation the supernatant was discarded and the sediments washed three times with physiologic saline. Equal volumes of sediment suspension containing 100,000-350,000 leukocytes per cu. mm. and Freund’s adjuvant were mixed. One ml. of the mixture was injected into the foot pad of rabbits subcutaneously, twice with a 1-week interval. One month after the last injection, rabbits were sacrificed to obtain antiserum. Globulin was fractionated from the antiserum by slowly adding two volumes of stock sodium sulfate solution (27 Gm. Na₂SO₄ per 100 ml. solution, brought to pH 8 with borate) to one volume of serum at room temperature. The crude globulin precipitate obtained by centrifugation was washed with a sodium sulfate solution containing two volumes of stock solution and one volume of borate buffer (pH 8), redissolved in 0.5 volume buffer reprecipitated with 0.5 volume of stock sodium sulfate solution, and taken up in a minimum volume of physiologic saline (brought to pH 8 with borate). The final products were dialyzed against 12 liters of buffered saline, absorbed with rat red cells, cleared by centrifugation, and lyophilized.

(b) Production of anti-bone marrow antibodies: Rats were completely perfused with physiologic saline and bone marrows were collected. Bone marrow cells were injected into
the foot pad of rabbits subcutaneously with Freund's adjuvant twice at intervals of one week. One month after the last injection, rabbits were sacrificed and antiserum was obtained. Anti-bone marrow globulin was prepared by the above mentioned method.

2. 1131 Labeling of Antibody

The method described by Pressman was used with slight modifications. Reagents were 1.0 N HCl, 1.0 N NaOH, 0.002 M KI, 0.02 M NaNO₂ and pH 8 borate buffer. The following solutions were prepared in three separate containers: (1) 1.0 ml. HCl, 0.2 ml. KI (for every 20 mg. protein), 200 μc. carrier free 1131 (diluted by water, usually to 1 mc. per 1 ml.); (2) 1.0 ml. NaOH, 5.0 ml. borate buffer; (3) 20 mg. of globulin dissolved in 2.0 ml. borate buffer. In rapid succession, 0.2 ml. NaNO₂ was mixed with solution (1) and then with solution (2). The resultant solution was then added with thorough and gentle shaking to solution (3). These procedures were carried out at 5 C. Unbound iodine was removed by dializing the resultant solution against 12 liters of physiologic saline brought to pH 8 by borate at 5 C. overnight.

3. Assay for Localization

One ml. portion of each 1131 labeled antibody was injected in the tail vein of five normal Wistar rats. Sixteen to 24 hours after injection, rats were weighed, heparinized, and perfused with physiologic saline. Tissue samples weighing less than 1.0 Gm. were used for determination of radioactivity. Usually the kidney and the spleen were counted as a whole, whereas the liver was counted from cut sections. Both femurs were counted and the bone marrow in both femurs was weighed. The weight of the total bone marrow was calculated as three per cent of the body weight. Heparinized blood from each animal was also assayed and, after separation to plasma and sediments, each portion was also counted.

4. Determination of Radioactivities

A well-type scintillation counter with a sodium iodide crystal and 3-inch lead shield was used. The counter had a normal background of less than 170 cpm. The least active tissue samples—that is, less than five to ten times normal background—were never counted. Samples of tissue were counted in the well for not less than 2 minutes but always long enough to accumulate 4,000 counts. Mean values of radioactivities determined in five rats were considered to represent the in vivo localization of each antibody sample.

RESULTS

1. Hematologic Changes due to Injection of Heterologous Anti-Leukocyte and Anti-Bone Marrow Antibodies (Fig. 1)

The intravenous injections of heterologous anti-leukocyte and anti-bone marrow antibodies into normal rats resulted in a marked decrease in the leukocyte count within 5 hours.

Reactive leukocytosis was seen 3 to 6 days after injection. A moderate decrease in the leukocyte count was observed 9 to 10 days after injection.

The examination of the bone marrow picture demonstrated a marked decrease of polymorphonuclear neutrophils and a remarkable increase of pro-myelocytes and myelocytes 24 hours after injection, demonstrating maturation arrest at the promyelocytic and myelocytic level. This maturation arrest was still seen 4 days after injection of antibodies.

2. In Vivo Localization of Normal Rabbit Globulin in Rats

The injection of 1131-labeled antibodies resulted in localization of radioactivity in various tissues. The total radioactivity localized in each organ was
the sum of specific localization due to radio-labeled antibody and non-specific accumulation of radio-labeled normal globulin. In order to determine the nonspecific localization, the in vivo localization of I\textsuperscript{131}-labeled normal rabbit globulin was determined in 35 rats. The mean and 95 per cent confidence limits in these rats were: bone marrow, 0.59 ± 0.1 per cent; liver, 1.4 ± 0.7 per cent; spleen, 0.14 ± 0.08 per cent; kidney, 0.3 ± 0.1 per cent; and lung, 0.2 ± 0.1 per cent; respectively.

These mean localizations of radio-labeled normal globulin were considered to represent the background localization. The specific localization (net localization) due to localizing antibody was determined by calculating differences between the observed total localization due to I\textsuperscript{131}-labeled antibodies and the above background localization. Net localization falling below the upper limits of the mean background localization for the particular tissue was considered to show absence of localization due to localizing antibody in that tissue.

3. In Vivo Localization of Heterologous Anti-Leukocyte Antibodies in the Bone Marrow

I\textsuperscript{131}-labeled heterologous anti-leukocyte antibodies were injected intravenously into rats and their localization in bone marrow was determined. The net localization of anti-leukocyte antibodies was as follows: bone marrow, 2.1–2.4 per cent; liver, 0.5–1.5 per cent; spleen, 0–0.3 per cent; lung, 0 per cent; and kidney, 0–0.2 per cent (table 1). Thus it is evident that anti-leukocyte antibodies contained localizing antibody which localized specifically in the bone marrow.

4. In Vivo Localization of Heterologous Anti-Bone Marrow Antibodies

In vivo localization of I\textsuperscript{131}-labeled anti-bone marrow antibodies was determined for three samples. As shown in table 2, the net localization was in: bone marrow, 1.7–3.6 per cent; liver, 0.4–0.6 per cent; spleen, 0.1–0.2 per cent;
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lung, 0-0.1 per cent; and kidney, 0.1-0.2 per cent. These results showed that anti-bone marrow antibodies localized specifically in the bone marrow.

5. In Vivo Combination of Heterologous Anti-Leukocyte and Anti-Bone Marrow Antibodies with Circulating Leukocytes

In order to determine the intravascular antigen-antibody reaction between injected anti-leukocyte and anti-bone marrow antibodies, and circulating leukocytes, the remaining radioactivity in whole blood, plasma and sediments was counted 24 hours after injection of I\(^{131}\)-labeled antibodies.

The radioactivity in whole blood of rats receiving injections of I\(^{131}\)-labeled normal rabbit globulin was 35 per cent of the injected dose. The remaining radioactivity in the blood of rats receiving injections of I\(^{131}\)-labeled anti-leukocyte antibodies was 33 per cent, while it was 34 per cent in rats receiving anti-bone marrow antibodies.

The heparinized blood was centrifuged and plasma and sediments were separated. Sediments were washed with physiologic saline five times. Then the radioactivity in sediments and plasma was determined separately.

The radioactivity in plasma was 99 per cent of that of heparinized whole blood in rats receiving injections of I\(^{131}\)-labeled normal globulin, leaving a trace radioactivity in sediments.

As for anti-leukocyte and anti-bone marrow antibodies, 1.3 per cent and 2.3 per cent, respectively, of the radioactivity of heparinized whole blood was present in sediments, demonstrating the intravascular combination of antibodies with circulating leukocytes.

6. Absence of Localizing Antibody in Serum Obtained 1 Hour after Injection of Radio-labeled Heterologous Antibodies

I\(^{131}\)-labeled heterologous anti-leukocyte and anti-bone marrow antibodies were injected intravenously into rats and they were sacrificed 1 hour later. After counting the radioactivity of pooled serum obtained from several rats, the serum was injected intravenously into test rats to determine its in vivo localization.

As for serum obtained from rats receiving injection of I\(^{131}\)-labeled anti-leukocyte antibodies, net localization was as follows: bone marrow, 0.3 per cent; liver, 0.4 per cent; spleen, 0.1 per cent; lung, 0.1 per cent; and kidney, 0.2 per cent. In the case of serum of rats receiving injection of I\(^{131}\)-labeled anti-bone marrow antibodies, net localization was: bone marrow, 0.4 per cent; liver, 0.8 per cent; spleen, 0.1 per cent; lung, 0.1 per cent; and kidney, 0.2 per cent.

These results demonstrated that no localizing antibody was present in serum obtained from rats 1 hour after injection of anti-leukocyte and anti-bone marrow antibodies.

7. Demonstration of Anti-Leukocyte and Anti-Bone Marrow Antibodies Attached to Bone Marrow Cells by the Fluorescent Antibodies Method

Rats were perfused completely with physiologic saline 24 hours after injection of anti-leukocyte and anti-bone marrow antibodies, and bone marrow
Table 1.—In Vivo Localization of I\textsuperscript{131}I-Labeled Heterologous Anti-Leukocyte Antibodies in Normal Rats

<table>
<thead>
<tr>
<th>Lot No. of Antibody</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>
<th>Lung total</th>
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<th>net</th>
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<td>0.6</td>
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<td>0.1</td>
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<td>0.2</td>
<td>0.2</td>
<td>0</td>
<td>0.5</td>
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<td>0.4</td>
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</tr>
</tbody>
</table>

Note: Total = total localization, showing the mean value of results determined in five rats; Bkgd = background localization; Net = net localization.

Table 2.—In Vivo Localization of I\textsuperscript{131}I-Labeled Heterologous Anti-Bone Marrow Antibodies in Normal Rats

<table>
<thead>
<tr>
<th>Lot No. of Antibody</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>
<th>Lung total</th>
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<th>net</th>
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<td>0.1</td>
<td>0.3</td>
<td>0.2</td>
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</tr>
</tbody>
</table>
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Table 3.—Remaining Radioactivity in Blood 24 Hours after Injection of I[131]-Labeled Antibodies

<table>
<thead>
<tr>
<th></th>
<th>Blood (diluted to 1:5)</th>
<th>Sediments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm/mL</td>
<td>% injected dose</td>
</tr>
<tr>
<td>Normal globulin</td>
<td>6539</td>
<td>35</td>
</tr>
<tr>
<td>Anti-leukocyte globulin</td>
<td>7028</td>
<td>34</td>
</tr>
<tr>
<td>Anti-bone marrow globulin</td>
<td>8297</td>
<td>33</td>
</tr>
</tbody>
</table>

Note: Sediments = sediments obtained from 2 ml whole blood; % blood = per cent of radioactivity in sediments to total blood radioactivity.

Table 4.—Absence of Localizing Antibody in Plasma Taken 1 Hour after Injection of Heterologous Anti-Leukocyte and Anti-Bone Marrow Antibodies

<table>
<thead>
<tr>
<th>Serum of rats receiving</th>
<th>Bone marrow</th>
<th>Liver</th>
<th>Spleen</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>total bgd net</td>
<td>total bgd net</td>
<td>total bgd net</td>
<td>total bgd net</td>
</tr>
<tr>
<td>Anti-L</td>
<td>0.9</td>
<td>0.6</td>
<td>0.3</td>
<td>1.8</td>
</tr>
<tr>
<td>Anti-BM</td>
<td>1.0</td>
<td>0.6</td>
<td>0.4</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Note: Anti-L = anti-leukocyte antibodies; Anti-BM = anti-bone marrow antibodies.

smears were prepared. These smears and smears of peripheral blood were stained with fluorescent anti-rabbit globulin antibodies.

As shown in figures 2 and 3, fluorescent dye was clearly demonstrated on the surface of bone marrow cells, showing the localization of anti-leukocyte and anti-bone marrow antibodies on these cells. The leukocytes in peripheral blood smears were also stained.

Discussion

It is well established that heterologous anti-leukocyte antibodies injected intravenously into experimental animals induce a rapid destruction of circulating leukocytes. Some authors23,24,31 have suggested their possible cytotoxic effect on the bone marrow. This is denied by others. Therefore, studies on the in vivo localization of heterologous anti-leukocyte antibodies in the rat bone marrow were undertaken in order to clarify their effect on immature granulocytic cells in the bone marrow.

The white cell count decreased markedly 5 hours after injection of heterologous anti-leukocyte antibodies in rats. This decrease in the leukocyte count was associated with a decrease in the number of lymphocytes as well. Three to 6 days after injection, reactive leukocytosis was seen. There was a moderate decrease in the white cell count 9 to 10 days after injection. Examination of the bone marrow picture demonstrated a marked decrease of polymorphonuclear neutrophils and a marked increase of promyelocytes and myelocytes 24 hours after injection, showing maturation arrest at the level
Fig. 2.—Peripheral blood smear stained by fluorescent anti-rabbit globulin antibody. Leukocytes stained brilliantly are seen.

Fig. 3.—Bone marrow smear stained by fluorescent anti-rabbit globulin antibody. Immature myeloid cells and granulocytes brilliantly stained by fluorescent dye are seen.

of promyelocytes and myelocytes. This maturation arrest was still seen 5 days after injection. An abrupt sharp decrease in leukocyte count may be due to the degradation induced by an intravascular reaction of anti-leukocyte antibodies with circulating leukocytes. The above reactive leukocytosis may coincide with promyelocytic bone marrow 24 hours after injection. However, the maturation arrest in the bone marrow picture suggests a cytotoxic effect.
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of anti-leukocyte antibodies on myeloid bone marrow cells in addition to the reaction with circulating leukocytes.

These changes in leukocyte count and bone marrow picture are similar to the observations of Chew,2a and Moeschlin.30 They considered that the changes in the bone marrow represented an active reaction to rapid leukocyte destruction. However, Cajano31 recently reported similar results in studies on immunomorphologic changes in the bone marrow due to the injection of heterologous anti-leukocyte antibodies, suggesting a possible cytotoxic effect on immature bone marrow cells.

The injection of I\(^{131}\)-labeled anti-leukocyte antibodies resulted in specific high localization of antibodies in bone marrow, indicating the presence of localizing antibody. The injections of I\(^{131}\)-labeled anti-bone marrow antibodies also resulted in specific high localization in bone marrow. These results indicate that heterologous anti-leukocyte antibodies show a cross-reaction with immature granulocytic bone marrow cells and exert some cytotoxic effects on them.

When P31-labeled normal rabbit globulin was injected into rats, no radioactivity was found in sediments of blood 24 hours after injection. However, relatively small amounts of radioactivities were always found in the sediments in the case of heterologous anti-leukocyte and anti-bone marrow antibodies, demonstrating the intravascular reaction of the injected antibodies with circulating leukocytes. This was also demonstrated by means of the fluorescent anti-rabbit globulin antibodies method.

The accumulation of clumps of leukocytes sensitized with leukoagglutinin, and their disintegration in lungs were reported in experimental allergic leukopenia.30 The accumulation of leukocytes sensitized with I\(^{131}\)-labeled heterologous anti-leukocyte antibodies should give a high radioactivity in lungs of rats receiving their injections. However, such a high in vivo localization of radioactivity in lungs was not demonstrated, in possible contrast to the above earlier concept of pulmonary emboli of clumped leukocytes.

There is a possibility that the high radioactivity in the bone marrow may be due to a nonspecific accumulation of leukocytes coated with I\(^{131}\)-labeled antibodies. An examination, therefore, was made to determine whether the high radioactivity in the bone marrow is due to the specific localization of antibodies or to the nonspecific accumulation of sensitized leukocytes. The bone marrow smears obtained from rats receiving heterologous anti-leukocyte antibodies were stained with fluorescent anti-rabbit globulin antibodies. It is well known that leukocytes show nonspecific fluorescence in the fluorescent antibodies method.37,38 However, the use of the fluorescent anti-rabbit globulin diluted to 1:64 diminished markedly the nonspecific autofluorescence of leukocytes in our experiments. The bone marrow cells, including not only mature granulocytes but also immature cells, were stained brilliantly with these diluted fluorescent antibodies. These results may indicate that the high radioactivity in the bone marrow is not due to the nonspecific accumulation of clumped leukocytes sensitized with radio-labeled antibodies.

When I\(^{131}\)-labeled anti-leukocyte and anti-bone marrow antibodies were
injected into rats, no localizing antibody was found in plasma 1 hour after injection. These results indicate that the injected localizing anti-leukocyte and anti-bone marrow antibodies disappear from plasma within 1 hour, fixing rapidly to peripheral leukocytes and bone marrow cells.

**Conclusion**

1. The intravenous injection of I\(^{131}\)-labeled heterologous anti-leukocyte and anti-bone marrow antibodies into rats resulted in a high specific in vivo localization in the bone marrow, indicating the presence of localizing antibody.

2. No in vivo localization in lungs was demonstrated, in possible contrast to the earlier concept of pulmonary emboli of clumped leukocytes sensitized with antibodies in experimental immunoleukopenia.

3. Heterologous anti-leukocyte and anti-bone marrow antibodies injected intravenously disappeared from plasma and fixed to peripheral leukocytes and bone marrow cells within 1 hour.

**SUMMARY IN INTERLINGUA**

1. Le injection intravenose de anticorpore, marcate con I\(^{131}\), anti leucocytos e anti medulla ossee ad in rattos resultava in un alte grado de specffic localisation in vivo in le medulla ossee, lo que indica le presentia de anticorpore localisante.

2. Nulle localisation in vivo esseva demonstrate in le pulmones—possibilemente in contrasto con le previe conception de embolos pulmonari de agglomerations de leucocytos sensibilisate con anticorpore in immunoleucopenia experimental.

3. Heterologe anticorpore anti leucocytos e anti medulla ossee, quando injicite per via intravenose, dispareva ab le plasma e se fixava a leucocytos peripheric e a cellulas de medulla ossee intra 1 hora.

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Akira Kurisu, M.D., Research Fellow, The Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Hongo, Bunkyo-ku, Tokyo, Japan.

Hajime Sugiyama, M.D., Research Fellow, The Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Hongo, Bunkyo-ku, Tokyo, Japan.
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