Effect of Leukocyte Antibodies on the Fate In Vivo of Indium-111-Labeled Granulocytes

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The effect of different leukocyte antibodies on the fate in vivo of granulocytes is not known. Thus, the optimum in vitro serologic tests to determine a safe and effective granulocyte transfusion or to diagnose immune destruction of granulocytes in other clinical situations have not been identified. We have studied the effect of granulocyte agglutinating (GA), granulocytotoxic (GC), and lymphocytotoxic (LC) antibodies on the intravascular recovery and half-life (t½) and the extravascular localization of Indium-111-granulocytes in 50 patients. GA antibodies caused reduced granulocyte recovery and t½ in three of three non-neutropenic patients (one with anti-NB1), increased sequestration of cells in the liver, and failure of granulocytes to localize at sites of infection in two of two patients (one with anti-NA1). In contrast, GC antibodies in five patients and LC antibodies in one patient did not cause reduced intravascular recovery or t½ of granulocytes. In nine patients with GC and six patients with LC antibodies, incompatible granulocytes localized at known sites of infection. It appears that GA, but not GC nor LC, antibodies alter the fate in vivo of granulocytes.

GRANULOCYTES contain antigens of the ABO system, some other red cell groups, HLA, and granulocyte-specific systems. Antibodies to non-red-cell antigens found on leukocytes can be detected by many techniques, but the comparative value of these different techniques for detection of immune destruction of granulocytes in different clinical situations is not known. Leukocyte antibodies are associated with transfusion reactions, autoimmune neutropenia, and isoimmune neonatal neutropenia, but it is not known which in vitro test relates best to the clinical situation. In granulocyte transfusion, early studies showed that when patients had leukoagglutinating or lymphocytotoxic antibodies, incompatible granulocytes had a decreased intravascular recovery and failed to localize at sites of infection. However, the clinical effectiveness of granulocyte transfusions was apparently not related to HLA compatibility, and leukocyte antibody testing did not protect the recipient from a transfusion reaction.

In red cell serology, understanding the clinical effect of antibodies that react under different conditions has allowed development of compatibility testing to ensure a safe and effective transfusion and also has contributed to understanding of pathologic processes, such as autoimmune hemolytic anemia and hemolytic disease of the newborn. Similar information about leukocyte antibodies is not available. In order to determine the effect of different leukocyte antibodies on the fate in vivo of granulocytes, we have studied the intravascular kinetics and extravascular localization of Indium-111-labeled granulocytes in patients with different kinds of leukocyte antibodies.

MATERIALS AND METHODS

On the morning of the indium study, blood was obtained from the recipients and from normal donors who met all the criteria of the American Association of Blood Banks for blood donation. A cross-match between recipient's serum and donor's granulocytes or lymphocytes was done that day.

For the granulocyte microagglutination assay (GA), a suspension of >95% granulocytes was prepared using the Ficoll-Hypaque double density gradient. The granulocytes were washed twice with 10 ml of phosphate-buffered saline (PBS, pH 7.3) and then twice with PBS containing 3% bovine serum albumin (BSA) and 0.4% EDTA solution. Cells were resuspended in 1 ml PBS solution containing 0.5% BSA and 0.5% EDTA and adjusted to give a granulocyte concentration of 5000/μl. Testing was carried out in Terasaki trays. Fifteen lambda of high viscosity mineral oil were placed in each well followed by 3 lambda of the recipient's serum. One lambda of the granulocyte suspension was added to the bottom of each well and the trays incubated at 30°C in a dry air incubator for 5-6 hr before the reactions were read using an inverted phase microscope.

Granulocytotoxicity (GC) was carried out essentially as described by Drew et al., except that blood for preparation of the cell suspension was collected in ACD and granulocytes were isolated using the double density Ficoll-Hypaque gradient and not papainized. The rabbit complement used (Grand Island Biological Company, Grand Island, N.Y.) was absorbed twice with human red cells at 0°C, and testing was carried out under oil in Terasaki trays as we have described. Granulocytes were incubated for 0.5 hr in the sensitization phase and 3 hr in the complement phase. Because the incubation temperature during the sensitization phase may be important, sensitization was carried out at 6°C, 22°C, and 37°C in

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different samples tested simultaneously. Cytotoxicity of at least 40% of the cells, as estimated by microscopic viewing, was considered a positive result.

Lymphocytotoxicity (LC) testing was done using standard techniques. Cytotoxicity of at least 20% of the cells was considered a positive result.

Granulocytes for $^{111}$In-labeling were prepared from the donors as we have previously described. The cell suspension for injection was approximately 95% granulocytes and had the following composition: 5 ml of saline containing $10^6$ granulocytes; approximately $5 \times 10^6$ red cells; $10^6$ mononuclear cells; and no platelets.

Indium-$^{111}$In-oxine was prepared by mixing $^{111}$Ind chloride with 50 $\mu$L of 8-hydroxyquinoline (oxine) (1 mg/ml ethanol) and 0.2 ml 0.3 M sodium acetate buffer (pH 5.5). After extraction with chloroform, the extract was evaporated to dryness and the residue taken up in 50 $\mu$L of ethanol and diluted with 0.2 ml of saline.

The granulocyte suspension was labeled with an average of 200 $\mu$Ci of $^{111}$In-oxine by incubation in a sterile vial at room temperature for 20 min with occasional gentle mixing. The labeled granulocytes were then withdrawn through a needle containing a filter and injected through an ordinary needle into the recipient. The small amount of labeled cell suspension remaining in the vial was used to determine labeling efficiency and the number of cells injected.

Blood samples were obtained from the recipient at approximately 10 min, 1, 2, and 4 hr after injection. Calculation of the percent of injected granulocytes recovered in the circulation and their intravascular half-life ($t_1/2$) was done as previously described. Patients were grouped based on the kind of antibody present, the presence of neutropenia, and the presence of infection. The mean value for recovery and $t_1/2$ for each group was determined. Each group of patients with antibodies was compared with a group of patients matched for the presence or absence of neutropenia and infection but with no antibodies. Differences between groups were evaluated using a $t$ test with 2–4 degrees of freedom. Differences were considered significant if $p > 0.05$, but very large differences in means would be necessary to achieve statistical significance in groups with small numbers of patients.

Extravascular localization of the injected granulocytes was determined by whole body imaging. Body scans were performed occasionally at 4 hr and always at 24 hr after injection, using either a whole body scanner or a scintillation camera. In normal subjects, the liver and spleen are apparent in the scan and the spine and ileosacral region are occasionally faintly visible; no other sites of localization of $^{111}$In-granulocytes are visualized in studies of normal subjects.

We have previously shown that granulocytes labeled with $^{111}$In-oxine have normal chemotactic response in vitro and normal migration into a skin wound. Additional evidence that $^{111}$In-leukocytes migrate to sites of infection is their 90% diagnostic accuracy in detecting occult infection in 405 patient studies at our institution.

The uptake of radioactivity by the liver and spleen was assessed in some patients using a scintillation camera interfaced with a computer. In previous studies of six normal subjects, the average percent of injected radioactivity in the spleen at 4 hr was 29% compared to 21% in the liver. In the present group of patients, the percentages were quite variable because of differences in dose and tissue attenuation of radioactivity between adults and children of various sizes. Therefore, we have calculated a liver to spleen (L/S) ratio as a means of comparison in different patients.

In order to evaluate the accuracy of the indium scan, the patients' subsequent clinical courses were monitored. X-ray and laboratory data and findings at surgery or autopsy were accumulated and a final decision was made whether, in retrospect, each patient had a localized infection at the time of the indium study. Each indium scan was then classified as a true-positive (if the patient was infected and granulocytes localized at the site of infection), true-negative (if the patient was not infected and granulocytes failed to localize anywhere other than the liver and spleen), false-positive (if granulocytes localized at a site that did not give evidence of infection), or false-negative (if granulocytes failed to localize at a known site of infection).

This study was approved by the University of Minnesota Committee on the Use of Human Subjects in Research. All participants gave informed consent. All donors of granulocytes met the criteria of the American Association of Blood Banks for ordinary blood donation and were HB, antigen negative.

RESULTS

We studied 25 children and 25 adults who had a variety of diseases, including acute and chronic leukemia, aplastic anemia, renal failure with rejection of a transplanted kidney, lymphoma, suspected abdominal abscess or wound infection, congenital neutropenia, and vasculitis. Some patients were receiving granulocyte or platelet transfusions; others were suspected of having an occult infection and an indium study was done for diagnostic purposes.

The 50 patients received 58 injections of $^{111}$In-granulocytes. Patients were neutropenic ($<$1000 PMNs/µl) during 36 of the 58 studies. In 26 studies, the granulocytes injected were compatible by the three antibody methods (GA, GC, LC); and in 32 studies, the granulocytes were incompatible in one or more assays. Body scans were obtained in all studies and quantitation of liver and spleen uptake was done in 21 studies. Intravascular recovery and $t_1/2$ of the labeled granulocytes were determined in 31 studies. Follow-up evaluation indicated 31 sites of infection in 25 studies and no localized infection in 33 studies.

In order to establish a base for comparison, patients who received granulocytes compatible in the GA, GC, and LC crossmatches will be considered first. Kinetics data were obtained in 15 and liver spleen ratios in 9 of the 31 patients. In the 6 non-neutropenic patients, the average intravascular recovery was 30.0% in the absence of infection and 31.7% in the presence of infection (Fig. 1), with an average $t_1/2$ of 8.8 hr and 4.8 hr, respectively (Fig. 2). These values are similar to the 30% recovery and 5.0-hr half-life we previously reported in normal subjects receiving autologous granulocytes. The three infected patients had decubitus ulcers, infected central venous pressure line, and numerous large boils. Neutropenic patients with no antibodies had significantly reduced recoveries when compared with similar non-neutropenic patients with no antibodies (Fig. 1: uninfected, 15.6% versus 30.0%; $p < 0.01$ and infected, 11.0% versus 31.7% $p < 0.01$).

There was no statistically significant difference among the $t_1/2$ for these groups of patients with no antibodies.

Liver spleen ratios in the 9 patients studied with no antibodies ranged from an average of 0.24 to 0.68 and did not indicate any influence of neutropenia or infec-
tion on the proportion of injected granulocytes that localized in these two organs.

Sixteen patients with leukocyte antibodies were studied, and the results are compared with the values for the 15 patients without antibody (Fig. 1 and 2). Four patients received granulocytes incompatible by GA. In the 3 non-neutropenic uninfected patients, the intravascular recovery (10.5%) and $t\frac{1}{2}$ (2.0 hr) were significantly less ($p < 0.01$) than in similar patients without antibodies (30%; 8.8 hr). In one of these patients, the GA antibody had anti-NB1 specificity, and NB1-positive donor granulocytes had a significantly reduced ($p < 0.05$) recovery (14.4%) and a $t\frac{1}{2}$ of 2.4 hr ($p > 0.05$). The intravascular recovery and $t\frac{1}{2}$ in the one neutropenic patient with GA antibody was not significantly different from similar patients without antibodies (6.4% versus 15.6%; $p > 0.05$). Studies in six patients with GC antibodies also showed differences between neutropenic and non-neutropenic patients, but none of the recovery or $t\frac{1}{2}$ values was different from similar patients with no antibodies (non-neutropenic 27.8% versus 30.0%; neutropenic uninfected 18.7% versus 15.6%; and infected 14.1% versus 11.0%). One neutropenic infected patient received granulocytes incompatible by LC. Although the recovery was less than those in patients without antibodies (5.1% versus 11%), the differences were not significant ($p > 0.05$). The one patient with both GA and LC antibodies also had a low recovery that was not statistically significant (4% versus 11%; $p > 0.05$). Four neutropenic uninfected patients who had both GC and LC antibodies had a recovery and $t\frac{1}{2}$ (12.3% and 5.5 hr) similar to those seen in neutropenic uninfected patients with no antibodies (15.6% and 4.4 hr).

The presence of GA antibodies affected the distribution of radioactivity between the liver and spleen,
The intravascular recovery (11.9%) and half-life (5.7 hr) in that patient were similar to other infected patients with no antibodies. Since the L/S ratios for the two uninfected and the other two infected patients with GC antibodies were normal, it does not appear that GC antibodies caused increased sequestration of cells in the liver.

Probably more important than survival in the intravascular space is the granulocyte's ability to migrate to a site of infection. Of the 58 indium studies reported here, 25 were done in patients with clinically proven infection. Twenty-three of these were detected by the indium scan and considered true-positives, but two were missed and thus were false-negatives (Table 1). Both of these patients had GA antibodies; one in combination with GC and one in combination with LC. The GA antibody that was present with the GC antibody had anti-NA1 specificity. This patient had osteomyelitis (confirmed by a bone scan) of the pelvis and an AK amputation stump. NA1-positive 111In-granulocytes did not localize at those sites. In the second patient with a false-negative scan, an LC antibody was present with the GA antibody. Granulocytes failed to localize at an area of clinically apparent submandibular cellulitis (Fig. 3).

None of the four patients with GA antibodies alone had localized infections, so that all of the scans in these patients were true-negatives. GC antibodies were present in ten studies. Five of these patients had known sites of infection and demonstrated localization of 111In-granulocytes at these sites (Table 1). One patient had pneumonia demonstrable by chest x-ray, one had oral and anal ulcers and pseudomembranous colitis (Fig. 4), one had an inflamed thermal burn, one had a lung abscess, and one was undergoing rejection of a transplanted kidney. There were no false-negative studies in patients who received GC incompatible granulocytes.

Six patients had LC antibody and two of these had proven infections (Table 1). Indium-111-granulocytes localized at infected sites in both patients. One patient had extensive oral ulcers and submandibular cellulitis (Fig. 4), and one had pneumonia demonstrable on chest x-ray. Four of eight patients with both GC and LC antibodies had proven infections (Table 1). Two patients had acute myelogenous leukemia; one with extensive cellulitis of the thigh and one with pneumonia. The other two patients had pneumonia and rejection of a transplanted kidney. Indium-111-granulocytes localized at all of these sites of infection (thighs, lungs, and kidney). Thus, 11 different patients with proven infection had localization of 111In-granulocytes at the infected site (true-positive scan) in the presence of either GC or LC antibodies alone or in combination. Two other patients with proven infection did not show localization of 111In-granulocytes at infected sites (false-negative scan), and both of these patients had GA antibodies (one combined with GC and one with LC).

It has been reported12 that the lower precomplement incubation temperatures increase the sensitivity of the GC assay. In two studies when the antibody was detected only at 6°C, the intravascular recovery and t½ were not different from values observed in comparable patients with no antibodies (GA, GC, or LC) detected. The nine patients with GC antibodies who had localization of granulocytes at sites of infection (Table 1) included patients whose antibodies reacted

### Table 1. Relationship of the Results of Indium Body Scans to Granulocyte Incompatibility Determined by Granulocyte Agglutination (GA), Granulocytotoxicity (GC), and Lymphocytotoxicity (LC)

<table>
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*A minus indicates no reaction between recipient's serum and donor cells, and thus, indicates a compatible transfusion, while a plus indicates incompatibility.

†The GA antibody in this patient was anti-NA1.
incompatible granulocytes failed to localize at known sites of infection. The in vivo effect of GA antibodies also was demonstrated in two patients who received granulocytes from both a compatible and an incompatible donor. Transfusions given in the presence of GA antibodies had markedly reduced intravascular recovery and $t/2$, but when compatible or autologous cells were used, granulocyte recovery and $t/2$ were normal. Thus, it appears that GA antibodies interfere with normal intravascular kinetics and the ability of granulocytes to localize at sites of infection.

GC antibodies were not associated with reduced...

at each of the different precomplement incubation temperatures. Thus, the temperature of reactivity of the GC antibodies did not affect the intravascular kinetics or ability of granulocytes to localize at sites of infection.

DISCUSSION

GA antibodies (including one example of anti-NB1) caused a statistically significant reduction in the intravascular recovery of incompatible granulocytes. In two patients with GA antibodies (one anti-NA1),...
incompatible in the LC assay was not expected to interfere with normal intravascular kinetics and possibly with migration of incompatible granulocytes to sites of infection, differences in methods might explain the lack of in vivo effect of GC antibodies in our study. We used the GC assay of Drew et al., which differs from the method Blashke et al. used to identify the patient with autoimmune neutropenia.

Although granulocytes contain HLA antigens, LC antibodies did not interfere with the fate of granulocytes in vivo. The intravascular recovery and $t_1/2$ of granulocytes incompatible in the LC assay was not significantly less than in similar patients without antibodies. In two patients with known infections and LC antibodies, incompatible granulocytes localized at the sites of infection. In four additional patients with both GC and LC antibodies, granulocytes also localized at sites of infection. In one patient who received two indium transfusions, cells localized at a site of infection when only LC antibody was present, but did not when GA antibody was also present. At one time, data suggested that HLA matching provided a better leukocyte increment following granulocyte transfusion, but that experience was not supported by further study. In one study, a poor increment following granulocyte transfusion may have been due to agglutinating, but not LC antibodies. Thus, it appears that LC antibodies and HLA matching may not be important for granulocyte transfusion.

In immune destruction of red cells and platelets, the amount of antibody on the cell surface affects the mechanism of cell removal. Destruction of cells in the liver becomes more prominent as cells are coated with a larger amount of antibody. Although we did not quantify the amount of antibody on the granulocytes in this study, the relative amount of granulocytes cleared by the liver and spleen did relate to the other observations of the in vivo fate of the cells. In normal subjects, the L/S ratio is approximately 0.7. Similar L/S ratios were found in this study in patients with no antibodies, with GC antibodies, or both GC and LC antibodies. This is consistent with the lack of effect of these antibodies on the intravascular recovery and $t_1/2$ and granulocyte localization of sites of infection. However, the L/S ratio was increased in patients with GA antibodies who had reduced recovery and failure of granulocytes to localize at sites of infection. Thus, it appears that the GA antibodies responsible for impaired circulation and migration of granulocytes are also associated with increased clearance of incompatible cells by the liver.

Patients had infections ranging from as small as an infected tip of a CVP catheter to abdominal abscesses or pneumonia. There was no statistically significant reduction in recovery or $t_1/2$ in these patients compared with similar uninfected patients as has been reported in animals. It also did not appear that patients with larger infections had lower recoveries or $t_1/2$. In our previous studies of autologous $^{111}$In-granulocytes in 10 normal subjects, the mean and standard error for recovery was $30\% \pm 5.9\%$ and for $t_1/2$ hr $\pm 1.6$. Thus, the variability in the method may preclude detection of rapid clearance of cells by a large infection or possibly none of the patients in this study had infections large enough to cause accelerated granulocyte removal from the circulation.

Our observation that neutropenia is associated with a decreased intravascular recovery, but normal $t_1/2$, is similar to results in rabbits and clinical observations in humans receiving granulocyte transfusions. Studies using subcutaneous sponges in rabbits indicate that the larger than usual portion of cells that do not enter the marginal pool are available to migrate to sites of infection, but the number of cells arriving at the site of infection is slightly reduced. We did not quantitatively compare migration to infected sites in neutropenic and non-neutropenic patients. However, in general, the scans on neutropenic patients did not show reduced uptake nor were they more difficult to interpret. It is possible that such information might be obtained by quantitating uptake of $^{111}$In at sites of infection, but problems of attenuation in different size patients make such studies difficult and the data quite variable.

Immunization of dogs by blood transfusion or skin grafting causes a reduced posttransfusion increment in granulocyte count. However, the serologic technique that most accurately reflects this immunization is not known. Usually a leukocyte rather than granulocyte suspension is used in the agglutination test, and the success of a granulocyte transfusion evaluated by the increase in count. These increases often average approximately 200/10$^6$ granulocytes transfused/sq m body surface area, which in an infected neutropenic patient may not be a suitable endpoint because of patient variables and the error of the granulocyte count in that range. Despite the variables introduced by the patients in our study, the $^{111}$In technique showed that GA antibodies altered the fate of granulocytes in vivo. Such alterations did not occur in the presence of GC or LC antibodies.

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