Inhibition of Granulocyte Erythrophagocytosis by HLA Antisera

By Jacob Nusbacher, James L. MacPherson, Ira Gore, Jr., and Rosa Gninberg

The ability of HLA antisera to inhibit granulocyte erythrophagocytosis (EP) of opsonized red blood cells (RBC) was evaluated. Human granulocytes (PMN) were separated from heparinized whole blood by the Ficoll-Isopaque technique and suspended in McCoy's medium. EP occurred when the PMN were incubated with opsonized RBC. For six HLA antibody specificities evaluated, prior incubation of PMN with HLA antisera resulted in significant inhibition of EP without cytolysis when the PMN donor was positive for the specific HLA antigen, but not when the donor was negative for the antigen. The inhibition was time- and dose-dependent. Prior absorption of HLA antisera with HLA-specific platelets reduced or abolished the inhibition. An example of anti-NB1 also inhibited EP in 4 individuals. These data suggest that HLA antibody may adversely affect granulocyte phagocytic function. Inhibition of EP might be useful in evaluating compatibility prior to granulocyte transfusion.

Several test methods have been developed for the study of granulocyte antigens and antibodies. These include leukoagglutination,1 antiglobulin consumption,2,3 granulocytotoxicity,4 immunofluorescence,5 and opsonic assays.6-8 It is not clear if these approaches are equally suitable for detecting all granulocyte antigenic specificities or different immunoglobulin classes of granulocyte antibodies. Such tests have been used in the diagnosis of patients with autoimmune9 or neonatal neutropenia10 and as compatibility tests prior to granulocyte transfusions with varying degrees of success.11,15

The effect of antibodies on granulocyte phagocytic function in vitro has been the subject of a number of studies. It has been demonstrated that (heterologous) rabbit anti-human-leukocyte serum inhibits phagocytosis by human granulocytes of starch granules or latex particles.16,17 Similarly, human granulocyte alloantibody has been shown to inhibit the ingestion of opsonized lipopolysaccharide-coated oil particles.18 Some of these systems may also have value as screening tests for the presence of granulocyte antibodies.

In this communication we present studies demonstrating the ability of HLA alloantisera to inhibit specifically granulocyte phagocytosis of opsonized erythrocytes (RBC), and this suggests an important role for HLA in granulocyte transfusion. The test method may also be useful in screening transfusion recipients for HLA and other antibodies prior to transfusion.

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MATERIALS AND METHODS

Blood Donors

Blood was obtained from healthy individuals who had taken no medication (except birth control preparations) for at least 7 days prior to testing.

RBC Suspension

Blood samples were drawn in heparin (10 units/ml blood) from group A, individuals. RBC were washed three times with phosphate-buffered saline, (PBS), pH 7.0, Dulbecco's (GIBCO, No. 404). Theuffy coat was aspirated and discarded with each wash. The RBC were suspended in PBS to a final concentration of 3%-5%.

Anti-A

High-titer (≥ 1:256 by indirect antiglobulin test) anti-A sera were obtained from group O donors who had never been transfused. The sera contained no irregular RBC antibodies. For each anti-A serum used, serial dilutions were performed. The greatest dilution still yielding high erythrophagocytosis scores was determined (vide infra) and used in all further test procedures.

Granulocyte (PMN) Suspensions

Ten milliliters of blood were collected in heparinized vacuum tubes and centrifuged at 200 g for 5 min. Without disturbing the buffy coat, approximately one-half of the settled RBC were drawn off with a Pasteur pipette. The remaining blood was inclined at a 45-deg angle at 37°C for 15-20 min. The plasma and buffy coat were drawn off and mixed with 2 ml of 0.9% NaCl solution. This mixture was carefully layered over 2.5 ml of Ficoll-Isopaque solution in culture tubes (13 X 100 mm) and centrifuged at 400 g for 30 min. The lymphocyte interface was removed and saved for lymphocytotoxicity studies. The lower two layers (RBC/PMN) were washed twice with and then resuspended in McCoy's 5a medium with 30% fetal calf serum (GIBCO, No. 166) to a white blood cell (WBC) count of 12-18 X 10⁹/liter. The final WBC preparation had at least 90% PMN.

Lymphocytotoxicity

All WBC donors were typed for HLA by lymphocytotoxicity using bulk antisera provided by the NIAID.

WBC Antibody

All HLA antisera were collected from multiparous women in citrate anticoagulants and recalcified to produce serum. Sera were stored at -80°C with no preservatives* or prior heat inactivation. Monospecificity was confirmed by lymphocytotoxicity, and screening for irregular RBC antibodies was negative. Six monospecific HLA antisera were chosen. Each specificity (A1, A2, A3, B5, B7, B12) was tested by lymphocytotoxicity against the WBC donors to confirm their HLA types.

Absorption of HLA Antisera

Removal of HLA antibody by absorption with platelets was performed according to the method of Rodey et al., with minor modifications.

Erythrophagocytosis (EP)

For opsonization, 0.1 ml of RBC suspension was added to 0.1 ml of guinea pig serum and 0.2 ml of the diluted anti-A serum. The mixture was incubated at 37°C for 30 min, washed three times, and resuspended to 0.6 ml with PBS at 22°C. The opsonized RBC were tapped vigorously to obtain a suspension visibly clear of aggregates. RBC, prepared in this manner, support an antiglobulin test with anti-IgG and anti-C.

*Sodium azide was found to interfere with results.
To determine EP, 0.2 ml of the PMN suspension was added to 0.2 ml of the test WBC antisera or McCoy’s medium (control). The mixture was incubated for 60 min at 37°C and then added to the opsonized RBC. After an additional incubation at 37°C for 60 min, the tubes were centrifuged for several seconds in a Serofuge™ (Clay Adams), and the supernatant was aspirated. The cell mixture was vortexed, and a large drop was smeared on a slide. After air drying and fixing with methanol, the slides were stained with Wright-Giemsa.

The slides were examined under oil immersion for percentage EP. At least 200 PMN were examined, and only strict engulfment was counted as positive. Any cell that had ingested RBC was considered positive; the number of RBC ingested per cell was not ascertained. Rosetting of RBC around PMN was not determined.

Viability

WBC counts and trypan blue/eosin dye exclusion tests were performed prior to and 1 and 2 hr after addition of HLA antisera to study the number and viability of PMN incubated with antibody.

Other Studies

PMN were incubated with HLA antisera for periods ranging between 15 and 180 min prior to EP testing. In three experiments the antisera was washed away prior to the second 60-min incubation with opsonized RBC. In three other tests the HLA antisera remained throughout the entire procedure, conforming to the standard procedure used.

Several dilutions of HLA antisera were incubated with PMN prior to incubation with opsonized RBC. Parallel lymphocytotoxicity tests were performed with the diluted sera against the PMN donor’s lymphocytes.

RESULTS

Viability

None of the HLA antisera affected the number or viability of PMN during the incubation period.

Titration Study

Figure 1 shows the effect on EP of anti-HLA-A2 tested in dilution against PMN from an antigen-positive donor. For the antibody chosen, EP returned to baseline levels at a 1:4 dilution. Lymphocytotoxicity was negative for all three individuals at a 1:2 dilution, but EP was still inhibited at this dilution.

Fig. 1. Dilutions of anti-HLA-A2 antiserum tested against PMN from HLA-A2 individuals (N = 3). Data expressed as mean ± SEM percentage of control EP (no antibody). Lymphocytotoxicity testing, performed in parallel, was negative at 1:2 dilution.
Incubation Time

Figure 2 shows that with increasing incubation time with HLA antiserum up to 60 min, inhibition of EP increased. Incubation past 60 min did not significantly increase inhibition. When antibody was not washed away during the 60-min EP testing period after preincubation for 60 min, as was the usual procedure for the tests below, there were no differences in EP scores as compared with scores in tests in which antibody was washed away.

Inhibition of EP

Table 1 shows the mean percentages of PMN ingesting opsonized RBCs in controls after incubation with antibody and after incubation with platelet-absorbed antibody for each specificity tested. Figure 3 illustrates the same data for HLA-A1 expressed as percent of control. When data from all specificities were displayed in this manner, the mean percent of control EP for antigen positive individuals was always less than 70% with nearly all individual values falling below 80%. While some donors who were antigen-negative for corresponding antisera also showed

![Graph showing relationship between incubation time and degree of inhibition of EP by anti-HLA-A2 antiserum.](Image)

**Table 1. Percentage Erythrophagocytosis by Granulocytes Without Antibody, With Antibody, With Specific HLA Antibody, and With HLA Antibody After Platelet Absorption**

<table>
<thead>
<tr>
<th>HLA of PMN Donor</th>
<th>Without Antibody (Control)</th>
<th>With Antibody</th>
<th>With Antibody†</th>
<th>With Antibody† Platelet-absorbed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>With Antibody No.</td>
<td>No.</td>
<td>No.</td>
</tr>
<tr>
<td>A1</td>
<td>pos</td>
<td>40 ± 3.9†‡</td>
<td>7 17 ± 1.8</td>
<td>7 34 ± 4.5</td>
</tr>
<tr>
<td></td>
<td>neg</td>
<td>34 ± 4.9</td>
<td>5 32 ± 5.1</td>
<td>5 44 ± 9.5</td>
</tr>
<tr>
<td>A2</td>
<td>pos</td>
<td>57 ± 2.2</td>
<td>25 33 ± 2.2</td>
<td>25 46 ± 6.5</td>
</tr>
<tr>
<td></td>
<td>neg</td>
<td>50 ± 3.9</td>
<td>12 46 ± 3.2</td>
<td>12 49 ± 6.0</td>
</tr>
<tr>
<td>A3</td>
<td>pos</td>
<td>36 ± 6.0</td>
<td>6 21 ± 4.9</td>
<td>6 32 ± 4.8</td>
</tr>
<tr>
<td></td>
<td>neg</td>
<td>46 ± 8.2</td>
<td>5 46 ± 9.1</td>
<td>5 46 ± 9.1</td>
</tr>
<tr>
<td>A5</td>
<td>pos</td>
<td>36 ± 4.7</td>
<td>6 16 ± 3.1</td>
<td>6 33 ± 1.3</td>
</tr>
<tr>
<td></td>
<td>neg</td>
<td>41 ± 7.0</td>
<td>5 42 ± 7.4</td>
<td>5 48 ± 13.0</td>
</tr>
<tr>
<td>A7</td>
<td>pos</td>
<td>48 ± 2.9</td>
<td>16 28 ± 2.6</td>
<td>16 41 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>neg</td>
<td>50 ± 2.8</td>
<td>11 43 ± 3.3</td>
<td>11 54 ± 4.1</td>
</tr>
<tr>
<td>B12</td>
<td>pos</td>
<td>40 ± 2.1</td>
<td>9 26 ± 1.7</td>
<td>9 31 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>neg</td>
<td>44 ± 3.3</td>
<td>9 40 ± 3.5</td>
<td>9 44 ± 5.1</td>
</tr>
</tbody>
</table>

* Determined by lymphocytotoxicity.
†With platelets from HLA-antigen-positive donor.
‡‡± SEM.
Fig. 3. Effect on EP of anti-HLA-A1 tested against PMN from antigen-positive and negative donors, with and without prior absorption with antigen-positive platelets.

Fig. 4. Effect of anti-NB1 antiserum on EP in four different individuals.
some reduction from control values, with few exceptions, values were higher than 80% of control. Differences between antigen-positive and antigen-negative donor groups were all significant ($p < 0.001$).

For each antiserum evaluated, several PMN preparations were tested against platelet-absorbed antisera. For antigen-positive individuals, there was correction to near control EP scores after absorption. Antigen-negative individuals also increased their EP scores somewhat following absorption. The platelet-absorbed sera gave negative lymphocytotoxicity tests when tested with cells from antigen-positive donors.

The NB1 leukoagglutinin inhibited phagocytosis by PMN (Fig. 4).

**DISCUSSION**

In 1958 Resnick and Klein showed that incubation of normal human polymorphonuclear leukocytes with rabbit anti-human-leukocyte plasma markedly depressed the phagocytosis of starch granules. They also noted that the inhibition was not related to cytolysis by antibody and that with prolonged incubation the lesion was partially reversible. Laleli et al. also demonstrated that phagocytosis-stimulated hexose monophosphate shunt activity (as measured by $^{14}$CO$_2$ release) was inhibited by rabbit anti-human-granulocyte antibody. However, Boxer and Stossel found inhibition of human granulocyte phagocytosis only at high concentrations of rabbit antibody, whereas lower concentrations had a stimulatory effect. Other studies have also demonstrated that xenogeneically derived antibody inhibits granulocyte phagocytic function.

Human antineutrophil alloantibodies have also been found to inhibit phagocytosis. Using anti-NA2 antiserum, Boxer and Stossel found inhibition of the ability of granulocytes to ingest opsonized lipopolysaccharide-coated oil particles at all antibody concentrations, which was in contrast with their findings with xenogeneic antisera. Bilezikian et al. also reported inhibition of human granulocyte phagocytosis by serum from a patient who had received many blood transfusions. Although the mechanism of inhibition is unknown, it is clear that antigranulocyte antibody of both xenogeneic and allogeneic origin can inhibit phagocytic function.

The studies presented here confirm previous observations for alloantibodies. HLA antisera obtained from multiparous women (which are usually used for HLA typing by lymphocytotoxicity) were found to inhibit granulocyte phagocytosis of opsonized RBC. Moreover, the inhibition was specific; granulocyte EP from antigen-positive individuals was inhibited by antisera of corresponding specificity but not by antisera of other specificities. The inhibition appeared to be related to the amount of antibody present and to the duration of exposure of granulocytes to antisera (Figs. 1 and 2); it was abolished or markedly reduced by prior absorption of the antisera with antigen-positive platelets. Granulocyte death was not a factor in the inhibition of EP, perhaps because an adequate source of complement was not part of the incubation mixture.

There was considerable variation in the amount of EP among individuals in the absence of HLA antisera. When tested repeatedly, some individuals gave consistently high EP scores, whereas others were consistently low, thus confirming
The reason for these differences is not known, although it has been suggested that plasma factors might play a role. The reason for these differences is not known, although it has been suggested that plasma factors might play a role. After incubation of granulocytes with HLA antisera, the degree of EP inhibition varied with different HLA specificities. From these data it is not certain whether this reflects merely differences in potency among the HLA sera or, perhaps, the degree of expression of antigen on the granulocyte. With regard to the latter, there is evidence that HLA is poorly expressed on granulocytes, as measured by their ability to absorb HLA antibody or by indirect immunofluorescence. Therefore, inhibition of EP may be a particularly sensitive method for studying granulocyte HLA antigens.

Granulocytes from individuals negative for a particular HLA specificity usually showed some inhibition of EP, as compared with controls. In only two cases (one with HLA-A1 and another with HLA-B12) was the inhibition greater than 20% of control EP values. This finding may result from the presence of other HLA specificities in these presumably monospecific antisera that are not detectable by lymphocytotoxicity testing but are effective in this system. Some antisera also may have specificities that cross-react with other HLA antigens. Also, it is likely that some of these antisera contain granulocyte-specific antibodies unrelated to HLA. Studies with anti-NBI antiserum have suggested that granulocyte EP is also sensitive to such specificities.

Absorption of the evaluated HLA antisera with antigen-positive platelets reduced the inhibition of EP, but in only one case (with HLA-A3) was removal complete. In some cases there was improved EP with platelet-absorbed sera even with granulocytes from antigen-negative individuals. These findings may result from some of the considerations suggested above and from dilutional effects of the absorption technique. One cannot rule out the possibility that with platelet absorption a material is released or generated that promotes phagocytosis by granulocytes.

Although much is known about the relationship of the HLA system to platelet transfusions, little information is available on the role of this system in granulocyte transfusion. Graw et al. have shown that increments in granulocyte counts after transfusion are correlated with the degree of HLA matching between donor and recipient. However, Higby and Hester found no relationship between the degree of HLA matching and posttransfusion granulocyte increment or the effect of the transfusion on the patient's clinical result. The data herein presented suggest that HLA antibody may inhibit in vivo a critical function of granulocytes, namely, phagocytosis, without necessarily causing cytolysis. Clinical studies are required before these speculations are verified.

The roles of other antigenic systems, particularly neutrophil-specific antigens, in granulocyte transfusion are unknown. Inhibition of EP may be of value in evaluating this question because it appears to be sensitive to neutrophil-specific antigens as well as HLA antigens, as evidenced by the studies with anti-NBI antiserum. These data are admittedly preliminary and incomplete. The NB1 antigen status of the granulocyte donors could not be determined because of limited quantities of antisera, although it is known that more than 80% of the population is NB1-positive. Prospective clinical studies will be required to ascertain whether this method is useful as a pretransfusion compatibility test for granulocytes.
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