Effect of Cytochalasin B on Human Monocyte Binding and Sphering of IgG-coated Human Erythrocytes

By Barton Herskovitz, Dupont Guerry IV, Richard A. Cooper, and Alan D. Schreiber

The ability of human mononuclear phagocytic cells to bind IgG-coated human erythrocytes (EA) and to cause bound EA to become osmotically fragile (sphered) was investigated in the presence of cytochalasin B, a known inhibitor of phagocytosis. Cytochalasin B inhibited the binding of EA to mononuclear cells in a dose-dependent fashion; 80% inhibition of binding was observed at a concentration of 5 μg/ml. This profound effect on EA binding together with presently available data suggested a role for IgG receptor mobility in the macrophage binding of IgG-coated erythrocytes. Cytochalasin B, however, had a minimal effect on the capacity of mononuclear cells to sphere adherent EA, suggesting that the processes involved in macrophage-induced spheroctysis may differ from those operable in phagocytosis.

HUMAN PERIPHERAL BLOOD MONOCYTES and tissue macrophages possess surface receptors responsible for the recognition of IgG-coated cells.1,2 These receptors play a major role in decreasing the survival of IgG-coated red cells (RBC) in immune hemolytic anemia and IgG-coated platelets in immune thrombocytopenia.3,4 The events following the binding of IgG-coated cells to the monocyte or macrophage surface, however, are incompletely understood. Phagocytosis of bound IgG-coated cells may result. However, LoBuglio et al. have observed that IgG-coated RBC that are bound to human monocytes are not necessarily phagocytosed.5 Rather, these monocyte-bound RBC may become deformed and develop increased osmotic fragility. The latter process is probably responsible for the spherocytes observed in the circulation of patients with autoimmune hemolytic anemia caused by IgG anti-RBC antibody.6,7 The relationship between the events causing RBC spheroctysis, on the one hand, and RBC phagocytosis, on the other, are unclear.

Cytochalasin B (CB), a fungal metabolite, effectively inhibits phagocytosis by polymorphonuclear leukocytes8,9 and macrophages.6,9 We have queried whether or not CB would modify the process of spheroctysis as it does phagocytosis. Therefore, we have employed an in vitro model using human mononuclear phagocytic cells, human erythrocytes, and human IgG anti-D antibody to examine the effect of cytochalasin B on mononuclear cell recognition and sphering of IgG-coated RBC.
MATERIALS AND METHODS

Preparation of human erythrocytes sensitized with IgG Anti-D (EA). Whole blood anticoagulated with heparin was obtained from D-positive donors and radiolabeled with $^{51}$Cr-sodium dichromate (New England Nuclear, Cambridge, Mass.) as previously described. The radiolabeled RBC were sensitized with anti-D antiserum (EA; Sera-Tec Biologicals, New Brunswick, N.J.) at 37°C in the presence of 0.01 M ethylenedinitrilotetraacetic acid (EDTA) and the number of IgG molecules per RBC was quantitated by Cl fixation and transfer, as described previously. After thorough washing, these EA were resuspended in Hank’s balanced salt solution (HBSS; Gibco, Grand Island, N.Y.) to a final concentration of $5 \times 10^7$ RBC/ml. In most studies erythrocytes were sensitized with 7200 molecules of IgG per RBC.

Preparation of mononuclear cell monolayers. Mononuclear cell monolayers were prepared as previously described. Human mononuclear cells were obtained by Ficoll-diatrizoate density gradient centrifugation of heparinized peripheral blood. The cells were diluted in HBSS and allowed to adhere to plastic tissue culture dishes (Falcon Plastics, Oxnard, Calif.) for 45 min at room temperature. Nonadherent cells were removed by thorough washing with HBSS. Monolayers contained greater than 90% mononuclear cells, of which approximately 75%, were peroxidase positive and capable of phagocytosing latex particles. Morphologically, 85%, of the cells appeared to be monocytes.

IgG receptor assay. Mononuclear cell IgG receptor activity was assessed in two assay systems:

1. Monolayers were preincubated for 15 min at 37°C with HBSS or HBSS containing 1.0% dimethylsulfoxide (DMSO) with or without CB (Aldrich Chemical, Milwaukee, Wis.). Radiolabeled EA were added in 1 ml ($5 \times 10^7$ RBC/ml) of CB-containing buffer and incubated at 37°C for 2 hr. Unbound EA were removed by thorough washing with HBSS and bound EA were quantitated by radioactivity, following H$_2$O lysis, in a gamma counter (Nuclear, Chicago, Ill.) or stained with Wright-Giemsa for light microscopy. Inhibition of mononuclear cell binding of EA was determined as follows:

$$1 - \frac{cpm \text{ of lysate (monolayer incubated with CB)}}{cpm \text{ of lysate (monolayer incubated with DMSO)}} \times 100$$

2. In this assay 1 ml of radiolabeled EA ($5 \times 10^7$ RBC/ml) was added to monolayers which were immediately centrifuged at room temperature for 4 min at 20 g. Monolayers were then washed thoroughly to remove nonadherent EA and bound EA were quantitated by radioactivity. In some experiments the monolayers were preincubated with CB before adding EA; in other experiments CB was added after EA were bound to the mononuclear cells.

Erythrocyte osmotic fragility. In the osmotic fragility experiments each monolayer with bound $^{51}$Cr-EA was sequentially exposed to 3 ml of pH 7.4 phosphate buffered saline containing three different NaCl concentrations: 0.9%, 0.45%, or 0.1%. Each NaCl solution was removed from the plate after 30 sec and centrifuged, and the radioactivity of the lysate supernatant was assessed. The percentage of red cells lysed was determined by comparing the radioactivity of each lysate to the total radioactivity present on each monolayer as determined following lysis with H$_2$O. No intact EA were left after exposure to the 0.1% solution.

RESULTS

IgG Receptor

The effect of CB on the mononuclear cell IgG receptor was studied by incubating mononuclear cells and EA in the presence of CB concentrations ranging from 0.018 to 10 µg/ml. CB inhibited the capacity of mononuclear monolayer cells to bind IgG-coated red cells in a dose-dependent fashion (Fig. 1). Eighty percent inhibition was observed at concentrations as low as 5 µg/ml. DMSO-containing buffer had no significant effect on mononuclear cell binding of IgG-coated cells and neither DMSO alone nor DMSO in combination with CB led to any significant loss of mononuclear cells from the monolayers. Cell viability as measured by the trypan blue dye exclusion technique revealed no differences between experimental and control cells, with less than 15% trypan
blue uptake in all experiments. Similar results were obtained when the number of RBC per mononuclear cell was quantitated visually by light microscopy (Table 1). CB (5 μg/ml) reduced the binding of EA from 2.5 to less than 0.5 RBC per mononuclear cell.

In order to examine the rapidity of the CB effect, monolayers were preincubated with CB or buffer for 15 min, after which EA were added rapidly onto the monolayer by centrifugation for 4 min (assay 2, Materials and Methods). This 15-min preincubation with CB (5 μg/ml) caused almost complete inhibition (>90%) of EA binding to the mononuclear cell monolayers. The effect of CB was reversed if the monolayers were washed free of CB prior to the addition of EA.

**Osmotic Fragility Studies**

Previous studies have shown that IgG-sensitized RBC that are bound to mononuclear cells become spheroid and increasingly osmotically fragile.\(^1\) The capacity of CB to modify this process was studied by examining the osmotic fragility of RBC which became bound to mononuclear cells despite the presence of CB. Mononuclear monolayers were preincubated with CB for 15 min at 37°C and EA were added for 2 hr in the presence of CB. The monolayers were washed and the osmotic fragility of the bound EA determined (Table 2).

### Table 1. Effect of Cytochalasin B on the Monocyte IgG Receptor

<table>
<thead>
<tr>
<th>Concentration of CB (μg/ml)</th>
<th>RBC/M*</th>
<th>Inhibition of EA Binding (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>0.15 ± 0.16</td>
<td>95</td>
</tr>
<tr>
<td>5</td>
<td>0.43 ± 0.11</td>
<td>83</td>
</tr>
<tr>
<td>Buffer</td>
<td>2.5 ± 0.71</td>
<td>-</td>
</tr>
</tbody>
</table>

*RBC bound per mononuclear cell (mean ± SEM).

### Table 2. Effect of Cytochalasin B on Mononuclear Cell (M)--Induced Increase in Erythrocyte Osmotic Fragility

<table>
<thead>
<tr>
<th>NaCl (%)</th>
<th>EA Incubated With M</th>
<th>EA Incubated Without M</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buffet 2.5 μg CB/ml</td>
<td>5 μg CB/ml</td>
</tr>
<tr>
<td></td>
<td>Buffet 2.5 μg CB/ml</td>
<td>5 μg CB/ml</td>
</tr>
<tr>
<td>0.9</td>
<td>11 ± 4</td>
<td>14 ± 17</td>
</tr>
<tr>
<td>0.45</td>
<td>51 ± 4</td>
<td>93 ± 4</td>
</tr>
</tbody>
</table>

*Mean ± SEM.

\(^1\)DMSO-containing buffer. Comparable results were observed in the absence of DMSO.
Table 3. Kinetics of Spherocytosis of Mononuclear Cell-bound IgG-sensitized Erythrocytes

<table>
<thead>
<tr>
<th>Incubation Time (min.)</th>
<th>Erythrocyte Lysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Buffer</td>
</tr>
<tr>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>30</td>
<td>27</td>
</tr>
<tr>
<td>90</td>
<td>40</td>
</tr>
<tr>
<td>120</td>
<td>53</td>
</tr>
</tbody>
</table>

\(^{31}\text{Cr EA were bound to mononuclear phagocytic cells in } 4\text{ min and incubated at } 37^\circ\text{C with buffer or CB. At the times indicated replicate monolayers were exposed to } 0.45\% \text{ NaCl to assess osmotic fragility and RBC lysis determined by radioactivity. The mean of two experiments is shown. Less than } 10\% \text{ lysis of bound EA was observed with } 0.9\% \text{ NaCl at all time points. Lysis of unbound EA incubated with CB was less than } 10\% \text{ with both } 0.45\% \text{ and } 0.9\% \text{ NaCl at all time points.} \)

the absence of CB, more than 50\% of the EA bound to mononuclear cells were lysed by 0.45\% NaCl, while insignificant lysis was observed with EA that were not exposed to mononuclear cells. CB at concentrations of 2.5 and 5.0 μg/ml did not inhibit this capacity of the mononuclear cells to make bound EA osmotically fragile. Control RBC incubated in the presence of CB and in the absence of mononuclear cells did not become osmotically fragile.

To study the time course of this process further, EA were bound to mononuclear cells by the rapid sedimentation method (assay 2, Materials and Methods; see Table 3). An incubation period of 30 min was required for EA bound to mononuclear cells to become osmotically fragile. At 2 hr more than 50\% of the bound EA developed an increase in osmotic fragility, both in the presence and absence of CB. Light microscopy revealed that mononuclear monolayer cells that bound and sphered RBC morphologically resembled monocytes. At 120 min bound EA appeared sphered, while at 0–15 min of incubation no visual sphering was observed. More RBC sphering was observed at 90 min of incubation than at 30 min.

DISCUSSION

The human mononuclear phagocytic cell (macrophage) is important in host defense because of its ability to recognize and damage cells coated with antibody or complement. Macrophages have specific surface receptors which serve to bind IgG-coated cells, whereupon these bound cells can be altered.\(^{1,2}\) A major mechanism by which the macrophage damages such cells is phagocytosis.\(^{13}\) However, the macrophage also has the ability to alter cell-bound RBC in a manner which results in spherocytosis, in the absence of significant phagocytosis.\(^{13}\) It is unclear as to whether the mechanism(s) involved in RBC phagocytosis and spherocytosis are identical. The fungal metabolite, CB, has been shown to inhibit the phagocytic process.\(^{6,9}\) Therefore, in order to understand better the relationship between spherocytosis and phagocytosis we have studied the effect of CB on the macrophage's ability to detect IgG-coated RBC and on its capacity to make bound RBC spheroidal. In these studies we have utilized monolayers of human peripheral blood mononuclear phagocytic cells, cells closely related to tissue macrophages,\(^{14}\) and EA coated with homologous IgG antibody.
Our data indicate that CB inhibits the ability of mononuclear phagocytic cells to bind IgG-coated RBC (EA) in a dose-dependent manner (Fig. 1; Table 1). Previous studies similarly suggest that CB interferes with the interaction between lymphocytes and target cells in homologous systems. Henney and Bubbers, in a mouse model, have observed decreased recognition of target cells by T lymphocytes in the presence of CB; Kalina and Hollander have demonstrated that CB alters rat lymphocyte binding of rat fibroblasts. However, in heterologous systems the effect of CB in altering macrophage recognition of target cells does not appear as marked. Studies with human systems are less numerous. Kersey et al. have observed that human peripheral blood lymphocytes do not bind sheep erythrocytes in the presence of CB. Phagocytosis of antibody- and/or complement-coated bacteria and particles by human polymorphonuclear leukocytes is also inhibited by CB.

Previous investigators have shown, however, that small particles (e.g., bacteria or latex) coated with antibody or complement are efficiently bound to CB-treated leukocytes and macrophages, although phagocytosis does not occur. We have observed that CB inhibits the IgG receptor activity of mononuclear phagocytic cells using the IgG-coated EA as an indicator cell. There is evidence that CB inhibits the movement of cell membrane constituents and cell mobility and that many IgG receptor sites on the macrophage surface may be required for effective EA binding. These observations suggest that the inhibition of EA binding with CB may not be due to an effect on individual IgG receptor sites, which may be able to bind small IgG-containing particles, but to the recruitment of IgG receptor sites necessary for binding IgG-coated larger cells. This effect of CB would explain its potency in inhibiting other cell-cell interactions as well.

EA that were cell bound in the presence of CB developed an increase in osmotic fragility (Table 2), consistent with their becoming sphered. When the kinetics of the spherocytic process were examined, only a small difference in the rate and extent of spherocytosis was observed in the presence of 10 μg/ml CB (Table 3). Thus, in our system CB poorly inhibited the spherocytosis of macrophage-bound EA. The capacity of CB-treated mononuclear phagocytic cells to sphere-bound EA suggested that the spherocytic process may proceed by a mechanism dissimilar to that necessary for phagocytosis. On the other hand, the binding of EA to the mononuclear cell surface by IgG receptor sites unaffected by CB may rapidly initiate a process sufficient for spherocytosis, but minimally inhibited by CB.

REFERENCES

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