Attachment of Particle-Bound IgG and Complement to Human Neutrophils

By William D. Lawrence, Charles H. Packman, Jacob M. Rowe, and Marshall A. Lichtman

The attachment of particle-bound IgG in a nonphagocytic system stimulates formation of a microfilament-rich, organelle-poor zone in the subjacent cytoplasm of human neutrophils. The attachment site is characterized by ruffling and invagination of the neutrophil membrane. Both IgG attachment and formation of the organelle-poor zone are inhibited by the microfilament inhibitor cytochalasin-B, but not by inhibitors of microtubules, such as colchicine or vinca alkaloids. In contrast, attachment of particle-bound complement is not inhibited by cytochalasin-B in doses known to disrupt actin filaments. There is no discernable change in the subjacent cytoplasm of the neutrophil in response to complement and the membrane attachment site is smooth, without ruffling or invagination. These studies disclose that both IgG-mediated attachment to neutrophils and its sequel, peripheral cytoplasmic reorganization, are mediated by cytochalasin-sensitive structures, possibly actin. Complement-mediated attachment to neutrophils is insensitive to high doses of cytochalasin, suggesting that actin integrity is not required.

THE PRESENCE of complement receptors and receptors for the Fc portion of the IgG molecule on the surface of human neutrophils has been known for over a decade.1,2 These receptors are thought to subserve different functions in immune-mediated phagocytosis by neutrophils.3,4 Activation of the complement (C3) receptor stimulates oxidative metabolism and aids in cell-to-particle adherence, whereas the IgG receptor appears to trigger ingestion. Rabbit alveolar macrophages appear to differ in that C3 alone may trigger ingestion.5 Because of the apparent difference in the relative abilities of IgG and complement receptors to stimulate ingestion by neutrophils, we investigated morphological and pharmacologic features of the interaction between human neutrophils and IgG- and complement-coated red cells. Our aim was to determine whether IgG and complement differ either in their ability to initiate processes related to phagocytosis or with regard to the mechanisms by which phagocytosis is initiated.

The presence of cytoskeletal disruptors on attachment of IgG and complement has been carefully investigated in rabbit and human monocytes and macrophages.7-11 Our studies of human neutrophils revealed both differences and similarities to monocytes with regard to the effects of cytoskeletal agents. We have also quantitated the influence of erythrocyte-bound IgG or complement on neutrophil cytoplasmic ultrastructure. Important differences in the morphology of IgG- and complement-mediated attachment correlate well with previous observations on the roles of these ligands in attachment and ingestion phases of phagocytosis by neutrophils.3,5

MATERIALS AND METHODS

Preparation of Neutrophil Suspension

Ten milliliters of heparinized (14 U/ml) human blood were mixed with 5 ml of 3% dextran in normal saline. After 30 min at 37°C, the supernatant plasma was placed in 16 × 100 mm plastic tubes and centrifuged at 250 g for 5 min at 4°C. The pellet, composed of leukocytes and contaminating erythrocytes, was subjected to hypotonic lysis (5 ml of 0.2% NaCl followed in 25 sec by 5 ml of 1.5% NaCl) and washed 3 times in M-199 (Gibco, Grand Island, N.Y.) at 4°C. Leukocytes were then resuspended to 107 cells/ml in M-199 and maintained at 4°C until use. The cells were approximately 85% neutrophils. The viability of these cells, greater than 90% by Trypan blue dye exclusion, remained unchanged by prolonged exposure to the agents tested. The neutrophils avidly ingested opsonized baker’s yeast at room temperature and 37°C. At room temperature, phagocytosis of IgG or complement-coated red cells was not noted.

Preparation of IgG Fractions

IgG, free of IgA and IgM by Ouchterlony double diffusion analysis, was prepared from human serum by column chromatography on diethylaminoethyl cellulose. Similarly, the IgG fraction of a rabbit antiserum to human erythrocytes (Cappel Laboratories, Cochransville, Pa.) was isolated.

Preparation of Erythrocytes

Aliquots of glycerinated group AB erythrocytes were maintained at −80°C until the day of use. After deglycerination and washing, the erythrocytes were resuspended to a final concentration of 2 × 109 cells/ml in veronal-buffered saline, pH 7.4, containing 1% gelatin, 1.5 mM calcium, and 0.5 mM magnesium (GVB + + ). Cells coated with IgG were prepared by mixing equal volumes of erythrocytes and a subagglutinating dilution (1:200 in GVB + + ) of rabbit IgG antibody to human erythrocytes. After incubating 20 min at room temperature and 3 washes in GVB + + , the cells were resuspended to 109 cells/ml in M-199 containing 2% bovine serum albumin at pH 7.2 (M-199–BSA).12

Complement-coated erythrocytes were prepared by a modification of the method of Jenkins et al.13 Group AB serum was dialyzed at 4°C for 8–12 hr against sucrose buffer (9.24% sucrose in 0.27 M
phosphate buffer, pH 6.1, containing 1.5 mM calcium and 0.5 mM magnesium) and stored at −80°C until the day of use. One milliliter of sucrose dialyzed serum was mixed with 0.8 ml of sucrose buffer and 0.2 ml of a 50% suspension of erythrocytes in GVB + + . After shaking at 37°C for 30 min, these cells were washed until the supernatant fluid was clear and then resuspended to 2 x 10⁶ cells/ml in M-199-BSA. To ascertain the specificity of these cells in the complement rosette assay, complement-coated erythrocytes were prepared using group O human erythrocytes and compatible C5-deficient human serum, generously provided by Dr. Stephen I. Rosenfeld. Complement-coated erythrocytes prepared from either normal serum or C5-deficient serum displayed no agglutination. Complement-coated erythrocytes exhibited no agglutination with a potent rabbit anti-human IgG reagent (gift of John P. Leddy). Cells prepared from normal serum also showed a 2+ agglutination reaction to rabbit anti-human C5 (prepared by injecting a rabbit with purified human C5⁺ in complete Freund's adjuvant), whereas cells prepared from C5-deficient serum displayed no agglutination. Complement-coated erythrocytes exhibited no agglutination with a potent rabbit anti-human IgG reagent (gift of John P. Leddy). Cells prepared from normal or C5-deficient serum formed similar percentages of neutrophil rosettes in the complement rosetting assay described below.

**Rosetting Assays**

Both assays described below are based on the methods of Wong and Wilson.¹³ To assay for IgG-mediated attachment, 10⁷ neutrophils were mixed with 10⁴ IgG-coated erythrocytes in 16 x 10⁶ mm glass tubes (step 1), centrifuged at 60 g for 10 min (step 2), and incubated 15 min at room temperature (step 3). We chose room temperature for this assay because at 37°C, fewer rosettes form and they are more easily disrupted.¹⁵ In some experiments the entire assay was performed at 4°C. The cells were gently resuspended, spun onto glass slides, and stained with Wright-Giemsa. In simultaneously performed control experiments, uncoated erythrocytes were substituted for IgG-coated erythrocytes. All assays were done in duplicate. A rosette was defined as three or more red cells attached to a neutrophil. Two-hundred neutrophils were counted and the percent rosetted cells calculated. In the negative controls, a small proportion (<5%) of rosettes was found. These rosettes were thought to be caused by spinning the cells onto slides, since they did not appear in simultaneously performed negative controls stained with brilliant cresyl blue and examined in a hemocytometer counting chamber. The values for control experiments were subtracted from the results of each assay. The addition of 2 mg/ml of human IgG to the assay prior to centrifugation inhibited rosette formation completely.

For assessing complement-mediated attachment, 6 x 10⁷ complement-coated erythrocytes and 5 x 10⁶ neutrophils were mixed and incubated in 10 x 75 mm plastic tubes at 37°C, or room temperature, for 15 min. Human IgG (2 mg/ml) had no inhibitory effect on complement rosette formation. For morphological comparisons by electron microscopy, the assay for complement-mediated attachment was performed using the protocol for IgG-mediated attachment to avoid differences due to time, temperature, and centrifugation.

**Effects of Microfilament and Microtubule Inhibitors**

The microfilament inhibitors, cytochalasin-B (Aldrich Chemical Co., Milwaukee, Wisc.) and dihydrocytochalasin B (Sigma Chemical Co., St. Louis, Mo.), were added to neutrophil suspensions in concentrations varying from 2 to 60 μM for periods of 3 min to 30 min prior to mixing with coated erythrocytes in the assay system (step 1). Colchicine (two sources tested: Aldrich Chemical Co., Minneapolis, Minn. and Eli Lilly and Co., Indianapolis, Ind.) in concentrations 10 nM - 100 μM and vindristine and vinblastine (Eli Lilly & Co.) in concentrations 1–100 μM were preincubated with neutrophils for up to 1 hr prior to assay. The initial concentration of the agent being tested was maintained throughout the assay by adding the appropriate amount to the erythrocyte suspension just prior to mixing with the preincubated neutrophils. The diluent for the cytochalasin, dimethyl sulfoxide, had no independent influence on the percent rosettes at the final concentration (0.5% v/v) used in the experiment. The diluents for the other agents were either normal saline or M-199.

The ability of cytochalasin-B to prevent or reverse the IgG-induced organelle-poor zone in neutrophils was evaluated. For these electron microscopic studies, cytochalasin-B (20 μM) was added to the pelleted rosettes immediately following centrifugation (step 2) or incubation (step 3) in the standard IgG rosette assay.

**Electron Microscopy**

Specimens from the rosette assay were gently swirled drop-by-drop into Sorensen's buffer containing 1% glutaraldehyde. For experiments testing the influence of cytochalasin-B, the agent was added to the fixative in appropriate concentration. Overnight fixation was at the temperature employed in the respective rosetting assay. The specimens were then postfixed in 1% osmium tetroxide. For observation of microfilaments, osmium postfixation was omitted. After sequential dehydration in serially increasing concentrations of ethanol (70%, 95%, 100%) and propylene oxide, the specimens were embedded in Durcupan (Polyscience, Inc., Warrington, Pa.) at 55°C for 48 hr and sectioned. After staining with lead citrate and uranyl acetate, consecutive neutrophils were photographed in a JEOL JEM 100B transmission electron microscope.

For assessment of the presence or absence of an organelle-poor zone, photographs were coded and the neutrophils (magnified 20,000 x ) were cut out of the photograph along the contours of their membranes, excluding evidence of attachment of red cells. The photographs were scored by two observers who did not have knowledge of the experimental conditions to which the neutrophils had been exposed. Statistical analysis was performed using the chi-square test. For some experiments the area of the organelle-poor zone was estimated semiquantitatively. The photographs in Fig. 1 were selected as examples of zero, two, and four-plus organelle-poor zones, and unknown photographs were compared and scored accordingly. The results were analyzed using the ranked sum test. Phagocytosis was noted in fewer than 1% of neutrophils.

**Fluorescence Microscopy of Actin**

Rhodamine-labeled heavy meromyosin (R-HMM) from rabbit skeletal muscle was the generous gift of Dr. Arthur Forer, Toronto, Ontario. The S-1 fragment of heavy meromyosin was isolated from rabbit skeletal muscle by Forer's modification of Cook's method.¹⁷ In negatively stained preparations, S-1 and R-HMM both formed typical arrowhead decoration of purified actin (Sigma Chemical Co., St. Louis, Mo.) by transmission electron microscopy. For analysis of actin distribution, IgG or complement rosettes or unrosetted neutrophils were centrifuged onto microscope slides, air dried, and fixed with ethanol for 10 min. Dilute R-HMM, 1.4 mg/ml final concentration, was layered over the cells and the slides were incubated at room temperature in a moist chamber for 12 hr. The R-HMM concentration selected was the lowest that gave easily detectable fluorescence of neutrophils. Slides were rinsed in 1 M NaCl for 1–2 hr, rinsed quickly with distilled water, and evaluated for fluorescence in a Nikon Fluophot fluorescence microscope. Photographs were taken on Kodak Tri-X film at ASA 1600.

Specificity of fluorescence for actin was confirmed by failure of

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Fig. 1. Gradations of peripheral organelle-poor zones in human neutrophils. Neutrophils were cut from photographs along the contours of their membranes (see Materials and Methods). The presence or absence of an organelle-poor zone was determined and the size of the zone estimated on a scale of 0–4. (A) Neutrophil without an organelle-poor zone. (B) Neutrophil with modest organelle-poor zone (2+). (C) Neutrophil with extensive organelle-poor zone (4+). (D) Neutrophil in C shown with rosetted red cells prior to cut-out along periphery.
neutrophils to fluoresce when R-HMM was diluted in sixfold molar excess of unlabeled S-I. Albumin did not inhibit fluorescence.

RESULTS
Attachment of Particle-Bound IgG
Approximately 70%-85% of neutrophils formed rosettes with IgG-coated erythrocytes. Cytochalasin-B (and dihydrocytochalasin-B, not shown) caused a dose-dependent inhibition of IgG-mediated attachment (Fig. 2). Maximum inhibition (80%) occurred at 20 \( \mu \)M. No further inhibition occurred at concentrations to 60 \( \mu \)M or with preincubation periods up to 30 min. The inhibitory effect could be reversed if the neutrophils were washed after incubation with the drug but prior to mixing and centrifuging the neutrophils and erythrocytes. Colchicine, vincristine, and vinblastine had no effect on IgG-mediated attachment, in doses up to 100 \( \mu \)M, and with preincubation periods up to 1 hr (Fig. 2).

Neutrophils rosetted to IgG-coated erythrocytes frequently exhibited an organelle-poor zone (Fig. 3B) subjacent to the membrane, often extending widely beyond sites of erythrocyte attachment, in contrast to control neutrophils (Table 1, experiments 1 and 2) and neutrophils attached to complement-coated red cells (see below). When osmium postfixation was omitted microfilaments could be seen in the organelle-poor zone of IgG rosetted neutrophils, near sites of red cell attachment (Fig. 4). However, fluorescent heavy meromyosin, which combines specifically with actin, failed to reveal any polarization of actin within control neutrophils, complement rosettes, or IgG rosettes (Fig. 5).

To determine the time course of appearance of the organelle-poor zone, neutrophils attached to IgG-coated erythrocytes were resuspended and fixed either immediately after centrifugation (step 2) or after 15-min incubation (step 3). The proportion of rosetted neutrophils was similar for incubated and unincubated specimens. However, a significantly higher proportion of incubated neutrophil rosettes exhibited an organelle-poor zone than did unincubated rosettes and control neutrophils (Table 1, experiment 3). The three experiments in Table 1 are representative of many, each of which gave similar results. Though the proportion of control cells showing an organelle-poor zone varied from 20%-50%, each experiment contained its own control and differences between IgG rosettes and control neutrophils were always statistically significant.

The relationship between IgG-mediated attachment and the development of the organelle-poor zone was further defined by two experiments. At 4°C, attachment still occurred but the organelle-poor zone did not develop (Table 2). In another experiment, 20 \( \mu \)M cytochalasin-B was added to the pelleted rosettes immediately after centrifugation (step 2) and incubation was continued at room temperature for 15 min. An organelle-poor zone was not evident in any neutrophil examined (Table 3). If cytochalasin-B was added to the pelleted rosettes after the initial 15-min incubation period (step 3), and the mixture incubated another 15 min, the organelle-poor zone was not seen, indicating that cytochalasin-B cannot reverse a preexistent organelle-poor zone. However, despite its effect on the organelle-poor zone, cytochalasin-B had no effect on IgG-mediated attachment when added at any time after the centrifugation step, i.e., the drug could not reverse preformed attachment (Table 3). This contrasts sharply with its inhibitory effect when added before attachment has occurred (Fig. 2). DMSO did not inhibit the development of the organelle-poor zone at the concentration used to permit cytochalasin to enter solution.

Blind analysis of electron micrographs disclosed a marked invagination and ruffling of neutrophil
Fig. 3. Human neutrophil rosettes. (A) Complement rosetted neutrophil. Complement rosettes were either not associated with a subjacent organelle-poor zone or with a very small zone similar to that of control neutrophils. The red-cell-neutrophil interface tended to be smooth. (B) IgG rosetted neutrophil. IgG rosettes were usually associated with a striking organelle-poor zone. The red-cell-neutrophil interface was often ruffled and invaginated.

Table 1. Formation of Submembrane Organelle-Poor Zone in Human Neutrophils: Influence of Particle-Bound IgG and Incubation Time

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Indicator Cell*</th>
<th>Incubation Following Centrifugation†</th>
<th>Neutrophil Rosettes (%)</th>
<th>Neutrophils Examined by TEM (No.)</th>
<th>Neutrophils With Organelle-Poor Zone (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>IgG-RBC</td>
<td>Yes</td>
<td>78</td>
<td>42</td>
<td>69†</td>
</tr>
<tr>
<td></td>
<td>Control RBC</td>
<td>Yes</td>
<td>0</td>
<td>18</td>
<td>33</td>
</tr>
<tr>
<td>2</td>
<td>IgG-RBC</td>
<td>Yes</td>
<td>78</td>
<td>36</td>
<td>69†</td>
</tr>
<tr>
<td></td>
<td>Control RBC</td>
<td>Yes</td>
<td>0</td>
<td>29</td>
<td>21</td>
</tr>
<tr>
<td>3</td>
<td>IgG-RBC</td>
<td>Yes</td>
<td>73</td>
<td>35</td>
<td>80†</td>
</tr>
<tr>
<td></td>
<td>IgG-RBC</td>
<td>No</td>
<td>76</td>
<td>35</td>
<td>54</td>
</tr>
<tr>
<td></td>
<td>Control RBC</td>
<td>No</td>
<td>0</td>
<td>37</td>
<td>51</td>
</tr>
</tbody>
</table>

*Indicator cells were either IgG-coated RBC (IgG-RBC) or uncoated RBC (control RBC).
†Following the scheme in Fig. 1, the neutrophils and red cells were centrifuged into a pellet and either (A) incubated for 15 min prior to fixation (yes) or (B) immediately resuspended and fixed for transmission microscopy (TEM) (no).
‡p < 0.02, chi-square method.
Fig. 4. Site of attachment of IgG-coated red cells to neutrophils showing microfilaments in the neutrophil cytoplasm. Microfilaments could not be seen in the interior of the cell, possibly because the cytoplasm is too crowded by organelles.

membrane at the site of IgG-mediated attachment but not in complement-mediated or control studies. This invagination was not seen in experiments conducted at 4°C or in which incubation (step 3) was omitted. Colchicine had no influence on the prevalence of invaginated neutrophils.

Attachment of Particle-Bound Complement

Approximately 60%–80% of neutrophils formed rosettes with complement-coated erythrocytes. Cytochalasin-B induced only minimal inhibition of complement-mediated attachment in doses ranging from 10 to 60 μM. Colchicine had no effect on complement-mediated attachment over a wide range of doses (10 nM to 100 μM). In contrast, vincaistine and vinblasine produced dose-dependent inhibition of complement-mediated attachment in doses from 1 to 100 μM. Inhibition by vinca alkaloids could not be reversed by washing the neutrophils prior to the rosetting assay. Longer preincubations up to 1 hr produced no greater inhibition of complement-mediated attachment than did brief preincubation. The results are shown in Fig. 6.

Neutrophils rosetted to complement-coated red cells exhibited an organelle-poor zone with a frequency equal to that of control neutrophils, and the attachment site was smooth (Fig. 3A). The ultrastructural differences between complement-rosetted and control neutrophils versus IgG-rosetted neutrophils was made more evident upon comparison of the amount or degree of organelle-poor zone noted in individual cells. The area of the organelle-poor zone was estimated for individual neutrophils in a blind fashion on a scale of 0–4, using for reference the examples shown in Fig. 1. The frequency distribution of the scores for IgG-rosetted, complement-rosetted, and control neutrophils are shown in Fig. 7. Using this system, which recognized minimal organelle-poor zones, all neutrophils rosetted to IgG-coated red cells exhibited an organelle-poor zone. The majority of neutrophils rosetted to complement-coated red cells, and control neutrophils exhibited minimal or no organelle-poor zone (Figs. 3A and 7). The proportion of rosetted neutrophils was comparable for IgG-coated (78%) and complement-coated (93%) red cells.

DISCUSSION

Using electron microscopy, we have quantitated the peripheral cytoplasmic reorganization of human neutrophils in response to particle-bound IgG and complement. Since phagocytosis did not occur under the conditions of our study, we were able to separate the events associated with binding from those associated with ingestion. Particle-bound IgG produced a striking morphological change in the neutrophil, consisting of a peripheral microfilament-rich zone containing few organelles. This zone is similar to the microfilament-rich zones seen in the periphery of spreading monocytes,20 lectin-stimulated monocytes and neutrophils,19 and subjacent to particles undergoing phagocytosis by monocytes20,21 and neutrophils.22 We have also noted neutrophil membrane ruffling and

Fig. 5. Actin distribution in neutrophils rosetted to IgG-coated red cells. Rhodamine-labeled heavy meromyosin causes the entire cytoplasm to fluoresce uniformly. Similar distribution of fluorescence was noted in complement rosetted and control neutrophils (not shown). Fluorescence could be specifically blocked by a six-fold molar excess of unlabeled S-1 fragment of heavy meromyosin, but not by albumin.
Table 2. Influence of Temperature on IgG-Mediated Attachment and Formation of Organelle-Poor Zone in Human Neutrophils

<table>
<thead>
<tr>
<th>Indicator Cell</th>
<th>Assay Temperature* (°C)</th>
<th>Neutrophil Rosettes (%)</th>
<th>Neutrophils Examined by TEM (No.)</th>
<th>Neutrophils With Organelle-Poor Zone (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG-RBC</td>
<td>25</td>
<td>82</td>
<td>35</td>
<td>80†</td>
</tr>
<tr>
<td>IgG-RBC</td>
<td>4</td>
<td>83</td>
<td>24</td>
<td>4</td>
</tr>
</tbody>
</table>

*Neutrophils and IgG-coated red cells were equilibrated at the reaction temperature indicated, prior to mixing. The remainder of the experiment, including fixation for transmission electron microscopy (TEM), was performed at that temperature.
†p < 0.01.

Table 3. The Influence of Cytochalasin-B on IgG-Mediated Formation of Organelle-Poor Zone in Human Neutrophils

<table>
<thead>
<tr>
<th>Indicator Cell</th>
<th>Cytochalasin-B Added*</th>
<th>Neutrophil Rosettes (%)</th>
<th>Neutrophils Examined</th>
<th>Neutrophils With Organelle-Poor Zone (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG-RBC</td>
<td>Before incubation</td>
<td>59</td>
<td>34</td>
<td>0†</td>
</tr>
<tr>
<td>IgG-RBC</td>
<td>After incubation</td>
<td>74</td>
<td>29</td>
<td>0</td>
</tr>
<tr>
<td>IgG-RBC</td>
<td>Not added</td>
<td>63</td>
<td>26</td>
<td>62</td>
</tr>
</tbody>
</table>

*Neutrophils and IgG-coated RBC were mixed and pelleted by centrifugation. Cytochalasin-B (20 μM) was then added to the pellet either (A) immediately (before incubation), (B) after 15-min incubation (followed by another 15-min incubation), or (C) not added. The remainder of the assay was performed as described in Materials and Methods.
†p < 0.02.

We took every precaution to achieve objective evaluation of the neutrophil cytoplasmic changes. In multiple experiments, hundreds of neutrophils, photographed consecutively, were cut from micrographs so that red cell attachment could not be seen. After randomization, the photographs were scored by two observers who were unaware of the experimental conditions to which neutrophils were exposed. Under these rigorous conditions of observation, visible, submembrane cytoplasmic organization and membrane ruffling in response to particle-bound complement were similar to control cells. Some control neutrophils and neutrophils attached to complement-coated red cells exhibited an organelle-poor zone. However, the proportion of cells exhibiting an organelle-poor zone was always significantly less than observed in IgG-stimulated neutrophils in the same experiment. Importantly, the area of organelle exclusion was very slight in control and complement-rosetted neutrophils, whereas it was prominent in IgG-rosetted neutrophils. In other studies we have observed that neutrophils in suspension in vitro may assume an amoeboid configuration with an organelle-

Fig. 6. Attachment of particle-bound complement to neutrophils. Influence of microtubule and microfilament inhibitors. Neutrophils were preincubated with vincristine, vinblastine, or cytochalasin-B for 3 min, and with colchicine for 1 hr prior to starting the assay. Data points represent the mean of the number of experiments shown in parentheses in the figure key. Standard error is indicated by the bars.

invagination at the site of attachment of IgG-coated red cells. In contrast to the localized nature of membrane ruffling and invagination, organelle-poor zones extended considerably beyond the site of red cell attachment in many cases, suggesting a more generalized stimulation of submembrane cytoplasmic structure.

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Fig. 7. Frequency histogram of the magnitude of the organelle-poor zone in control neutrophils and in neutrophils attached to IgG or complement-coated erythrocytes. The areas of organelle-poor zones in individual neutrophils were estimated on a scale of 0–4 (see Fig. 1) by two observers who had no knowledge of the experimental conditions employed. Neutrophils incubated with control (uncoated) or complement-coated erythrocytes had similar magnitudes of organelle-poor zones. Neutrophils incubated with IgG-coated erythrocytes exhibited significantly larger organelle-poor zones (p < 0.01, ranked sum test).

Area of Organelle-poor Zone

IgG AND COMPLEMENT BINDING TO NEUTROPHILS

Microfilaments are visible by electron microscopy in the peripheral organelle-poor zones of IgG-stimulated neutrophils but not in the inner portion of the cells. This could be due to actin redistribution or simply unmasking of actin microfilaments by movement of organelles from the peripheral cytoplasm. Microfilaments may not be seen in central areas because of the obscuring effect of the crowding of organelles. Indeed, the experiments with fluorescent heavy meromyosin indicate that actin is present throughout the cytoplasm of IgG-stimulated, complement-stimulated, and control neutrophils, and that no gross redistribution of actin has occurred. We used the lowest possible concentration of labeled heavy meromyosin to maximize the sensitivity in detecting shifts in actin distribution. Other investigators demonstrated by direct protein measurements that there was only minimal gradient of actin from the cell center to peripheral organelle-poor zones of rabbit alveolar macrophages attached to nylon wool; however, the control proteins, myosin and actin-binding protein, redistributed to the cell periphery. Since actin constitutes a major proportion of neutrophil protein (about

poor zone at the ruffled edge of the cell. Thus, cytoplasmic organization can be induced by nonspecific activation of neutrophils in vitro, which may account for the slight degrees of peripheral organelle clearing observed in complement-mediated attachment and controls. Particle-bound IgG was a far more potent stimulus for cytoplasmic organization and the extensive organelle-poor zones developed under this condition were not seen in control or complement-rosetted neutrophils.

IgG-mediated attachment and subsequent formation and maintenance of the organelle-poor zone are both dependent on cytochalasin-sensitive structures. The data suggest that these two cytochalasin-sensitive events may be mediated or controlled by different mechanisms. First, attachment occurs almost immediately upon contact between IgG-coated red cells and neutrophils, whereas clearing of organelles from the cell periphery requires more time. Furthermore, in this system, IgG-coated red cells attach to neutrophils equally well at room temperature and 4°C but the organelle-poor zone forms only at room temperature. Finally, whereas cytochalasin-B can inhibit but not reverse IgG-mediated attachment, the drug can both prevent formation of the organelle-poor zone after attachment has occurred and reverse the organelle-poor zone after it has already formed.
10%, it seems reasonable that actin would not need to redistribute, but merely to reorganize itself locally to begin the motile processes concerned with ingestion. Such reorganization may be difficult to observe by electron microscopy or fluorescence techniques.

The mechanisms by which pharmacologic agents affect changes in neutrophil function are sometimes difficult to ascertain. There is abundant evidence that cytochalasin-B influences actin structure and the interactions between actin, actin-binding proteins, and myosin in dosage range used in these experiments. Many biologic effects of cytochalasin-B, especially those related to motile processes, may be mediated either directly or indirectly through effects on actin and its cofactors. Cytochalasin-B also inhibits cellular sugar transport, but at much lower doses than required for these experiments; furthermore, dihydrocytochalasin-B, which does not inhibit sugar transport, was equal in effectiveness to cytochalasin-B in blocking IgG-mediated attachment. Nonetheless, the cytochalasin-sensitive structures involved in IgG-mediated attachment cannot be definitely identified as actin by these experiments. However, cytochalasin-B, in doses known to depolymerize actin in vitro, failed to significantly inhibit complement-mediated attachment, suggesting that such attachment is not actin dependent.

In the dosage ranges employed for these studies, colchicine inhibits microtubule assembly and depletes neutrophils of microtubules. The finding that IgG and complement-mediated attachment occurred in the presence of 100 μM colchicine suggests that an intact microtubule system is not necessary for these functions. We used colchicine from two companies to guard against the possibility of an inactive lot. The mechanism by which vinca alkaloids inhibit complement-mediated attachment is probably not related to microtubule inhibition, since high-dose colchicine failed to inhibit.

The lack of development of an organelle-poor zone following complement rosetting could represent incomplete or incorrect (C5, C3bi, or C3d, instead of C3b) complement receptor activation, rather than the inability of activated C3b receptors to trigger peripheral cytoplasmic organization. Since similar amounts of bound C3 were noted (by agglutination of red cells with anti-C3), and similar percentages of neutrophils attached to indicator red cells prepared from either normal or C5 deficient serum, our assay measures primarily C3 rather than C5 receptor activity.

Furthermore, C3bi or C3d would be expected to mediate no more than 15% rosetting formation with mature neutrophils. An attempt was made to maximize the amount of rosetting, but the actual percentage of complement receptors engaged cannot be determined.

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Attachment of particle-bound IgG and complement to human neutrophils

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