Pertussis Toxin Partially Inhibits Phagocytosis of Immunoglobulin G-Opsonized *Staphylococcus aureus* by Human Granulocytes but Does Not Affect Intracellular Killing

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The aim of the present study was to determine whether pertussis toxin (PT)-sensitive GTP-binding proteins (G proteins) are involved in the signal transduction pathway(s) used for phagocytosis and intracellular killing of bacteria by human granulocytes. Treatment of granulocytes with PT resulted in decreased phagocytosis of immunoglobulin G (IgG)-opsonized *Staphylococcus aureus* but did not affect subsequent intracellular killing of these bacteria. PT also caused a decrease in the extracellular release of superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) by granulocytes in response to *S. aureus* opsonized by IgG. However, neither the phagocytosis nor the intracellular killing of *S. aureus* opsonized by fresh serum was affected by PT, and the release of O$_2^-$ was partially inhibited. The release of O$_2^-$ in response to serum-treated zymosan, opsonized mainly by complement components, was also only partially inhibited by PT. It is therefore possible that PT inhibits responses mediated through complement receptors to a lesser extent than those mediated via Fcy receptors. The results of this study indicate that PT-sensitive G proteins are involved in the signal transduction pathways that mediate the phagocytosis of IgG-opsonized bacteria and the accompanying respiratory burst.

Important functions of human granulocytes are the phagocytosis and subsequent killing of microorganisms that have penetrated the body. A variety of surface receptors on the granulocytes, including receptors that recognize the Fc part of immunoglobulins and those recognizing bound C3 fragments, play a role in these antimicrobial functions (20). After binding of the ligands to the receptors, signal transduction is required for processes such as phagocytosis and intracellular killing to occur. G proteins are known to act as signal intermediaries in signal transduction pathways in human granulocytes (19). Pertussis toxin (PT) inactivates certain G proteins because of its ability to induce ADP-ribosylation of the α-subunit of these G proteins (1, 19). Substrates for PT are present in the plasma membrane, cytosol, and specific granules but not in azurophilic granules of granulocytes (17, 21). Treatment of granulocytes with PT results in inhibition of such responses as chemotaxis, degranulation, and the respiratory burst of granulocytes to various stimuli such as f-Met-Leu-Phε (fMLP), C5a, leukotriene B4, platelet activating factor, or antibodies to the Fcy receptor type II (FcyRII) (1, 7). In contrast, phagocytosis of fluorescent latex beads, Candida albicans opsonized with heat-inactivated serum, or antibody-coated erythrocytes by granulocytes is not inhibited by PT (11), and ingestion of immunoglobulin G (IgG)- or IgG3-opsonized yeast particles is only partially inhibited by PT (6). The aim of the present study was to investigate whether PT affects the phagocytosis and intracellular killing of opsonized bacteria by granulocytes.

**MATERIALS AND METHODS**

**Opsonins.** Human serum (containing 7.8 mg of IgG per ml) was obtained from a healthy AB donor, and agammaglobulinemic serum (containing <0.1 mg of IgG per ml) was obtained from a patient with agammaglobulinemia. The sera were stored in aliquots at −70°C. IgG was isolated from pooled normal human serum by ammonium sulfate precipitation and anion-exchange chromatography on DEAE-Sephael (Sigma Chemical Co., St. Louis, Mo.).

**Treatment of granulocytes with PT.** Human granulocytes were isolated as described previously (8), and residual erythrocytes were removed by hypotonic shock in distilled water. Granulocytes, 10⁷ per ml, were incubated with 2 μg of PT per ml (Sigma) in modified Hanks’ balanced salt solution (HBSS) containing 50 U of DNase I (7) per ml for 2 h at 37°C under slow rotation (4 rpm). Control granulocytes were incubated with buffer alone. Next the granulocytes were washed in phosphate-buffered saline (PBS; pH 7.4) containing 0.5 U of heparin per ml (PBS-heparin). Viability of the granulocytes, as judged by trypan blue exclusion, was not affected by PT treatment. PT was only present during treatment of the granulocytes and not during the various assays, since the inhibitory effect of PT on granulocyte functions is irreversible for at least the duration of the phagocytosis and intracellular killing assays, as found in preliminary studies.

**Phagocytosis and intracellular killing assays.** *Staphylococcus aureus* (type 42 D) was cultured overnight at 37°C in nutrient broth no. 2 (Oxoid Ltd., Basingstoke, United Kingdom) (13). The bacteria were then washed three times in PBS and resuspended in HBSS containing 0.1% (wt/vol) gelatin (gelatin-HBSS).

Killing of *S. aureus* during continuous phagocytosis was determined by measuring the decrease in the total number of viable extracellular and cell-associated bacteria after incubation of granulocytes with opsonized bacteria (4). *S. aureus* (10⁷ bacteria per ml) was opsonized by incubation with 1 mg of IgG per ml or 10% (vol/vol) serum in gelatin-HBSS for 30 min at 37°C under rotation. Next the bacteria were washed and resuspended in gelatin-HBSS. Opsonized *S. aureus* (5 × 10⁶ bacteria) was incubated with 5 × 10⁶ granulocytes in a final volume of 1.1 ml of gelatin-HBSS containing 1 mg of IgG or 100 μl of serum under rotation (4 rpm) at 37°C. At selected times, 100-μl samples were taken.

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added to 900 μl of distilled water containing 0.01% (wt/vol) bovine serum albumin, and mixed vigorously to disrupt the granulocytes. The number of viable bacteria was determined as the number of CFU obtained after plating serial dilutions.

The phagocytosis and intracellular killing of S. aureus were also investigated separately (13). Phagocytosis of IgG-opsonized S. aureus was studied by incubation of 5 × 10⁶ granulocytes and 5 × 10⁶ preopsonized S. aureus in 1 ml of gelatin-HBSS containing 1 mg of IgG at 37°C under slow rotation (4 rpm). Phagocytosis of serum-opsonized S. aureus was studied in the presence of 10% (vol/vol) serum. At selected times, samples were taken and phagocytosis was determined after differential centrifugation for 4 min at 110 × g by assessing the decrease in the number of viable intracellular bacteria (13).

Intracellular killing of ingested S. aureus by granulocytes was studied after phagocytosis of IgG-opsonized S. aureus for 5 min at 37°C at a bacterium/granulocyte ratio of 1:1 (13). Following phagocytosis, noningested bacteria were removed by washing, and 5 × 10⁶ granulocytes containing ingested bacteria were reincubated in 1 ml of gelatin-HBSS containing 1 mg of IgG at 37°C under slow rotation (4 rpm). At selected times, samples were taken and diluted with distilled water containing 0.01% (wt/vol) bovine serum albumin. After disruption of the granulocytes by vigorous mixing, the number of residual viable bacteria was determined. To study the intracellular killing of serum-opsonized S. aureus, granulocytes were allowed to ingest bacteria for 3 min at 37°C, since after this time period the maximum number of viable intracellular bacteria was observed. Intracellular killing of serum-opsonized S. aureus was studied in the presence of 10% (vol/vol) serum or in gelatin-HBSS alone.

Superoxide anion (O₂⁻) and hydrogen peroxide (H₂O₂) production. The release of O₂⁻ was determined by measuring the reduction of ferricytochrome c (9), and H₂O₂ release was determined by measuring the horseradish peroxidase-mediated H₂O₂-dependent oxidation of homovanillic acid (18). Granulocytes (10⁶ per ml) were stimulated with fMLP, phorbol myristate acetate (PMA; Sigma), opsonized heat-killed (30 min at 100°C) S. aureus, or serum-treated zymosan in PBS containing 0.9 mM CaCl₂, 0.5 mM MgCl₂, and 5.6 mM D-glucose (PBS-Ca⁺⁺-Mg⁺⁺-glucose) for 15 min at 37°C under slow rotation (4 rpm). O₂⁻ production was measured by incubation of 500 μl of washed heat-killed bacteria (10⁶ per ml) with 125 μl of serum, 125 μl of heat-inactivated serum (30 min at 56°C), or 10 mg of IgG per ml for 30 min at 37°C under slow rotation (4 rpm) in PBS-Ca⁺⁺-Mg⁺⁺-glucose and subsequently washed twice in the same buffer. Serum-treated zymosan was prepared by incubating 10 μg of zymosan (Sigma) in 1 ml of serum for 30 min at 37°C. Excess serum was removed by two washes in PBS-Ca⁺⁺-Mg⁺⁺-glucose.

RESULTS

Effect of PT on O₂⁻ and H₂O₂ release by stimulated granulocytes. First the concentration of PT that has the maximum effect on the function of human granulocytes was determined. For this purpose the effect of PT on the O₂⁻ production induced by opsonized S. aureus was compared with the effect on that induced by stimulation with fMLP, since the response of granulocytes to fMLP is known to be inhibited by PT (1). The inhibition of fMLP-induced O₂⁻ release was optimal at PT concentrations of 0.5 to 2 μg/ml (data not shown). Therefore, in further studies a concentration of 2 μg of PT per ml was used. Granulocytes treated with 2 μg of PT per ml showed an 86% reduction in their response to fMLP, but the response to PMA was not affected (Table 1). When granulocytes were stimulated with S. aureus opsonized by serum (resulting in coating with immunoglobulins and complements components), a partial inhibition (44%) of the O₂⁻ production was observed. The response to serum-treated zymosan (opsonized mainly with complement components) was also partially inhibited by PT. The same degree of inhibition by PT was observed when zymosan was opsonized only by complement components, using agamaglobulinemic serum (data not shown). The O₂⁻ response to S. aureus opsonized by immunoglobulins (using heat-inactivated serum or purified IgG) was inhibited to a greater extent by PT (69% inhibition for heat-inactivated serum and 81% for IgG) (Table 1). Determination of the release of H₂O₂ (instead of O₂⁻) in response to the various stimuli revealed similar degrees of inhibition by PT (PMA, no inhibition; fMLP, 87% inhibition; serum-opsonized S. aureus, 37%; IgG-opsonized S. aureus, 74%).

Killing of S. aureus by PT-treated granulocytes during continuous phagocytosis. During continuous phagocytosis, the decrease in the number of extracellular and intracellular IgG-opsonized S. aureus achieved with PT-treated granulocytes was less than that found for control granulocytes (P < 0.02; Wilcoxon signed rank test) (Fig. 1). However, when serum-opsonized S. aureus was used, there was no difference in the decrease in the number of bacteria between control and PT-treated granulocytes.

Phagocytosis and intracellular killing of S. aureus by granulocytes after treatment with PT. The observation that the decrease in the number of viable extracellular and intracellular IgG-opsonized S. aureus achieved with PT-treated granulocytes is less than that found for control granulocytes can be due to an effect of PT on phagocytosis or intracellular killing or both. Therefore, these two processes were investigated separately. After treatment of the granulocytes with PT, phagocytosis of S. aureus opsonized by IgG was significantly decreased compared with that of control cells (P < 0.02; Wilcoxon signed rank test) (Fig. 2). In contrast, phagocytosis of S. aureus preopsonized by 10% (vol/vol) serum was not affected.

Intracellular killing of ingested IgG-opsonized S. aureus

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>nmol of O₂⁻/10⁶ granulocytes per 15 min (no. of exps)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.9 ± 1.8 (10)</td>
<td>6.6 ± 1.1 (10)</td>
</tr>
<tr>
<td>PMA</td>
<td>36.8 ± 7.8 (10)</td>
<td>38.6 ± 8.0 (10)</td>
</tr>
<tr>
<td>fMLP</td>
<td>16.1 ± 8.1 (10)</td>
<td>1.7 ± 0.9 (10)</td>
</tr>
<tr>
<td>Serum-opsonized</td>
<td>37.0 ± 12.5 (7)</td>
<td>20.3 ± 7.0 (7)</td>
</tr>
<tr>
<td>S. aureus</td>
<td></td>
<td>44 ± 18</td>
</tr>
<tr>
<td>Heat-inactivated serum-opsonized S. aureus</td>
<td>14.6 ± 6.4 (5)</td>
<td>4.0 ± 1.7 (5)</td>
</tr>
<tr>
<td>IgG-opsonized S. aureus</td>
<td>15.8 ± 7.5 (4)</td>
<td>2.4 ± 0.7 (4)</td>
</tr>
<tr>
<td>Serum-treated zymosan</td>
<td>20.2 ± 6.7 (5)</td>
<td>10.4 ± 3.0 (5)</td>
</tr>
</tbody>
</table>

*Granulocytes were incubated in the presence or absence (control) of PT and stimulated with PMA (100 ng/ml), fMLP (10⁻⁹ M), opsonized S. aureus (10⁶ per ml), or serum-treated zymosan (0.5 mg/ml).

†O₂⁻ production was measured in the presence of 5 μg of cytochalasin B per ml. Values are means ± standard deviations.

*Not significantly different from control granulocytes (Student’s t test); P > 0.3.

‡Significantly different from control granulocytes; P < 0.01.

§Significantly different from control granulocytes; P < 0.02.
The cells with PT did... respectively; with... were incubated... intracellular bacteria... of S. aureus... S. granulocytes... (4.4... studied in the... was... standard deviations of... 10;... 

FIG. 1. Killing of serum- or IgG-opsonized S. aureus by PT-treated granulocytes during continuous phagocytosis. Granulocytes were incubated with 2 μg of PT per ml for 2 h at 37°C. Controls were incubated with buffer alone. Granulocytes, 5 × 10⁶, were incubated in a final volume of 1.1 ml with 5 × 10⁶ serum-opsonized S. aureus in the presence of 100 μl of serum (control) or with 5 × 10⁶ IgG-opsonized S. aureus in the presence of 1 mg of IgG (control; PT treated). After incubation, the total numbers of extracellular and intracellular bacteria were determined. Means ± standard deviations of three (for serum-opsonized S. aureus) or four (for IgG-opsonized S. aureus) independent experiments are shown.

was studied in the presence of IgG. The number of cell-associated bacteria at the start of the intracellular killing assay did not differ significantly between PT-treated and control granulocytes [(4.4 ± 2.8) × 10⁶ and (4.7 ± 3.1) × 10⁶ per ml, respectively; Student's t-test: P > 0.3]. Treatment of the cells with PT did not affect the rate of intracellular killing of S. aureus (Fig. 3). In addition, the intracellular killing of serum-opsonized S. aureus in the presence of 10% (vol/vol) serum (Fig. 3) or in gelatin-HBSS (data not shown) alone was not affected by PT.

DISCUSSION

The present study demonstrates that the phagocytosis of IgG-opsonized S. aureus by human granulocytes and the accompanying respiratory burst are inhibited by PT, whereas the intracellular killing of IgG-opsonized S. aureus is not affected. In contrast, the phagocytosis of serum-opsonized S. aureus is not inhibited by PT. Optimal intracellular killing of S. aureus by granulocytes is known to be oxygen dependent (14, 15). Since PT does not affect intracellular killing but does inhibit the release of O₂⁻ or H₂O₂ in response to stimulation with IgG-opsonized S. aureus, either PT does not cause a decrease in the intracellular generation of reactive oxygen metabolites or such a decrease does not affect intracellular killing.

The finding that the respiratory burst of granulocytes stimulated with fMLP was markedly inhibited by PT, whereas the response to PMA (which bypasses receptor-mediated processes) was not affected, is consistent with earlier studies (10, 12). It was found that the respiratory response to particles opsonized by complement components (serum-treated zymosan) or antibodies and complement components (serum-opsonized S. aureus) was inhibited less than the response to particles opsonized by antibodies only (S. aureus opsonized by heat-inactivated serum or IgG). This indicates that the respiratory response following stimulation of complement receptors is less sensitive to inhibition by PT than that following stimulation of FcγR receptors (FcγR). It is important to note that stimulation of the respiratory burst by zymosan opsonized by agamagglubulinemic serum is not only due to opsonization by iC3b, since zymosan itself binds to a site on the membrane receptor CR3, distinct from the iC3b binding site on CR3 (16).

In addition to these findings on the effect of PT on the respiratory burst, in the present study it was found that PT also inhibits the phagocytosis of IgG-opsonized S. aureus and not that of serum-opsonized S. aureus. This indicates that, like the respiratory response, phagocytosis mediated via complement receptors alone or via complement and Fcγ receptors is less sensitive to inhibition by PT than phagocytosis mediated via Fcγ receptors alone.

Previous studies have shown that stimulation of the respiratory burst with antibodies directed against either of the two receptors for the constant region of IgG that are present.
on resting neutrophils (FcγRII and FcγRIII) is inhibited by PT (5, 7). In addition, the response to soluble IgG immune complexes (5) or soluble IgG aggregates (2) is inhibited. However, stimulation of the respiratory burst with insoluble IgG immune complexes (5, 22) or surface-bound IgG (2) is insensitive to PT. In the present study, a marked inhibition by PT of the \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) response to insoluble IgG-opsonized \( \text{S. aureus} \) was found. This indicates that the characteristics of the IgG-containing stimulus used (e.g., insoluble immune complexes, surface-bound IgG, or IgG-opsonized \( \text{S. aureus} \)) determine whether a PT-sensitive or a PT-insensitive signal transduction pathway is employed to activate the respiratory burst.

In the present study, for the first time the effect of PT on the phagocytosis and subsequent intracellular killing of bacteria was studied. It was found that PT partially inhibits the phagocytosis of IgG-opsonized \( \text{S. aureus} \) and strongly inhibits the accompanying respiratory burst but does not affect intracellular killing of these bacteria. It is therefore possible that the signal transduction pathways mediating the respiratory burst, phagocytosis, and intracellular killing differ in their involvement of PT-sensitive G proteins.

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REFERENCES