Involvement of Guanine Nucleotide Binding Proteins in Neutrophil Activation and Priming by GM-CSF

By Shaun R. McColl, Christophe Kreis, John F. DiPersio, Pierre Borgeat, and Paul H. Naccache

Pre-incubation of human neutrophils with pertussis toxin significantly inhibited the neutrophil-directed biologic actions of granulocyte-macrophage colony-stimulating factor (GM-CSF) in three separate assays: the induction of c-fos mRNA, the enhancement of both platelet-activating factor-induced mobilization of intracellular calcium, and stimulation of leukotriene synthesis by the calcium ionophore A23187. Cholera toxin did not have an effect on the latter two assays. Pre-treatment of human neutrophils with pertussis toxin did not affect the binding of GM-CSF to its surface receptor. These results provide the first evidence that a pertussis toxin substrate plays an important mediatory role in the mechanism of action of GM-CSF.

© 1989 by Grune & Stratton, Inc.

MATERIALS AND METHODS

The human neutrophils used in this study were isolated from peripheral blood following dextran sedimentation on Ficoll-Paque cushions as previously described. Following hypotonic lysis, the cells were resuspended in Hank's Balanced Salt Solution (GIBCO, Grand Island, NY) for the experiments on calcium mobilization and leukotriene synthesis, or RPMI supplemented with 10% FCS (GIBCO, Grand Island, NY) for the c-fos and binding experiments. Neutrophils represented at least 97% of the cell suspensions. Cell viability, as estimated by trypan blue exclusion, was better than 98%. rHGM-CSF was a generous gift from the Genetics Institute (Cambridge, MA). Intracellular free calcium was monitored using the fluorescent probe fura-2 as described. Leukotriene synthesis was determined by RP-HPLC as previously described in detail.

Total RNA was extracted from 1 x 10⁶ neutrophils as previously described. Integrity of RNA and equal loading was verified by ethidium bromide staining and by hybridization with a cDNA probe that non-specifically anneals with ribosomal RNA. Whole cell blotting was performed as described. Briefly, 10⁶ cells were applied onto Hybond-N membranes (Amersham, Oakville, Ontario) using a Bio-Rad (Mississauga, Ontario) dot blot apparatus. The cells were lysed with sodium dodecyl sulfate (SDS) and denatured with formamide and formaldehyde. Hybridization and post-hybridization washes were done under stringent conditions. A cDNA clone (pc-fos-1) was used for all hybridizations and was a generous gift of Dr R.J. Matusik (University of Manitoba, Winnipeg, Canada).

The experimental protocol used for the receptor binding assay has been described in detail previously. Data from the binding experiments were analyzed by the weighted non-linear least squares curve fitting program developed by Munson and Rodbard. Non-specific binding was treated as a parameter subject to error and was fitted simultaneously with other parameters.

RESULTS AND DISCUSSION

Pertussis toxin, by virtue of its ability to catalyze the ADP-ribosylation of specific G₁ and G₂-like proteins (including the G-protein implicated in neutrophil activation), has proven to be an invaluable tool in studies directed at the cell-physiologic role of the latter. However, in view of the “priming” nature of the effects of GM-CSF on neutrophil functions, it was necessary to select assay systems which, while being enhanced by the colony stimulating factor, were themselves insensitive to the bacterial toxin in order to validate the use of pertussis toxin as a probe of the involvement of G-proteins in the mechanism of action of GM-CSF. The calcium mobilizing properties of platelet-activating factor, the A23187-induced leukotriene synthesis and the expression of c-fos mRNA (see below) were chosen as test assays as they all satisfied the above criteria.
INVolvement of G-PROTEINs in GM-CSF Action

Table 1. Effect of GM-CSF and Pertussis Toxin on the Calcium Mobilizing Ability of Platelet-Activating Factor in Human Neutrophils

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Increase in Cytoplasmic Free Calcium (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated control</td>
<td>354 ± 9 (5)</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>530 ± 43 (5)</td>
</tr>
<tr>
<td>Pertussis toxin</td>
<td>338 ± 21 (5)</td>
</tr>
<tr>
<td>GM-CSF + pertussis toxin</td>
<td>387 ± 14 (5)</td>
</tr>
</tbody>
</table>

*The cells were treated with pertussis toxin for two hours at 37°C, and with 200 pmol/L GM-CSF for 30 minutes at 37°C.†Mean ± sem of increase in cytoplasmic free calcium induced by 1 nmol/L platelet-activating factor. The numbers in parenthesis represent the number of experiments.§Significantly different from untreated control (P < .01).§§Significantly different from cells treated with GM-CSF alone (P < .01). There were no significant differences between untreated controls, pertussis toxin-treated cells, and (GM-CSF + pertussis toxin)-treated cells.

The effects of pertussis toxin on the enhancement of calcium mobilization by platelet-activating factor and leukotriene synthesis by the A23187 are summarized in Table 1 and Fig 1. As previously reported,12 GM-CSF enhanced these two responses. In addition, neither the platelet-activating factor induced mobilization of calcium nor the A23187 stimulated synthesis of 5-lipoxygenase products was affected by pre-incubation with pertussis toxin. In contrast, the enhancing effect of GM-CSF on both parameters of neutrophil activation was significantly reduced in the cells treated with pertussis toxin. The efficacy of pertussis toxin was verified in parallel experiments by demonstrating that it inhibited, as previously observed,26 the calcium responses to fMet-Leu-Phe and leukotriene B4 (results not shown).

Exposure of human neutrophils to GM-CSF for 30 min-

Fig 1. Effect of GM-CSF and pertussis toxin on the production of 5-lipoxygenase products by human neutrophils in response to A23187. Neutrophils (10^7 cells/mL) were stimulated with 1.0 µmol/L A23187 for one minute following pre-incubation at 37°C under the following conditions: (A) diluent control for 120 minutes; (B) diluent control for 90 minutes followed by GM-CSF for 30 minutes; (C) pertussis toxin for 120 minutes; and (D) pertussis toxin for 90 minutes followed by GM-CSF for 30 minutes. The synthesis of 5-lipoxygenase products was determined by RP-HPLC as previously described.26 Values represent mean ± sem of four separate experiments, each performed in triplicate. (1) Significantly different from column A at P < .01; (2) significantly different from column B at P < .01; and there was no significant difference between columns 1, 3, and 4.

Fig 2. Effect of GM-CSF and pertussis toxin on the induction of c-fos mRNA by human neutrophils. Panel I, whole cell RNA blotting. Lane A, untreated controls; lane B, GM-CSF treated cells; lane C, pertussis toxin-treated cells; lane D, pertussis toxin and GM-CSF treated cells. The incubation conditions were as described in Fig 1. No signal was detected with doubling dilutions of t-RNA. Representative of three experiments. Panel II, Northern blot analysis. Lane A, untreated cells; lane B, GM-CSF treated cells. RNA extracted from fibroblasts was used as control and gave no signal.

Fig 3. Scatchard analysis of the binding of GM-CSF to control and pertussis toxin-treated human neutrophils. The conditions depicted are control (filled squares) and pertussis toxin (open squares). The experimental protocol used for the receptor binding assay has been described in detail previously.13 The number of binding sites detected in this particular experiment were 239 and 236 sites per cell in control and pertussis toxin-treated cells, respectively. The Kd of the binding sites were 28 and 32 pmol/L for the control and pertussis toxin-treated cells, respectively. These values correspond to previously published data.13 The data are from a single experiment representative of four.
utes at 37°C led, as reported earlier, to an enhanced level of c-fos mRNA over that observed in control cells (Fig 2, panel I). Pre-incubation of the cells with pertussis toxin for two hours had no effect on the level of c-fos mRNA in cells untreated with GM-CSF. However, the toxin significantly inhibited the increase in expression of c-fos mRNA stimulated by GM-CSF. Northern blot analysis of c-fos mRNA performed to confirm the specificity of the whole cell blot technique showed that a single band hybridized with the probe and further confirmed the enhanced expression of c-fos mRNA by cells pre-incubated with GM-CSF (Fig 2, panel II).

The potential interference of pertussis toxin with the binding of GM-CSF to its surface receptors was tested by direct binding experiments. The latter were performed on neutrophil suspensions pre-incubated for four hours at 37°C with 0.5 μg/mL pertussis toxin. The data from a representative experiment shown in Fig 3 demonstrate that the toxin had no effect on the number or affinity of the GM-CSF binding sites. These results indicate that pertussis toxin inhibits the potentiation of neutrophil functions by GM-CSF by interference with elements of the excitation-response coupling sequence distal to the binding sites of the colony stimulating factor. Furthermore, since it has been shown previously that pertussis toxin does not raise cellular levels of cAMP and that cholera toxin increases cellular levels of cAMP, but did not inhibit the actions of GM-CSF in the present report, it is unlikely that pertussis toxin indirectly inhibits the effect of GM-CSF by altering cellular cAMP levels.

The above data provide the first evidence that the GM-CSF receptors may be coupled to a pertussis toxin-sensitive G-protein and that the latter provides a critical link in the transduction of the signals initiated by the growth factor in human neutrophils. This conclusion is based upon the observations that pre-incubation of human neutrophils with pertussis toxin inhibited three independent experimental indices of the neutrophil-directed activities of GM-CSF, in the absence of any detectable effect on the binding of the CSF. From the current studies, we are unable to tell if the ability of pertussis toxin to inhibit the effects of GM-CSF should be interpreted as a disruption of the direct coupling of a G-protein to the GM-CSF-receptor or, additionally, as a secondary or indirect requirement of the GM-CSF receptor for a pertussis toxin-sensitive G-protein in order to produce its characteristic enhancement of cell responses.

Recent reports implicating a pertussis toxin substrate in the induction by interleukin-3 (IL-3) and CSF-1 of marrow cell proliferation and in the mitogenic responses to bombesin and platelet-derived growth factor (PDGF) support the possibility that other biologic actions of GM-CSF may also be mediated by one or more G-proteins. Finally, the precise identity of the G-protein(s) and the effector system(s) involved in the actions of GM-CSF remain to be determined, although recent evidence dissociated the mechanism of action of GM-CSF from the stimulation of the phosphatidylinositol 4,5-bisphosphate hydrolysis pathway and a requirement for the activation of protein kinase C. It is possible, however, that a pathway leading to the up-regulation of guanylate cyclase activity may be involved.

REFERENCES

17. Grynkiewicz G, Poenie M, Tsien RY: A new generation of...


26. Molski TFP, Naccache PH, Marsh ML, Kermode J, Becker EL, Sha'afi RI: Pertussis toxin inhibits the rise in the intracellular concentration of free calcium that is induced by chemotactic factors in rabbit neutrophils. Possible role of the "G proteins" in calcium mobilization. Biochem Biophys Res Commun 124:644, 1984


31. He BY, Hewlett E, Temeles D, Quesenberry P: Inhibition of interleukin 3 and colony-stimulating factor 1-stimulated marrow cell proliferation by pertussis toxin. Blood 71:1187, 1988


Involvement of guanine nucleotide binding proteins in neutrophil activation and priming by GM-CSF

SR McColl, C Kreis, JF DiPersio, P Borgeat and PH Naccache