Inhibition of Interleukin 3 and Colony-Stimulating Factor 1-Stimulated
Marrow Cell Proliferation by Pertussis Toxin

By Yixin He, Erik Hewlett, Daniel Temeles, and Peter Quesenberry

Pertussis toxin (PT) catalyzes the ADP-ribosylation of several guanine nucleotide-binding (G) proteins that are involved in the transduction of cell surface receptor-mediated signals. Involvement of such G-proteins in regulation of hematopoiesis by at least two growth factors, colony-stimulating factor-1 (CSF-1) and interleukin 3 (IL 3), was investigated using pertussis toxin. Continuous or pulse exposure of murine bone marrow cells to pertussis toxin inhibited CSF-1 or IL 3-induced colony formation by ~50%. Pertussis toxin inhibition was also demonstrated against partially separated marrow from 5-fluorouracil-treated mice. The toxin effect was blocked by heating (95°C for 30 minutes), by antitoxin antibody and was not associated with increased cAMP levels in target cells. In experiments with murine marrow, toxin-mediated inhibition appeared to involve predominantly the macrophage lineage. IL 3 stimulation of proliferation of the murine marrow-derived factor-dependent cell line FDC-P1, as measured by 3H-TdR incorporation, and CSF-1 stimulation of pure populations of murine bone marrow derived macrophages, as measured by DNA content and cell number, was also inhibited. Analysis of the effects of pertussis toxin on the growth of single cells stimulated by IL 3 demonstrated that this inhibition involved a decreased growth rate rather than a toxic ablation of cells. Phorbol myristate acetate (PMA) stimulated FDC-P1 cells and was able to abrogate the PT inhibition of IL 3 stimulation of these cells, suggesting but not establishing that IL 3 may mediate its proliferative effects through activating protein kinase C.

Vol 71, No 5 (May), 1988: pp 1187-1195

From the Departments of Internal Medicine and Pharmacology, University of Virginia School of Medicine, Charlottesville.

Supported in part by Grants No. 5 ROI AM27424, 5 ROI CA47465, AM22125 from the National Institutes of Health, Bethesda, MD, and ACS Grant CH-280 and the Pratt Bequest to the University of Virginia.

Address reprint requests to Peter J. Quesenberry, MD, Professor and Chief, Division of Hematology/Oncology, University of Virginia, School of Medicine, Box 502, Charlottesville, VA 22908.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

© 1988 by Grune & Stratton, Inc.
a predominant cell type. Megakaryocyte colonies (>3 cells) were also enumerated in the morphological assessment, although these may not have been included in the total colony counts since only aggregates of >50 cells were enumerated for total colony counts done prior to slide preparation and morphological assessment.

**Anti-PT antibody.** Clonal soft-agar stem-cell cultures were established as described abovea with 5 x 10^5 ICR murine marrow cells and with 182 U CSF-1/mL with either PT or HIPT at 20 ng/mL added at the start of the cultures. In each situation, either purified rabbit IgG anti-PT (antibody) or control nonimmune rabbit IgG (1gG) at a final concentration of 0.05 mg/mL or 4.5% media diluent (media) was included in the cultures. Culture plates were then incubated at 37°C, 5% CO₂, for 7 days, and colonies of >50 cells were counted with an inverted Olympus microscope.

**Pulse exposure to PT.** ICR murine marrow was cultured at 4 x 10⁵ cells/mL in Fischer’s media and 5% fetal calf sera (FCS) with 20 ng/mL PT, 20 ng/mL HIPT, or Fischer’s media diluent for 24 hours at 37°C in 5% CO₂. Cells were then washed three times in Fischer’s media plus 5% FCS and cultured in a double-layer soft agar culture system with a final concentration of 190 U CSF-1/mL or IL 3 1.9 ng/mL.

**Bone marrow-derived macrophages.** Bone marrow cells of C/H-Hej mice (Jackson Labs, Bar Harbor, ME), 8 weeks old, were seeded into 25-cm² flasks (Corning, New York) at cell concentrations of 0.8 x 10⁶/mL in 10 mL Dulbecco’s modified essential medium eagle (DMEM) with 15% FCS and 500 U stage-1 CSF-1/mL as described by Stanley and co-workers. Stage 1 CSF-1 was prepared by calcium phosphate absorption of L cell conditioned media as described by Stanley and Jubinsky. Cells were incubated at 37°C in 10% CO₂. There were then two separate 48-hour culture periods with 500 U CSF-1/mL and harvesting of the nonadherent cells and then an additional culture period ≥2 days, after which the nonadherent cells were discarded and the adherent cells used as CSF-1-dependent marrow-derived macrophages. Tushinski and Stanley demonstrated previously that these cells were dependent on CSF-1 for growth and that 95% had CSF-1 receptors. We have independently confirmed the CSF-1 dependence of these cells, showing that after 1 and 4 days of CSF-1 deprivation macrophage cell numbers are decreased by 2.5-fold and 15.3-fold, respectively. These adherent bone marrow-derived macrophages (6 days of growth, 2 days of adherence) were then washed three times with PBS and set up with DMEM, 15% FCS, and 500 U CSF-1/mL. The conditioned media were produced by a tenfold increase in PT concentration to 833 ng/mL. PMA alone with added IL 3 and with HIPT and PT was also evaluated for its effect on ³H-TdR incorporation.

**Analysis of effects of PT on IL 3 stimulation of single cells in culture.** FDC-P1 cells were washed, as described above, and then plated in Terasaki dishes under limiting dilution conditions. Wells with 1 cell/well were visually identified under an inverted microscope, and cells per well were then visually counted daily for 5 to 6 days of growth with IL 3 (10 ng/mL) and either PT or HIPT at 100 ng/mL.

**ADP-ribosylation of FDC-P1 membranes.** FDC-P1 membranes were prepared and extracted with cholate, and the resulting extract was incubated with PT and [³²P-NAD as described previously. The ADP-ribosylation reaction products were then separated by PAGE and evaluated by autoradiography.

**cAMP assay.** FDC-P1 cells were washed, resuspended in RPMI plus FCS without WEHI-3 CM, incubated for 2 hours at 37°C, and then washed and resuspended in RPMI, 0.5% bovine serum albumin (BSA) with IL 3 and either HIPT or PT media. These tubes were incubated at 37°C in 5% CO₂ for varying time periods. Cell pellets were collected after centrifugation, mixed with 0.1 N HCl at 37°C for 15 minutes and then recenterfuged, and the resulting supernatants were stored at −20°C until assay for cAMP by automated radioimmunoassay as described by Brooker et al. cAMP levels were expressed as pmol per milligram of cellular protein.

**Statistics.** Data were analyzed statistically using the Wilcoxon two-sample test (normal approximation with continuity correction of 0.5).

**RESULTS**

Colony formation of ICR murine marrow cells in soft agar clonal cultures was stimulated by varying concentrations of purified, L cell-derived CSF-1 or IL 3 prepared from WEHI-3 cell CM. PT at both 20 and 84 ng/mL inhibited the development of colonies induced by each stimulus (Figs 1 and 2). In six separate experiments, PT (20 and 84 ng/mL) reduced CSF-1–stimulated colony formation to a mean of 51% ± 4% of HIPT control. The PT-mediated inhibition noted with CSF-1 at all concentrations was significant at P values varying from 0.001 to 0.017. The PT inhibition of IL 3-dependent colony formation was significant at each level with P values ranging from 0.018 to 0.0045. Inhibitions achieved with these concentrations of PT appeared to be maximal in that similar levels of inhibition of both CSF-1–stimulated and IL 3-stimulated colony formation was produced by a tenfold increase in PT concentration to 833 ng/mL. Morphological assessment of colony types was carried out by preparing whole agar slide preparations and staining them as described in the Materials and Methods section. Megakaryocyte colonies (>3 cells) were enumerated in the morphological assessment although these may not have been included in total colony counts since only aggregates of >50 cells were enumerated for total colony counts done prior to slide preparation. In each of three experiments, morphology was assessed from one slide at CSF levels ranging from 18 to 284 U/mL. At all CSF-1 levels, the percentage of macrophage/monocyte colonies ranged from 95.8 to 100, indicating no significant shift in differentiation lineage with exposure to HIPT or PT. Morphological evaluation of colonies from the IL 3 experiments is shown in Table 1. PT inhibited IL 3–induced macrophage-monocyte colonies...
Effect of pertussis toxin on CSF-1-stimulated and IL 3-stimulated hematopoietic colony formation. Marrow cells were cultured at a final concentration of 0.5 x 10⁶/mL with 20 ng/mL PT or HIPT or, alternatively, with the media diluent and with varying concentrations of IL 3 or CSF-1. Data are expressed as the number of colonies (designated colony-forming unit-culture or CFU-C) stimulated per 10⁶ marrow cells ± 1 SEM. Each data point is derived from counts of three separate culture dishes in one representative experiment from three separate experiments, in which full dose-response curves were carried out for each regulator (total 6 experiments). In one of these other experiments assessing CSF-1 stimulation, 84 ng/mL (rather than 20 ng/mL) PT and HIPT showed similar levels of inhibition when PT was compared to HIPT, but there was ~20% inhibition when HIPT was compared to media control over 36 to 284 U CSF-1/mL.

Over an IL 3 dose range of 0.454 to 1.82 ng/mL (P < .003), but granulocyte colony formation over the same dose levels was not significantly inhibited (P = .78). When the 1.82 ng/mL concentration of IL 3 was considered separately, inhibition of granulocyte colony formation was significant (P = .016) (Table 1). Mixed granulocyte colonies were variably affected by PT, and there was no inhibition of megakaryocyte colony formation.

Specificity of PT inhibition of marrow stem cell proliferation induced by CSF-1 or IL 3 was demonstrated by experiments in which specific rabbit anti-PT toxin immunoglobulin was used. When toxin was mixed with antitoxin before addition to the cells, the toxin effect was abolished relative to buffer control and to toxin mixed with nonimmune rabbit immunoglobulin (Fig 3).

---

**Table 1. Effect of PT on IL 3-Stimulated Colony Subtypes Expressed as a Percentage of HIPT Control**

<table>
<thead>
<tr>
<th>IL 3 ng/mL</th>
<th>Granulocyte</th>
<th>Macrophage-Monocyte</th>
<th>Megakaryocyte</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.82 *</td>
<td>63 ± 3.7</td>
<td>46.3 ± 6.5</td>
<td>91 ± 3</td>
</tr>
<tr>
<td>0.9</td>
<td>109.5 ± 39.9</td>
<td>29.8 ± 7.3</td>
<td>108.5 ± 13.5</td>
</tr>
<tr>
<td>0.454</td>
<td>133 ± 16</td>
<td>28.9 ± 3.2</td>
<td>112 ± 15</td>
</tr>
</tbody>
</table>

*At the 1.82 ng/mL level the PT groups consisted of 19.8% granulocyte, 50.2% macrophage-monoctye, 16.9% mixed granulocyte-macrophage and 13.5% megakaryocyte colonies, and the HIPT groups had 17% granulocyte, 64% macrophage-monoctye, 10.8% mixed granulocyte-macrophage, and 8.2% megakaryocyte colonies. Results with PT effect on mixed granulocyte-macrophage colonies were quite variable and are not included in the data.

---

**Fig 1.** Effect of pertussis toxin on CSF-1-stimulated and IL 3-stimulated hematopoietic colony formation. Marrow cells were cultured at a final concentration of 0.5 x 10⁶/mL with 20 ng/mL PT or HIPT or, alternatively, with the media diluent and with varying concentrations of IL 3 or CSF-1. Data are expressed as the percentage of CFU-C/10⁶ cells in PT groups and (lgG) at a final concentration of 0.05 mg/mL or 4.5% media diluent (media) was included in the cultures. The results are expressed as a mean of CFU-C/10⁶ cells in PT groups and (lgG) at a final concentration of 0.05 mg/mL or 4.5% media diluent (media) was included in the cultures. The results are expressed as a mean of CFU-C/10⁶ cells in PT groups and (lgG) at a final concentration of 0.05 mg/mL or 4.5% media diluent (media) was included in the cultures. The results are expressed as a mean of CFU-C/10⁶ cells in PT groups and (lgG) at a final concentration of 0.05 mg/mL or 4.5% media diluent (media) was included in the cultures. The results are expressed as a mean of CFU-C/10⁶ cells in PT groups and (lgG) at a final concentration of 0.05 mg/mL or 4.5% media diluent (media) was included in the cultures. The results are expressed as a mean of CFU-C/10⁶ cells in PT groups and (lgG) at a final concentration of 0.05 mg/mL or 4.5% media diluent (media) was included in the cultures. The results are expressed as a mean of CFU-C/10⁶ cells in PT groups and (lgG) at a final concentration of 0.05 mg/mL or 4.5% media diluent (media) was included in the cultures. The results are expressed as a mean of CFU-C/10⁶ cells in PT groups and (lgG) at a final concentration of 0.05 mg/mL or 4.5% media diluent (media) was included in the cultures.

**Fig 2.** Effect of PT on CSF-1-stimulated and IL 3-stimulated hematopoietic colony formation expressed as a percentage of HIPT control. Experiments were carried out as described for Fig 1. Data are presented as a mean of CFU-C/10⁶ cells in PT groups and expressed as a percentage of HIPT controls at levels of CSF-1 and IL 3 in which inhibition was apparent and the level of colony formation was such that inhibition could be reliably interpreted. These data are from 9 experiments, 6 for CSF-1 and 3 for IL 3.

**Fig 3.** Effect of purified rabbit anti-PT antibody on PT inhibition of CSF-1-induced colony formation. Clonal soft-agar stem cell cultures were established with 5 x 10⁶ ICR murine marrow cells and with 182 U CSF-1/mL with either PT or HIPT at 20 ng/mL added at the start of the cultures. In each situation, either purified rabbit Igg anti-PT (antibody) or control nonimmune rabbit Igg (IgG) at a final concentration of 0.05 mg/mL or 4.5% media diluent (media) was included in the cultures. The results are expressed as a percentage of HIPT control ± 1 SEM for media, antibody, and IgG groups. Numbers in parentheses represent number of separate experiments for each group.
As has been demonstrated in experimental animals and other cultured cells, a transient exposure to PT elicits a sustained effect. When unseparated marrow cells were exposed to PT, HIPT, or medium for 24 hours and were then washed and assayed with the appropriate growth factor, the inhibition was equivalent to that which occurred with continuous exposure to PT (Fig 4).

The effect of PT could be mediated by a direct effect on marrow stem cells or by an effect on accessory regulatory cells. This latter possibility seems unlikely, since inhibition was noted on the plateau region of the dose–response curve for both CSF-1 and IL 3.

To address this issue further, we evaluated light-density nonadherent marrow cells. This latter possibility seems unlikely, since inhibition was noted on the plateau region of the dose–response curve for both CSF-1 and IL 3. To address this issue further, we evaluated light-density nonadherent marrow cells from mice treated with 5-fluorouracil (5-FU) 150 mg/kg body weight 3 days prior to marrow harvest (Table 2). Post-5-FU marrow represents a stem cell population depleted of conventional stem cells, but relatively enriched in high proliferative potential marrow-renewing cells. Metrizamide density-gradient separation removes most granulocytes, whereas the sequential adherence technique depletes the marrow predominantly of monocytes. PT inhibition was also observed with this partially depleted cell population. In addition, these data indicate that similar degrees of inhibition are evident at longer time intervals of in vitro soft agar culture (14 and 21 days), indicating that the observed effects are not the result of a prolonged lag period preceding the growth phase.

The IL 3-responsive murine marrow cell line, FDC-P1, provides an alternative model system, apparently free of accessory helper or inhibitor cells, in which to study the effects of PT on IL 3-induced cell proliferation. IL 3-induced proliferation of this homogeneous population of responder cells was markedly inhibited by PT over 24 hours at levels of a 100 ng/mL (Fig 5) or 1,000 ng/mL (Fig 6). Culture periods of 52 and 77 hours with both levels of PT gave similar results. Evaluation of the effect of PT treatment on ADP-ribosylation of the target protein (Fig 7) documents the presence of a PT-sensitive substrate in FDC-P1 cells. Together, these data indicate that the PT effect is directly on the IL 3-responsive cell. In addition, the phorbol ester PMA directly stimulated FDC-P1 cells (data not shown; Fig 8) and was able to bypass the PT inhibition (Fig 8), suggesting but not proving that the PT effect acted at a site proximal to protein kinase C activation. An alternative interpretation would simply be that PMA and IL 3 act on separate pathways in stimulating FDC-P1 cells.

**Table 2. Effect of PT on CSF-1 Stimulation of Post-5-FU and Nonadherent Light-Density (NALD) Post-5-FU Murine Marrow**

<table>
<thead>
<tr>
<th>Days of Culture</th>
<th>CFU-C/10⁶ Marrow Cells (% HIPT Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Post-5-FU Marrow</td>
</tr>
<tr>
<td>7</td>
<td>57 ± 11 (4)</td>
</tr>
<tr>
<td>14</td>
<td>74 ± 8 (2)</td>
</tr>
<tr>
<td>21</td>
<td>56 ± 13 (2)</td>
</tr>
</tbody>
</table>

Marrow was obtained from ICR mice 3 days after 150 mg/kg 5-fluorouracil (5-FU) by tail vein and either cultured (post-5-FU marrow) or subjected to separation of light-density cells (>1.055 g/cm³) on a metrizamide gradient (47) followed by two sequential 1-hour adherence separations on plastic Petri dishes at 5% CO₂, 37°C (nonadherent light density post-5-FU or NALD post-5-FU marrow) and then cultured at 37°C, 5% CO₂ in double-layer soft agar cultures. Colonies of >50 cells were then counted after 7, 14, and 21 days of culture. Numbers in parentheses represent number of separate experiments carried out for each data point. (CSF-1 was included at concentrations ranging from 18 to 71 U/mL in different experiments.)

**Fig 4. Effect of a 24-hour pulse exposure of ICR murine marrow to PT on subsequent CSF-1-stimulated and IL 3-stimulated colony formation.**

ICR murine marrow was cultured at 4 x 10⁶ cells/mL in Fischer’s media and 5% FCS in the presence of 20 ng/mL PT, 20 ng/mL HIPT, or Fischer’s media diluent for 24 hours at 37°C in 5% CO₂. Cells were then washed three times in Fischer’s media plus 5% FCS and were then cultured in a double-layer soft agar culture system with a final concentration of 190 U CSF-1/mL or IL 3 1.9 ng/mL. Colonies of >50 cells were counted after 7 days, and data are presented as the mean number of colonies per 10⁶ cells or per incubation tube and expressed as a percentage of the control. SEM. Numbers in parentheses are number of separate experiments from which these data are pooled. In three separate experiments, the mean absolute recovery per incubation of colony-forming cells stimulated by CSF-1 for PT and HIPT were 9,961 ± 3,319 and 15,871 ± 4,311, respectively; for CFU-C stimulated by IL 3, the mean absolute recoveries for the PT and HIPT groups were 5,842 ± 2,580 and 13,073 ± 7,192, respectively. HIPT, as compared to the media group, did not have asignificant effect on IL 3 or CSF-1 CFU-C recovery per incubation (107.5% ± 15.6% and 101.4% ± 20% of media control, respectively).
single cell wells were identified in the PT group; in 5 of these no cells were found by day 2 (failure rate 16.7%). These no-growth groups are not included in the calculations in Table 3. Data from all other groups (34 wells for HIPT and 25 for PT) are included in Table 2. There was progressive growth in all these wells with the exception of two HIPT wells and one PT well. These data indicate that PT did not exert its effect by killing or lysing the target cell but rather slowed its growth rate, as indicated by the altered cumulative doubling times.

In a similar manner, we studied the effect of PT on homogeneous populations of CSF-1-responsive (and dependent) marrow-derived macrophages. These cells have been previously established to be >95% pure CSF-1-responsive macrophages as determined by CSF-1 receptor binding, histochemistry, and cell morphology.15 We have confirmed the results on CSF-1 dependence and morphological homogeneity of these cells. PT reduced the proliferation of CSF-1-stimulated marrow-derived macrophages as measured by cell number and DNA content (Table 4 and Fig 9) and also altered the morphological appearance of the marrow-derived macrophages, causing the cells to assume a more rounded configuration (Fig 9).

Because PT treatment elevates cAMP modestly in a few cell types, we directly assessed this possible mechanism by studying FDC-P1 cells treated with PT. Results are pre-

**Fig 5.** Effect of PT (100 ng/mL) on IL-3-stimulated proliferation of FDC-P1 cells. FDC-P1 cells were cultured for 24 hours with varying levels of IL-3 and with either PT at 100 ng/mL (PT), HIPT at 100 ng/mL (HIPT), or media diluent (media). The cultures were pulsed with 3H-TdR at 24 hours; after a 5-hour labeling period, they were harvested and 3H-TdR incorporation was determined and expressed as cpm. These data are from 1 experiment but were replicated in 2 other separate experiments (total experiments). □ CSF-1; □ IL-3.

**Fig 6.** Effect of PT (1,000 ng/mL) on IL-3-stimulated proliferation of FDC-P1 cells. This experiment was carried out as part of the experiments described for Fig 5; details are provided in this legend.

**Fig 7.** Autoradiogram of PAGE-separated ADP-ribosylation reaction products. FD cells were cultured with buffer, PT (100 ng/mL), or HIPT for 24 hours. After homogenization, membranes were prepared and extracted with cholate, and the resultant extract was incubated with PT and 32P-NAD as described previously.24 Lane A is extract from control cell membranes; lane B is extract from membranes of PT-treated cells; and lane C is extract from membranes of cells treated with HIPT.
concentration of FDC-P1 cells were stimulated by varying levels of IL-3. PT and HIPT were included at a final concentration of 100 ng/mL and PMA at 120 nmol/mL. These data are from a representative experiment after 24 hours of growth. Similar results were obtained at 72 hours of FDC-P1 growth in this experiment and at 1, 2 and 3 days of growth in a separate experiment. Pertussis Toxin –○―○; Heat Inactivated PT ——; Heat Inactivated PT + PMA —— ○; HIPT + PMA [——]. These latter data indicate that PT did not kill or lyse FDC-P1 cells but rather acted to slow their growth rate.

The observation that PT inhibits IL-3-stimulated and CSF-1-stimulated marrow colony formation on the plateau region of each stimulators dose–response curve suggests that the inhibitory action of PT is probably direct. Inhibition of partially separated post-5-FU marrow provides further indirect evidence for such an effect. The observed PT inhibition of proliferation of the IL-3-responsive FDC-P1 cells and the CSF-1-dependent marrow-derived macrophages, both homogeneous cell populations, provide strong evidence that the PT effect is directly on the factor-responsive cell. The studies on individual FDC-P1 cells further substantiates this conclusion. These latter data indicate that PT did not kill or lyse FDC-P1 cells but rather acted to slow their growth rate.

Although receptors for CSF-1 and IL-3 have been identified, isolated, and characterized, the mechanism of signal transduction from these receptors has not yet been determined. One of the actions of PT is to modify guanine nucleotide binding G proteins by ADP-ribosylation, thereby blocking transduction of signals to second messenger pathways. The present data suggest that both CSF-1 and IL-3 receptor-mediated transduction occurs through these proteins since PT is capable of at least partially abrogating the stimulatory effect of both of these hemopoietic growth factors. In vivo exposure to PT blocked in vitro ADP-ribosylation of PT in cultured FDC-P1 cells (Fig 7), although the lack of effect of PT on cyclic AMP levels in IL-3-stimulated FDC-P1 cells argues against either a direct or indirect effect of PT on the cyclic nucleotide system. Pertussis toxin inhibits a variety of activities in target cells, apparently by ADP-ribosylation of PT-sensitive G proteins. The effects of PT include inhibition of cardiac potassium channel activation by muscarinic cholinergic agonists, reduction of phosphoinositide turnover and arachidonate release, attenuation of adenylate cyclase inhibition, and suppression of calcium channel conductance. Although the mechanism is not clear, PT interferes with the effects of peptide growth factors. Johnson et al showed that PT inhibited EGF-stimulated calcium mobilization and cytosolic protein phosphorylation in rat hepatocytes. Angiotensin-mediated effects in the same PT-treated cells were unaffected. More recently, Hildebrandt et al showed that PT-mediated ADP-ribosylation of an M, = 40,000 cell, inducing proliferation of murine pluripotent cells and promoting the establishment of IL-3-dependent cell lines from murine marrow. In addition, IL-3 possesses "synergistic" activity with the capacity to augment the effects of erythropoietin on erythroid stem cells ("burst-promoting activity") and CSF-1 on high proliferative potential macrophage stem cells (synergistic activity or hemopoietin 2). These latter actions are presumed to occur through modification or increased expression of receptors for erythropoietin or CSF-1.

The inhibition of CSF-1-stimulated marrow proliferation by PT selectively involved the monocyte-macrophage lineage, as would be expected from the previously established action of this growth factor on this pathway. PT inhibition of IL-3-stimulated marrow growth affected predominantly monocyte-macrophage colony formation, although at higher IL-3 levels granulocyte formation was also influenced.

The data we present show sustained attenuation of CSF-1 and IL-3-stimulated FDC-P1 cells, indicating that PT is a component of IL-3-stimulated proliferation of mouse bone marrow stem cells. CSF-1 selectively stimulates monocyte-macrophage proliferation, whereas IL-3 is a multi-lineage hemopoietic growth factor stimulating basophil, granulocyte, macrophage, and megakaryocyte differentiation. IL-3 also acts on more primitive hematopoietic stem cells, inducing proliferation of murine pluripotent cells and promoting the establishment of IL-3-dependent cell lines from murine marrow. In addition, IL-3 possesses "synergistic" activity with the capacity to augment the effects of erythropoietin on erythroid stem cells ("burst-promoting activity") and CSF-1 on high proliferative potential macrophage stem cells (synergistic activity or hemopoietin 2). These latter actions are presumed to occur through modification or increased expression of receptors for erythropoietin or CSF-1.

The inhibition of CSF-1-stimulated marrow proliferation by PT selectively involved the monocyte-macrophage lineage, as would be expected from the previously established action of this growth factor on this pathway. PT inhibition of IL-3-stimulated marrow growth affected predominantly monocyte-macrophage colony formation, although at higher IL-3 levels granulocyte formation was also influenced.

The observation that PT inhibits IL-3-stimulated and CSF-1-stimulated marrow colony formation on the plateau region of each stimulators dose–response curve suggests that the inhibitory action of PT is probably direct. Inhibition of partially separated post-5-FU marrow provides further indirect evidence for such an effect. The observed PT inhibition of proliferation of the IL-3-responsive FDC-P1 cells and the CSF-1-dependent marrow-derived macrophages, both homogeneous cell populations, provide strong evidence that the PT effect is directly on the factor-responsive cell. The studies on individual FDC-P1 cells further substantiates this conclusion. These latter data indicate that PT did not kill or lyse FDC-P1 cells but rather acted to slow their growth rate.

Table 3. Effect of PT on IL-3-Stimulated Growth of Single FDC-P1 Cells in Liquid Culture

<table>
<thead>
<tr>
<th>Day</th>
<th>Cell/Wall</th>
<th>PT</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1 (34)</td>
<td>1 (25)</td>
</tr>
<tr>
<td>1</td>
<td>2.2 + .14 (34)</td>
<td>1.8 + .14 (25)</td>
</tr>
<tr>
<td>2</td>
<td>7.56 + .6 (34)</td>
<td>4.72 + .49 (25)</td>
</tr>
<tr>
<td>3</td>
<td>25.4 + 1.7 (34)</td>
<td>12.6 + 1.3 (25)</td>
</tr>
<tr>
<td>4</td>
<td>93.7 + 8.45 (34)</td>
<td>42.5 + 4.85 (24)</td>
</tr>
<tr>
<td>5</td>
<td>313.7 + 32.5 (34)</td>
<td>123.8 + 13.4 (24)</td>
</tr>
<tr>
<td>6</td>
<td>660 + 141 (10)</td>
<td>198.9 + 31 (5)</td>
</tr>
</tbody>
</table>

Data are expressed as mean + SEM. Numbers in parentheses represent number of individual wells followed. FDC-P1 cells were stimulated with 10 ng/mL IL-3. PT and HIPT were included at a final concentration of 100 ng/mL.
Table 4. Effect of PT on CSF-1-Stimulated Bone Marrow-Derived Macrophage Growth

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell/Petri Dish x 10^4</th>
<th>DNA µg/Petri Dish</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>1</td>
</tr>
<tr>
<td>CSF-1</td>
<td>2.8,2.5</td>
<td>12.7,11.2</td>
</tr>
<tr>
<td>CSF-1 + PT</td>
<td>2.8,2.5</td>
<td>7.0,6.5</td>
</tr>
<tr>
<td>CSF-1 + HIPT</td>
<td>2.8,2.5</td>
<td>11.2,9.4</td>
</tr>
</tbody>
</table>

Adherent C3H/HeJ murine bone marrow-derived macrophages were studied after 2 days of adherent growth (6 days of culture). At this time, (time 0), cells in DMEM, 15% FCS, and 500 U stage-I CSF-1/mL were exposed to 44 ng/mL PT (PT), 44 ng/mL HIPT (HIPT), or media diluent (media) and cultures were re-fed daily with the same media plus added reagents. Cells per Petri dish and DNA (microgram) per dish were then assessed daily. Results are from representative experiments. Values from two flasks are given for each group at each data point. These results were replicated in three experiments.

Fig 9. The effect of PT on CSF-1 stimulation of bone marrow-derived macrophages. Representative photomicrographs from one of the experiments presented in Fig 7. PT at 44 ng/mL (A); HIPT at 44 ng/mL (B); numbers 1, 2, and 3 represent 1, 2, and 3 days, respectively, of exposure to PT or HIPT.

Table 5. Effect of PT on cAMP Levels in FDC-P1 Cells Stimulated by IL 3

<table>
<thead>
<tr>
<th>Group</th>
<th>cAMP (pmol/mg Protein) at Different Times of PT Exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min</td>
</tr>
<tr>
<td>IL 3* + media</td>
<td>32.5 ± 2.7</td>
</tr>
<tr>
<td>IL 3* + PT†</td>
<td>34.3 ± 5.3</td>
</tr>
<tr>
<td>IL 3* + HIPT†</td>
<td>31.2 ± 3.9</td>
</tr>
</tbody>
</table>

Cell level 0.75 × 10^6/mL/tube. Results confirmed at 30 minutes and 24 hours in one additional experiment. Similar results also obtained at different cell concentrations at 1 hour (two experiments) and 3 hours (one experiment).

*IL 3 at 10 ng/mL.
†PT and HIPT at 100 ng/mL.

Protein (protein) in Swiss 3T3 cells is associated with suppression of serum-dependent proliferation. Luttrell et al. have observed an inhibition of insulin effects (including the proliferative response as measured by 3H-TdR incorporation) in BC3 H1 myocytes.

In each of the studies in which PT was shown to affect proliferation, the inhibition was partial (40% to 60%), as it was in our studies. These data suggest that either PT blocks only one of several pathways responsible for the proliferative response or that the PT-induced defect is on an amplification pathway that is not essential for the basal response to occur. These two alternatives cannot be distinguished with present data.

Recent data by Farrar and colleagues have indicated that IL 3 stimulation of FDC-P1 cells causes a translocation...
of protein kinase C (PKC) to the membrane with subsequent phosphorylation of a 68k substrate. These data suggest further that IL 3 stimulation of proliferation may be mediated through PKC activation presumptively through activation of phosphoinositol pathway and generation of diacylglycerol. In the present studies, the decrease in cell proliferation caused by PT was reversed with exposure to the PKC activator PMA. Admittedly, the effect of PMA could be quite separate from the mechanisms whereby IL 3 stimu-
lates this cell population. The above noted data on PKC activation by IL 3, however, suggest otherwise.

In summary, these data indicate that a PT-sensitive G protein may be involved in transduction of the signals from CSF-1 and IL 3 receptors to the initiation of proliferation on target bone marrow cells. In that no changes are detected in cAMP levels, the phosphoinositol turnover-diacylglycerol generation pathways represent the principle possibility for the site of this toxin-induced defect.

REFERENCES

4. Moski TFP, Nacache PH, Marsh ML, Kermode J, Becker EL, Sha‘aﬁ RL: 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. Biochim Biophys Res Commun 124:644, 1984
31. Bartelmez SH, Sacca R, Stanley ER: Lineage specific recep-
Inhibition of interleukin 3 and colony-stimulating factor 1-stimulated marrow cell proliferation by pertussis toxin

YX He, E Hewlett, D Temeles and P Quesenberry