Priminig Effects of Granulocyte-Macrophage Colony-Stimulating Factor Are Coupled to Cholera Toxin-Sensitive Guanine Nucleotide Binding Protein in Human T Lymphocytes

By Ala Al-Aoukayt, Adel Gaid, Clive Sinoff, Anthony D. Ho, and Azzam A. Maghazachi

In addition to the mobilization of neutrophils and monocytes, granulocyte-macrophage colony-stimulating factor (GM-CSF) also mobilizes lymphocytes into peripheral blood. We examined the ability of GM-CSF to induce the proliferation of purified human T-cells (CD3+CD4+CD56+CD16- B1+MO2-) in two major aspects: (1) the mechanisms of GM-CSF interaction with interleukin-2 (IL-2) causing T-cell proliferation, and (2) the intracellular signals transmitted by GM-CSF in T lymphocytes. We observed that concentrations of GM-CSF between 0.01 ng/mL and 10 ng/mL had a synergetic effect with concentrations of IL-2 between 1 U/mL and 10 U/mL in stimulating T-cell proliferation. This effect of GM-CSF was maximal when it was added at the start of the culture. In situ hybridization showed the presence of mRNA for GM-CSF receptors in T-cells. Further analysis showed that GM-CSF induced the expression of IL-2 receptor (IL-2R) on the surface of T lymphocytes. These events coincide with the ability of GM-CSF to increase the intracellular levels of both cyclic 3',5'-adenosine monophosphate (cAMP) and cyclic 3',5'-guanosine monophosphate (cGMP) in T-cells, to increase the binding of [γ-35S]-GTP to T-cell membranes, and to enhance GTPase activity as determined by increased 32P-GTP, IL-2 also induced IL-2R expression, cyclic nucleotide secretion, and G-protein activation. However, the presence of IL-2 reduced GM-CSF induction of these activities. Addition of antibodies to the α and β subunits of IL-2R permitted the activation of G protein by GM-CSF even when IL-2 was present. Furthermore, GTP binding and GTpase activity induced by GM-CSF or IL-2 were inhibited by the addition of cholera toxin (CT), but not pertussis toxin (PT). Cumulatively, these results suggest that in T lymphocytes, receptors for GM-CSF or IL-2 may be coupled to the same CT-sensitive G protein, although other possibilities may exist. The role that G proteins play in mediating the intracellular signaling pathways induced by GM-CSF or IL-2 in human T-cells is supported by adenosine diphosphate-ribosylation of a 44-kD or a 39-kD G protein in T-cell membranes by CT and PT, respectively.

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HUMAN granulocyte-macrophage colony-stimulating factor (GM-CSF) is a hematopoietic cytokine that stimulates the formation of granulocyte, macrophage, eosinophil, and neutrophil colonies. Recent studies have focused on the intracellular signaling pathways mediated by GM-CSF. In human neutrophils, GM-CSF does not directly induce Ca2+ mobilization, but it primes these cells for formyl-methionyl-leucyl-phenylalanine (fMLP)- or platelet-activating factor (PAF)-induced Ca2+ mobilization, or increased release of arachidonic acid and leukotriene B4 synthesis.

The priming effect of GM-CSF in neutrophils has been found to be mediated through a pertussis toxin (PT) substrate guanine nucleotide-binding (G) protein, but not through a choleratoxin (CT) substrate G protein. Furthermore, GM-CSF receptors expressed on neutrophils were found to be coupled to a PT-sensitive G protein. In contrast, both PT and CT inhibit GM-CSF-induced proliferation of the AML-193 cell line. These results suggest that GM-CSF interaction with its receptors in different cell types varies in sensitivity to PT and CT.

The heterotrimeric G proteins are composed of three subunits: α, β, and γ. The most extensively studied include transducing (Gz), stimulatory (Gq), inhibitory (Gi), Gα11, and Gβ. In the nonactivated form, the α subunit of G protein binds guanosine diphosphate (GDP). However, when ligands bind to receptors that are coupled to G proteins, activation of the α subunit occurs. Guanosine triphosphate (GTP) that is abundant in the plasma membranes will then displace the bound GDP, resulting in the dissociation of the α subunit from the β and γ subunits. The α subunit of G proteins contains sites that are covalently modified by bacterial toxins. Consequently, CT activates Gi whereas PT inhibits Gα14-17 resulting in increased synthesis of cyclic adenosine 3',5'-monophosphate (cAMP).

GM-CSF, through its action on membrane receptors and G proteins, modulates the intracellular levels of cyclic nucleotides. In human neutrophils, high levels of GM-CSF induced a twofold increase in cyclic 3',5'-guanosine monophosphate (cGMP), but not cAMP level. In contrast, when murine hematopoietic cells were examined, GM-CSF increased the synthesis of endogenous cAMP.

The effect of GM-CSF on human lymphocytes has not been thoroughly examined. Santoli et al. showed that GM-CSF amplifies interleukin-2 (IL-2)-driven human T-cell proliferation. In patients with non-Hodgkin's lymphoma, it has been previously shown that an increase in the absolute lymphocyte count, and particularly in activated lymphocytes, occurs after the administration of GM-CSF. In this study we investigated the role of GM-CSF in stimulating the proliferation of purified human T-cells (CD3+CD4+CD56+CD16- B1+MO2-) in two major aspects: (1) the mechanisms of GM-CSF interaction with interleukin-2 (IL-2) causing T-cell proliferation, and (2) the intracellular signals transmitted by GM-CSF in T lymphocytes. We observed that concentrations of GM-CSF between 0.01 ng/mL and 10 ng/mL had a synergetic effect with concentrations of IL-2 between 1 U/mL and 10 U/mL in stimulating T-cell proliferation. This effect of GM-CSF was maximal when it was added at the start of the culture. In situ hybridization showed the presence of mRNA for GM-CSF receptors in T-cells. Further analysis showed that GM-CSF induces the expression of IL-2 receptor (IL-2R) on the surface of T lymphocytes. These events coincide with the ability of GM-CSF to increase the intracellular levels of both cyclic 3',5'-adenosine monophosphate (cAMP) and cyclic 3',5'-guanosine monophosphate (cGMP) in T cells, to increase the binding of [γ-35S]-GTP to T-cell membranes, and to enhance GTPase activity as determined by increased 32P-GTP. IL-2 also induced IL-2R expression, cyclic nucleotide secretion, and G-protein activation. However, the presence of IL-2 reduced GM-CSF induction of these activities. Addition of antibodies to the α and β subunits of IL-2R permitted the activation of G protein by GM-CSF even when IL-2 was present. Furthermore, GTP binding and GTPase activity induced by GM-CSF or IL-2 were inhibited by the addition of cholera toxin (CT), but not pertussis toxin (PT). Cumulatively, these results suggest that in T lymphocytes, receptors for GM-CSF or IL-2 may be coupled to the same CT-sensitive G protein, although other possibilities may exist. The role that G proteins play in mediating the intracellular signaling pathways induced by GM-CSF or IL-2 the intracellular signaling pathways induced by GM-CSF or IL-2 in human T-cells is supported by adenosine diphosphate-ribosylation of a 44-kD or a 39-kD G protein in T-cell membranes by CT and PT, respectively.

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proliferation of purified T lymphocytes, especially in relation to the synergy with IL-2, and examined the intracellular signals activated by this cytokine.

MATERIALS AND METHODS

Reagents. Recombinant human GM-CSF and IL-2 were supplied by Behringwerke AG (Marburg, Germany) and Cetus Corp (Emeryville, CA), respectively. Unconjugated monoclonal mouse antihuman IL-2 receptor 1 (IL-2R1) (CD25) and fluorescein isothiocyanate (FITC)-conjugated monoclonal mouse antihuman T11 (CD2), CD3, T4 (CD4), T8 (CD8), NK11 (CD56), B1, and MO2 antibodies were purchased from Coulter Corp (Hialeah, FL). Antibodies to human IL-2Rβ and FcyRIII (CD16) were purchased from Cederlane Laboratories (Hornby, Ontario, Canada). Pertussis toxin (PT), cholera toxin (CT), leupeptin, aprotinin, pepstatin A, phenyl methyl sulfonyl fluoride (PMSF), thymidine, diethiothreitol (DTT), EDTA, EGTA, MgCl2, KCl, bovine serum albumin (BSA), adenosine triphosphate (ATP), adenosine 5′-[γ-35S]-triphosphate (App[NHP]), Tris HCl, phosphate creatine, creatine phosphokinase, dextran, charcoal (Norit A), 3-isobutyl-1-methylxanthine (IBMX), paraformaldehyde, and sodium citrate were purchased from Sigma Chemical Co (St Louis, MO).

Culture medium (CM). CM consisted of RPMI-1640 supplemented with 10% human AB serum (NABI, Miami, FL), 10 U/mL penicillin, 100 µg/mL streptomycin, 1 mmol/L L-glutamine, 1% nonessential amino acids (GIBCO Laboratories, Burlington, Ontario, Canada), and 5 x 10−5 mol/L 2-mercaptopethanol (Sigma).

Preparation of T cells. Buffy coats of normal human volunteers were obtained from the Canadian Red Cross Blood Bank (Sudbury, Ontario, Canada). The cells were first layered over Ficoll-Hypaque carefully collected, extensively washed, and then examined. The cells were first layered over Ficoll-Hypaque (Pharmacia Chemicals, Quebec, Canada) and centrifuged for 25 minutes at 500 x g to remove the red blood cells (RBCs). The erythrocyte-depleted cell preparation was incubated on a nylon-wool column (NCW) for 1 hour at 37°C in a 5% CO2 incubator to remove B cells and macrophages. NCW-nonadherent cells were then layered over a discontinuous Percoll (Pharmacia) density gradient. Four layers of Percoll were used: 37%, 44%, 52%, and 60% as previously described. After centrifugation, cells of the 60% fraction were carefully collected, extensively washed, and then examined.

Immunofluorescence and flow cytometric analysis. Cells (5 x 10^5/100 µL) were incubated with the appropriate FITC-conjugated monoclonal antibody (MoAb) at 4°C for 45 minutes. The cells were washed with buffer containing phosphate-buffered saline (PBS) plus 1% BSA and 0.02% sodium azide, and then analyzed for fluorescence using the EPIC Elite flow cytometer (Coulter). The expression of IL-2R was done by incubating 5 x 10^4/100 µL T cells with either mouse antihuman IL-2Rα, or mouse antihuman IL-2Rβ for 45 minutes at 4°C. The cells were then washed with the buffer and incubated with FITC-conjugated, affinity-purified, and human absorbed goat antinouse Fab(2) IgG antibody (Cederlane Laboratories) for an additional 30 minutes at 4°C. The cells were washed twice and tested for the expression of IL-2R using the EPIC Elite flow cytometer.

Proliferation assay. This method has been described elsewhere. In brief, T cells (1 x 10^4) were incubated in triplicate with various concentrations of IL-2 at 37°C. Four hours before harvesting the cultures, 1 µCi (PH) thymidine (TrH; NEN/ Du Pont, Mississauga, Ontario, Canada) was added to the cultures. The cultures were harvested on fiberglass papers using a cell harvester (LKB Wallac, Turku, Finland).

Measurement of cyclic nucleotides. This was done as previously described. T cells (2 x 10^6/mL) were incubated with 1 ng/mL GM-CSF, 10 U/mL IL-2, a combination of 1 ng/mL GM-CSF and 10 U/mL IL-2, or 5 µg/mL CT for various time intervals in the presence of 100 µmol/L IBMX to inhibit the activity of the phosphodiesterases. The cells were then harvested, washed with CM, and resuspended in 1.0 mL of the assay buffer (Tris/EDTA). The metabolic activity was stopped by three rounds of freezing and thawing. The cells were incubated in a boiling water bath for 4 minutes to precipitate the protein and were centrifuged for 10 minutes at 13,000g in the cold. The supernatant was collected and divided into two parts: one for the measurement of cAMP and the other for the measurement of cGMP, using radioimmunoassay (Amersham, Oakville, Ontario, Canada) according to the manufacturer's specification.

Preparation of T-cell membranes. T-cell membranes were prepared in a lysis buffer containing 10 mmol/L HEPES pH 7.5, 3 mmol/L MgCl2, 2 mmol/L EDTA, 40 µg/mL phenylmethylsulfonyl fluoride (PMSF), 10 µg/mL leupeptin, 2 µg/mL pepstatin A, and 2 µg/mL aprotonin. After homogenization (Brinkman Homog-nizer, Switzerland) and 1-minute sonication (Branson Sonifier, Danbury, CT), the membranes were centrifuged at 1,000g for 10 minutes. The supernatant was transferred into Beckman tubes (Beckman, Mississauga, Ontario, Canada) and ultracentrifuged at 150,000g for 45 minutes at 4°C. The membranes were transferred into a buffer containing 10 mmol/L HEPES, 3 mmol/L MgCl2, and 2 mmol/L EDTA. Protein concentration was determined according to the method described by Bradford.

Activation of bacterial toxins. PT was activated in 100 µL of buffer containing 7.5 µg PT (60 µL), 20 µL of 100 mmol/L DTT, and 20 µL of 100 mmol/L HEPES for 10 minutes at 37°C. For CT, a buffer containing 50 µg (60 µL) CT, 20 µL of 100 mmol/L DTT, and 20 µL of 50 mmol/L PBS was used to activate the toxin.

Adenosine diphosphate (ADP)-ribosylation. This was performed as described. In brief, activated PT or CT was added to 200 µg membranes in the presence of 20 mmol/L thymidine, 1 mmol/L ATP, 1 mmol/L GTP, 1 mmol/L EDTA, and 20 mmol/L HEPES. The reaction was started by adding 1 to 3 µmol/L of 32P-NAD, and was incubated for 45 minutes at 30°C. The reaction was stopped by incubation on ice, and the mixture was washed with a buffer containing 20 mmol/L HEPES, 1 mmol/L EDTA, and 1 mmol/L DTT. For CT, GTP was omitted and 1 mmol/L MgCl2 was added. About 100 µg membranes were suspended in 15 µL sample buffer, heated for 5 minutes, and then loaded on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli.

GTP-binding assay. This was performed according to described methods. The binding buffer consisted of 50 mmol/L TrisHCl, 2 mmol/L MgCl2, 1 mmol/L EDTA, 5 mmol/L DTT, and 100 µg/mL BSA. GM-CSF (1 ng/mL) was added to 50 µg membranes in the presence of 1.6 µCi GTP32P (specific activity 1,202 Ci/mmol; NEN/DuPONT). The mixture was incubated for 15 minutes at 37°C. Nonspecific binding was determined by adding 1 µmol/L of cold GTP32P. Specific binding was calculated by subtracting nonspecific binding from total binding. The reaction was terminated by adding 900 µL of cold buffer. After centrifugation at 16,000g in the cold and two rounds of washing, the pellet was dissolved in 150 µL of liquid scintillation cocktail (Beckman Ready Instrument). All assays were performed in triplicate.

GTPase assay. This was performed by measuring the release of 32P from (γ-32P) GTP as described. The reaction consisted of 50 µg membrane and 1 ng/mL GM-CSF in a total volume of 100 µL of buffer containing 25 mmol/L Tris HCl, 1 mmol/L EDTA, 1 mmol/L MgCl2, 138 mmol/L KCl, 0.1 µg/mL BSA, 0.5 mmol/L App (NH)p, 1 mmol/L DTT, 0.25 mmol/L ATP, 5 mmol/L phosphate creatine, and 50 µU/mL creatine phosphokinase. (γ-32P)GTP (specific activity 30 Ci/mmol) was then added and the mixture was incubated for 15 minutes at 37°C. The reaction was terminated by adding the reaction mixtures to scintillation vials.
GM-CSF OR IL-2 ACTIVATES G PROTEIN IN T CELLS

Fig 1. GM-CSF potentiates IL-2-induced T-cell proliferation. \( ^\text{3}H \)-thymidine incorporation assay was conducted after 7 days of culture. Results are shown as mean ± SD from triplicate determinations of different three donors. Data not shown, see Fig 2. The magnitude of the response was about twofold to fivefold, depending on the donor. Although all doses of GM-CSF tested potentiated IL-2-induced T-cell proliferation, for simplicity we used 1 ng/mL GM-CSF plus 10 U/mL IL-2 for further study, because of apparent plateau at this concentration of GM-CSF.

Effect of GM-CSF on T-cell phenotype. To determine whether there was any change in the surface markers of T lymphocytes, the phenotype of the same T-cell preparation was examined before and after incubation with GM-CSF and/or IL-2. As shown in Table I, there is a slight, but significant, increase in the percentage of CD3 and CD4 \( (P < .05) \), but not of CD8, cells after culture with GM-CSF. IL-2 alone was able to induce a similar increase.

GM-CSF must be added at the onset for optimal synergy with IL-2. The maximal response to IL-2 occurred when IL-2 was added at the onset of the culture (day 0) compared with its addition at day 1, 2, or 3 \( (P < .02) \). GM-CSF alone had little effect in increasing T-cell proliferation (Fig 2). However, in the presence of IL-2, GM-CSF substantially increased T-cell proliferation. GM-CSF was added at day 3 and IL-2 at day 0, significantly less enhancement was observed \( (P < .05) \) compared with the addition of GM-CSF and IL-2 at day 0. However, when GM-CSF was added at day 0 and IL-2 at day 1 or 2, greater T-cell proliferation was demonstrated compared with the addition of IL-2 alone (no GM-CSF) at day 1 or 2 \( (P < .01) \) or with the simultaneous addition of IL-2 and GM-CSF at day 0 \( (P < .05) \). Delayed addition of GM-CSF to day 1, 2, or 3 resulted in lower synergy with IL-2. These results suggest that addition of GM-CSF at the start of the culture is important for optimal T-cell proliferation.

GM-CSF receptor mRNA is present in T lymphocytes. The above results suggest that T cells may express receptors for GM-CSF. To investigate this possibility, we used the in situ hybridization technique to demonstrate the presence of GM-CSF receptor mRNA in T lymphocytes. Hybridization signals shown by presence of silver grains were either clustered (Fig 3, A and B) or evenly distributed around the nu-

RESULTS

GM-CSF potentiates IL-2-induced T-cell proliferation. Addition of GM-CSF to purified T cells did not induce their proliferation (data not shown, see Fig 2). However, addition of GM-CSF to T cells incubated with IL-2 for 7 days augmented IL-2-induced T-cell proliferation (Fig 1). Concentrations of GM-CSF between 0.01 and 10 ng/mL enhanced T-cell proliferation induced by 1 to 10 U/mL of IL-2 (Fig 1).
Table 1. Phenotypic Expression of Cells Before and After Culture

<table>
<thead>
<tr>
<th></th>
<th>CD3</th>
<th>CD4</th>
<th>CD8</th>
<th>CD56</th>
<th>CD16</th>
<th>B1</th>
<th>MO2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before activation</td>
<td>76.6±1.4</td>
<td>50.4±5.3</td>
<td>28.7±6.9</td>
<td>&lt;.1</td>
<td>&lt;.1</td>
<td>&lt;.1</td>
<td>&lt;.1</td>
</tr>
<tr>
<td>After activation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cells incubated with:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CM</td>
<td>75.5±2.6</td>
<td>53.8±3.4</td>
<td>21.8±0.2</td>
<td>&lt;.1</td>
<td>&lt;.1</td>
<td>&lt;.1</td>
<td>&lt;.1</td>
</tr>
<tr>
<td>IL-2</td>
<td>86.3±4.2*</td>
<td>63.9±1.0*</td>
<td>25.0±3.4</td>
<td>&lt;.1</td>
<td>&lt;.1</td>
<td>&lt;.1</td>
<td>&lt;.1</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>83.7±1.6*</td>
<td>61.6±3.9*</td>
<td>25.3±3.4</td>
<td>&lt;.1</td>
<td>&lt;.1</td>
<td>&lt;.1</td>
<td>&lt;.1</td>
</tr>
<tr>
<td>IL-2 + GM-CSF</td>
<td>85.3±1.6*</td>
<td>65.6±1.0*</td>
<td>24.0±1.8</td>
<td>&lt;.1</td>
<td>&lt;.1</td>
<td>&lt;.1</td>
<td>&lt;.1</td>
</tr>
</tbody>
</table>

T cells were cultured in the presence of culture medium (CM), 10 U/mL IL-2, 1 ng/mL GM-CSF, or both for 7 days. The cells were collected and stained with various MoAbs. The results are shown as mean ± SD of three to five experiments. CD3 is a T-cell marker, CD4 is a T-helper cell marker, CD8 is a cytotoxic T-cell marker, CD56 and CD16 are natural killer cell markers, B1 is a B-cell marker, and MO2 is a monocyte marker.

* P < .05 compared with control values (cells incubated with CM only).

The majority of T lymphocytes showed moderate level of grain deposits. Specificity of hybridization signals was confirmed by presence of low signal in cells digested with RNase before hybridization with the antisense probe (Fig 3D).

**GM-CSF induces the expression of IL-2R on human T lymphocytes.** Table 2 shows that both IL-2 and GM-CSF increased the expression of IL-2Rβ 1 day after culture. Whereas the increase in IL-2Rβ remains constant after 2 days incubation with IL-2 (about 16% of the cells express this receptor), a maximum expression (about 46% of T cells express IL-2Rβ) occurred on the second day of incubation with GM-CSF.

Incubating T cells with both factors substantially reduced the expression of IL-2Rβ 2 days after incubation (about 22% of the cells express this receptor) compared to incubation with GM-CSF alone. In contrast, at day 3 of culture there was an enhancement of the expression of IL-2Rα by either IL-2 or GM-CSF (Table 2).

**Effect of GM-CSF and IL-2 on the level of cyclic nucleotides.** Table 3 shows that 1 ng/mL GM-CSF significantly increased the endogenous level of cGMP after 5 or 30 minutes of incubation with T lymphocytes. IL-2 also increased the intracellular level of cGMP 30 minutes after incubation. However, combining GM-CSF with IL-2 attenuated the enhancement of cGMP level induced by GM-CSF. Surprisingly, GM-CSF also increased the intracellular level of cAMP 5 minutes after culture (Table 3). As expected, CT increased the synthesis of cAMP but not cGMP in T cells after incubation for 5 and 30 minutes.

**GM-CSF enhances GTP binding to T-cell membranes.** Figure 4 shows that 1 ng/mL GM-CSF significantly increased the binding of GTPγ35S to T-cell membranes compared with basal binding in the absence of GM-CSF. To investigate whether a bacterial toxin-sensitive G protein is coupled to GM-CSF receptor, we incubated T-cell membranes with 1 ng/mL GM-CSF in the presence of PT or CT. The results shown in Fig 4 demonstrate that CT (1 μg/mL), but not PT (5 μg/mL), inhibited GM-CSF enhancement of GTPγ35S binding to T-cell membranes.
GM-CSF or IL-2 activates G protein in T cells

To examine whether there was any synergistic effect on GTP binding between GM-CSF and IL-2, these cytokines were added alone or in combination to T-cell membranes. Figure 5 shows that GM-CSF or IL-2 enhanced GTP binding (P < .001 and P < .005, respectively, compared with basal binding). Surprisingly, combining GM-CSF and IL-2 resulted in lower GTP binding compared with the addition of GM-CSF alone (P < .05), but similar to the response observed in the presence of IL-2 only (P not significant). CT inhibited IL-2-induced (P < .01) or GM-CSF-induced (P < .05) GTP binding (Fig 5). CT also inhibited GTP binding in the presence of GM-CSF plus IL-2; however, the level of GTP binding reached that observed in the presence of CT plus IL-2, but was significantly lower than that obtained in the presence of GM-CSF plus CT (P < .01).

The above results suggest that IL-2 or GM-CSF receptors may be coupled to the same G protein that is CT-sensitive, and that simultaneous addition of IL-2 and GM-CSF resulted in the ability of IL-2 (which appears to have the upper hand) to stimulate this G protein, thus preventing GM-CSF from activating it. To examine this possibility, we blocked IL-2R expressed on T cells by the addition of antibodies to the IL-2Rα and β. For control, T cells were pretreated with IgG antibody only. Membranes were prepared from these cells, and GTP binding was measured after the addition of IL-2, GM-CSF, or their combination. Figure 6 shows that addition of anti–IL-2 receptor antibodies or a control IgG antibody to T cells resulted in higher basal GTP binding. This could be caused by the activation of G protein through the Fc receptor, but this possibility was not further
GTPase activity. Figure 7 also shows that increased GTPase activity after incubating T-cell membranes with GM-CSF resulted in a response similar to that obtained in the presence of CT plus IL-2. GM-CSF-induced GTP binding or GTPase activity is caused by the addition of GM-CSF, whereas PT ADP-ribosylates a 39-kD G protein. Activity to that obtained in the presence of CT plus IL-2 was inhibited by 1 mL CT but not by 0.2 mL CT. We also observed that addition of CT to T-cell membranes incubated with GM-CSF alone resulted in similar GTPase activity after incubating T-cell membranes with GM-CSF, but was higher than that obtained in the presence of IL-2 only (P < .05), confirming our earlier finding (Fig 5). However, when both cytokines were added to membranes of T cells pretreated with anti-IL-2R antibodies, the level of GTP binding was similar to that obtained in the presence of GM-CSF, but was higher than that obtained in the presence of IL-2 only (P < .05).

**GM-CSF enhances GTPase activity in T-cell membranes.** Figure 7 shows that GM-CSF increased the hydrolysis of GTP, indicating that GM-CSF activates the intrinsic GTPase activity. Figure 7 also shows that increased GTPase activity after incubating T-cell membranes with GM-CSF was inhibited by 1 μg/mL CT but not by 0.2 μg/mL PT.

IL-2 alone was able to enhance GTPase activity (Fig 8; P < .002 compared with basal binding). Combining IL-2 with GM-CSF resulted in a response similar to that obtained after incubating membranes with IL-2 only, but was lower than that observed in the presence of GM-CSF alone (P < .02). Furthermore, addition of CT to T-cell membranes incubated with IL-2 plus GM-CSF resulted in similar GTPase activity to that obtained in the presence of CT plus IL-2.

**Bacterial toxins ADP-ribosylate G proteins in T-cell membranes.** Figure 9 shows that CT ADP-ribosylates a 44-kD G protein, whereas PT ADP-ribosylates a 39-kD G protein. To clearly establish whether the ability of CT to inhibit GM-CSF-induced GTP binding or GTPase activity is caused by the modification of G proteins, T cells were intoxicated with either CT or PT, membranes were prepared from intoxicated cells, and then challenged with freshly activated CT or PT in the presence of (32P)NAD. Figure 9 shows that membranes prepared from intoxicated cells pretreated with CT or PT incorporated less (32P)NAD than membranes of intact cells.

**DISCUSSION**

Although GM-CSF activates various cell types,14 little information is available regarding its effects on T lymphocytes. In our study, concentrations of GM-CSF between 0.01 ng/mL and 10 ng/mL were able to augment IL-2-induced proliferation of purified human T cells. CD3+ CD4+ was the major T-cell subpopulation activated by GM-CSF. This result supports an earlier finding showing that there was a significant increase in CD4+ T cells in patients with malignant lymphoma who received GM-CSF.21

The optimal proliferative response occurred when GM-CSF was added at the start of the culture as opposed to delayed exposure (Fig 2); addition of GM-CSF at day 1, 2, or 3 of a 7-day culture resulted in lower synergy with IL-2. It was also clear that addition of IL-2 and GM-CSF together at the start of the culture resulted in lower synergism compared with the addition of GM-CSF 1 or 2 days before the addition of IL-2. The above results suggest that T lymphocytes may express receptors for GM-CSF that have not been previously detected in these cells. Using in situ hybridization, we showed the presence of mRNA for GM-CSF receptors in T cells. This result, combined with a recent observation showing that GM-CSF induces the generation of T-derived lymphokine activated killer cells, clearly indicates that immune cells, particularly T lymphocytes, express receptors for GM-CSF, which facilitate the induction of various biologic activities upon binding GM-CSF.

The effect of GM-CSF, at least in part, is mediated through the induction of IL-2R expression on human T lymphocytes. There are at least three subunits of IL-2R: IL-2Ra(p55), IL-2RP (~70), and IL-2Ra(p70). Table 2 shows the effect of GM-CSF on IL-2R expression.

**Table 2. Effect of GM-CSF on IL-2R Expression**

<table>
<thead>
<tr>
<th>% Positive Cells</th>
<th>CM</th>
<th>IL-2</th>
<th>GM-CSF</th>
<th>IL-2 + GM-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Before activation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>IL-2Ra (p55)</td>
<td>3.3 ± 0.7</td>
<td></td>
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<tr>
<td>IL-2Ra (p70)</td>
<td>6.9 ± 2.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>After activation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>3.4 ± 0.9</td>
<td>6.7 ± 2.0</td>
<td>3.4 ± 0.8</td>
<td>5.7 ± 1.4</td>
</tr>
<tr>
<td>IL-2Ra (p55)</td>
<td>6.7 ± 2.0</td>
<td>15.6 ± 0.4</td>
<td>18.8 ± 0.1</td>
<td>17.1 ± 3.1</td>
</tr>
<tr>
<td>Day 2</td>
<td>2.0 ± 2.0</td>
<td>6.8 ± 0.8</td>
<td>4.6 ± 0.7</td>
<td>9.8 ± 6.1</td>
</tr>
<tr>
<td>IL-2Ra (p55)</td>
<td>6.8 ± 0.8</td>
<td>16.0 ± 2.4</td>
<td>46.2 ± 3.8</td>
<td>22.6 ± 2.1</td>
</tr>
<tr>
<td>Day 3</td>
<td>1.9 ± 1.7</td>
<td>11.2 ± 3.7</td>
<td>7.8 ± 0.8</td>
<td>8.7 ± 4.2</td>
</tr>
<tr>
<td>IL-2Ra (p55)</td>
<td>0.2 ± 0.0</td>
<td>17.2 ± 8.11</td>
<td>28.4 ± 8.31</td>
<td>19.5 ± 3.11</td>
</tr>
</tbody>
</table>

Cells were incubated with CM, 10 U/mL IL-2, 1 ng/mL GM-CSF, or their combination. Between 1 and 3 days later, the cells were collected and tested for the expression of IL-2Ra(p55). The results are shown as mean ± SD of three experiments. *P < .01, **P < .001 compared with control values (cells incubated with CM only).

**Table 3. GM-CSF Elevates the Intracellular Levels of Cyclic Nucleotides in T Cells**

<table>
<thead>
<tr>
<th>Nucleotides</th>
<th>cGMP Level (pmol/2 × 10⁷ cells at time after incubation)</th>
<th>cAMP Level (pmol/2 × 10⁷ cells at time after incubation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min</td>
<td>30 min</td>
</tr>
<tr>
<td>CM</td>
<td>0.5 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>2.3 ± 0.6*</td>
<td>1.2 ± 0.1*</td>
</tr>
<tr>
<td>IL-2</td>
<td>0.4 ± 0.2</td>
<td>1.2 ± 0.2*</td>
</tr>
<tr>
<td>IL-2 + GM-CSF</td>
<td>0.6 ± 0.4</td>
<td>0.6 ± 0.21</td>
</tr>
<tr>
<td>CT</td>
<td>0.3 ± 0.2</td>
<td>0.7 ± 0.3</td>
</tr>
</tbody>
</table>

T cells (2 × 10⁶) were incubated with either CM, 1 ng/mL GM-CSF, 10 U/mL IL-2, a combination of GM-CSF and IL-2, or 5 μg/mL CT for 5 or 30 minutes at 37°C in the presence of 100 μmol/L IBMX. The cells were extensively washed and cyclic nucleotides levels were measured as described in Materials and Methods. The results are shown as mean ± SD of two experiments. *P < .02, **P < .05 compared with control values (cells incubated with CM only).

1P < .05 comparing IL-2 + GM-CSF with GM-CSF.
GM-CSF OR IL-2 ACTIVATES G PROTEIN IN T CELLS

Fig. 4. CT inhibits GM-CSF–induced GTP binding to T-cell membranes. T-cell membranes were exposed to either buffer (basal GTP binding), 1 ng/mL GM-CSF, 1 ng/mL GM-CSF plus 1 μg/mL CT, 1 ng/mL GM-CSF plus 5 μg/mL PT, CT or PT alone. The membranes were incubated for 15 minutes in the presence of [γ-35S]GTP as described in Materials and Methods. Results are shown as mean ± SD of triplicate determinations. P < .001 comparing the GTP binding after GM-CSF stimulation versus basal control value. P < .01 comparing the GTP binding after stimulation with GM-CSF alone versus stimulation with GM-CSF in the presence CT. P not significant comparing the GTP binding after stimulation with GM-CSF alone versus stimulation with GM-CSF in the presence PT.

2Rα, IL-2Rβ, and IL-2Rγ. These subunits bind each other to form the high-affinity IL-2R. Addition of GM-CSF to T lymphocytes resulted in increased expression of IL-2Rβ 1 day after the culture is started, with a maximum expression 2 days after culture. GM-CSF to a lesser extent also increased the expression of IL-2Rα 3 days after culture. The latter coincides with reduced expression of IL-2Rβ. Whether GM-CSF regulates this pattern of cross-expression or whether there is a cross-talk between IL-2R subunits remains to be seen.

To further understand the ability of GM-CSF to enhance IL-2–driven T-cell proliferation, we investigated the intracellular signaling pathways. It has been shown that GM-CSF induces the synthesis of cGMP, but not cAMP, in human neutrophils. Surprisingly, GM-CSF induced the synthesis of not only cGMP but also cAMP in human T lymphocytes (Table 3). IL-2 alone induced the synthesis of intracellular cGMP in T cells. However, addition of GM-CSF reduced this effect. The same combination (GM-CSF and IL-2) also resulted in reduced expression of IL-2Rβ on T lymphocytes (Table 2). Although it is conjectural at the present time, it is possible that upregulation of IL-2R expression may be a consequence of increased synthesis of intracellular cGMP (and perhaps cAMP) after GM-CSF incubation with T lymphocytes. Confirming this proposal is our observation showing that although GM-CSF augments IL-2–induced T-cell proliferation when both factors were added at the start of the culture, addition of GM-CSF 1 or 2 days before the addition of IL-2 resulted in greater synergy. During this period, cyclic nucleotides may induce the expression of IL-2 receptors, allowing T cells to respond to IL-2. In this regard, Vitte-Mony et al. observed that cAMP upregulates the surface ex-

Fig. 5. Effect of the combination of IL-2 and GM-CSF on GTP binding. T-cell membranes were incubated with buffer (basal binding), 10 U/mL IL-2, 1 ng/mL GM-CSF, 10 U/mL IL-2 plus 1 ng/mL GM-CSF with or without 1 μg/mL CT. The membranes were incubated for 15 minutes at 37°C in the presence of [γ-35S]GTP. Results are shown as mean ± SD of triplicate determinations.
pression of IL-2R in normal human natural killer cell clones.

The secretion of endogenous cAMP is linked to the activation of G protein, known as G stimulatory (Gs). Activation of G proteins results in enhanced binding of GTP to the cell membranes and increased GTPase activity. Various cytokines such as tumor necrosis factor (TNF), TGF-\(\beta\), IL-2, or IL-8 stimulate GTP binding and GTPase activity in various cell types. Our results showed that GM-CSF may belong to this family of cytokines because it enhances GTP binding and GTPase activity in human T-cell membranes. Both of these activities were inhibited by CT but not by PT, showing that GM-CSF activates G protein in human T-cell membranes which is CT-sensitive, but not PT-sensitive.

In addition, IL-2 stimulated both GTP binding and GTPase activity, confirming previous findings. However, addition of IL-2 plus GM-CSF to T-cell membranes resulted in lower GTP binding and GTPase activity than those obtained in the presence of GM-CSF alone. Furthermore, CT inhibited GTP binding and GTPase activity when added to T-cell membranes incubated with GM-CSF plus IL-2. The levels of these responses were similar to those obtained in the presence of IL-2, but were significantly lower than those obtained in the presence of GM-CSF. Cumulatively, these results suggest that IL-2 and GM-CSF receptors may be coupled to the same G protein, which is a CT-sensitive. Thus, when both factors are added together, IL-2 that appears to have the upperhand activates this G protein, uncouples it from the receptors, and consequently prevents GM-CSF
GM-CSF OR IL-2 ACTIVATES G PROTEIN IN T CELLS

Fig 8. Effect of the combination of IL-2 and GM-CSF on GTPase activity. T-cell membranes were either incubated with buffer (basal GTPase activity), 10 U/mL IL-2, 1 ng/mL GM-CSF, or their combination with or without 1 μg/mL CT in the presence of (32P) GTP for 15 minutes at 37°C. Results are shown as mean ± SD of triplicate determinations.

from using the G-protein pathway. This may explain why T cells showed lower expression of IL-2R, and lower secretion of cyclic nucleotides after incubation with both cytokines. Although addition of both factors at the start of the culture resulted in GM-CSF augmentation of IL-2-induced T-cell proliferation, addition of GM-CSF before the addition of IL-2 resulted in higher responses. However, the ability of GM-CSF to augment IL-2-induced T-cell proliferation when both cytokines were added at the onset of the cultures (and were left until the termination of these cultures 7 days later) is probably caused by the dissociation, and re-association of the various subunits of G protein during this time, that makes it possible for GM-CSF to activate this protein.

If the hypothesis that IL-2 and GM-CSF receptors are coupled to the same G protein is correct, and that IL-2 and GM-CSF compete for the same G protein, then blocking IL-2R should allow GM-CSF to activate G proteins even in the presence of IL-2. Indeed, addition of antibodies to the IL-2R resulted in the activation of G protein by GM-CSF in the presence of IL-2. In addition, GM-CSF–induced or IL-2–induced GTP binding and GTPase activity were inhibited by CT, and when this toxin was used to intoxicate T cells, it inhibited the transfer of ADP-ribose into T-cell membranes. Because PT was unable to inhibit GM-CSF–induced GTP binding and GTPase activity in T-cell membranes, our results indicate that a CT-sensitive but not a PT-sensitive G protein is coupled to GM-CSF receptors. IL-2R are also coupled to the same G protein. This is not surprising in light of previous findings showing that various growth factor receptors can be coupled to the same G protein. However, this is the first report which suggests that IL-2 and GM-CSF receptors may be coupled to the same CT-sensitive, PT-insensitive G protein.

In summary, our results showed that in vitro proliferation of human T cells induced by the administration of GM-CSF and IL-2 is maximal when GM-CSF is added to the culture 1 or 2 days before IL-2. It appears that GM-CSF receptors are associated with a CT (but not a PT) sensitive G protein.

Fig 9. Bacterial toxin-dependent ADP-ribosylation of G proteins in T-cell membranes. (Left) CT-catalyzed ADP-ribosylation of G protein. Lane 1, membranes were incubated in the presence of 32P-NAD only (no toxin). Lane 2, membranes were incubated in the presence of activated CT and 32P-NAD. Lanes 3 and 4, membranes were prepared from T cells intoxicated for 2 hours with 10 ng/mL or 100 ng/mL CT, respectively. The latter were challenged with freshly activated CT and 32P-NAD. (Right) PT-catalyzed ADP-ribosylation of G protein. The lanes are similar to those described in the left panel, except that PT was used instead of CT. The arrows indicate the G proteins ADP-ribosylated by the toxins (kD 44 in case of CT, and kD 39 in case of PT). The numbers to the left represent the approximate molecular weight of marker proteins.
Binding of GM-CSF to its receptors results in increased secretion of intracellular cyclic nucleotides, and in increased expression of IL-2R, which in turn is activated when bound by IL-2. In the presence of GM-CSF–primed cells, even low concentration of IL-2 causes T cells to proliferate. The ability of GM-CSF to activate G protein is an additional mechanism by which this cytokine transduces various intracellular signaling pathways. Other studies have shown that GM-CSF induces the phosphorylation of various proto-oncogene products.39,41

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