Release of Interleukin-1 and Interleukin-6 From Human Monocytes by Antithymocyte Globulin: Requirement for De Novo Synthesis

By Pranela Rameshwar and Pedro Gascón

Antithymocyte globulin (ATG) is an effective treatment in patients with severe aplastic anemia (SAA). Its mechanism of action remains unclear, although it has been assumed to be immunosuppressive. However, ATG has also been shown by several laboratories to be immunostimulatory. Recently, interleukin-1 (IL-1) production has been found to be decreased in lipopolysaccharide-stimulated peripheral blood monocytes obtained from SAA patients. We have investigated the ability of ATG to function as an immunostimulatory agent via the production of IL-1 and IL-6 by normal human monocytes in vitro. Supernatants from ATG-stimulated monocytes were assayed for biologically active and immunoreactive IL-1 and IL-6. We have found that ATG, via its F(ab')2 fragment, is a powerful inducer of IL-1 and IL-6 production. Furthermore, ATG induction of both cytokines from normal monocytes required de novo synthesis, as determined by 35S-methionine incorporation. Because these two cytokines synergize with other cytokines at both the stem cell and progenitor levels, these stimulatory properties of ATG may be relevant to the treatment of SAA. This would favor the hypothesis of a bimodal mechanism for ATG as an inducer of hematopoietic growth factors and as an immunosuppressive agent.

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(PBS; Cellgro; Mediatech) supplemented with 2% FCS (deadherent medium). Adherent cells were subsequently removed by flushing the dishes with cold deadherent medium. Greater than 95% of the adherent cells (monocytes) were positive for non-specific esterase and expressed the CD14 antigen, as determined by FACS analysis.

**Stimulation of monocytes.** Normal human monocytes were plated in 24-well tissue culture dishes (Falcon 3047) at 1 × 10⁶ cells/mL in serum-free RPMI 1640 medium. Cells were stimulated for 8 hours with nonimmune horse IgG (10 μg; nonimmune IgG; US Biochemical Co, Cleveland, OH), ATG (10 μg; gift of Upjohn, Kalamazoo, MI), lipo polysaccharide (LPS), Salmo nella typhosa (2 μg; Sigma), polymyxin B, and nonimmune IgG, polymyxin B and ATG, or polymyxin B and LPS. Similar concentrations of monocytes incubated with medium alone were used as controls. After incubation, supernatants (SNs) were cleared via centrifugation (4,000g for 5 minutes), aliquoted, and stored at −22°C until ready to be assayed.

**Cytokine level estimation.** IL-1 and IL-6 activities were determined in biologic assays and by the enzyme-linked immunosorbent assay (ELISA).

IL-1 activity in the SNs was determined based on the ability to support the growth of concanavalin A (Con A; Sigma)-stimulated mouse helper T-cell clone, D10.G4.1. Maintenance of the D10.G4.1 cell line has been previously described. Briefly, cells were cultured in the presence of Con A-stimulated rat spleen cells SN and then expanded via activation by egg white conalbumin (US Biochemical) in the context of 𝜀-irradiated I-A¹ (AKR/J spleen) feeder cells. Assays were performed at least 8 days after stimulation (quiescent cells). SNs (0.1 mL) in triplicate were serially diluted in Click's medium (Irvine Scientific, Santa Ana, CA) containing 5% FCS and 2.5 μg/mL Con A (assay medium) in microtiter wells (Falcon 3072). For each assay, a standard curve was established using serial dilutions of rhIL-1α or rhIL-1β at 500 U/mL Quiescent cells (2 × 10⁶), resuspended in 0.1 mL assay medium, were added to each well. After 65 hours (according to ATCC recommendations), wells were pulsed for 4 hours with 1 μCi tritiated thymidine ([3H]TdR, 2 Ci/mmol; New England Nuclear, Boston, MA). Cells were harvested onto glass fiber filters with a PHD cell harvester. For all bioassays, 1 cytokine unit was equivalent to one half-maximal growth of the cells as determined by [3H]TdR incorporation in the standard curves (quadruplicate cytokines were kindly provided by Dr Craig W. Reynolds, National Cancer Institute, National Institutes of Health, Bethesda, MD). The presence of biologically active cytokines was quantitated by a modification of the sandwich ELISA as previously described. Capture and second antibodies were added at 4 μg/mL in Tris buffer (pH 8.9), and PBS containing 3% bovine serum albumin (BSA), respectively. Binding of the second antibody was detected by incubating with a specific biotinylated tagged antibody amplified with Avidin D (20 μg/mL; Vector), followed by incubation with biotinylated conjugated alkaline phosphatase (0.2 U/mL; Vector). Bound enzyme was detected with Sigma 104 phosphatase substrate, which was dissolved in 0.5 mmol/L diethanolamine buffer containing 0.5 mmol/L magnesium chloride, and 0.02% sodium azide (all from Sigma). Color development was measured at 405 nm on a Mdl 450 microplate reader (BioRad, Richmond, CA).

**Western blots.** Protease inhibitors phenylmethylsulfonyl fluoride (PMSF; 1 mmol/L), leupeptin (5 μmol/L), chymostatin (1 μg/mL), and (3-[3-Cholamidopropyl]-dimethylammonio)-2-hydroxy-1-propanesulfonate (0.1 mmol/L) (all obtained from Sigma) were added to SNs, which were then concentrated 10× with a Centricon 10 microconcentrator (Amicon, Danvers, MA). Immunoblotting was performed at room temperature using a modification of the method described by Towbin et al. Briefly, proteins were separated on 16% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred to Immobilon PVDF transfer membrane (Millipore, Bedford, MA) for 45 minutes at a constant current of 400 mA and 50 V. Protein-bound membranes were incubated with PBS containing 2% BSA (US Biochemical) for 2 hours and then incubated overnight with the following sequence of primary antibodies: 100 neutralizing units (NU) rabbit anti–hIL-1α or anti–hIL-1β or 10 μg goat anti–hIL-6. Sixteen to 24 hours later, excess antibodies were washed three times (15 minutes each) with 2% BSA in PBS. IL-1- and IL-6-complexed membranes were incubated for 1 hour with horseradish peroxidase-conjugated goat antirabbit IgG (BioRad) and alkaline phosphate-conjugated swine antigoat IgG (Boehringer-Mannheim, Indianapolis, IN), respectively. Color was developed with the appropriate substrate. For all gels, the molecular weights (MWs) of the developed bands were compared with pre-stained low MW standards (Diversified Biotech, Newton Centre, MA).

**De novo synthesis.** Monocytes (1 × 10⁶/mL) were resuspended in methionine-free RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 1 μCi/μL [35S]methionine (1,000 Ci/mmol, ICN, Irvine, CA) and 1 × serum supplement (GMSA; GIBCO). Cells were stimulated for 16 hours with nonimmune IgG or ATG, or cultured in medium alone. SNs were cleared by centrifugation (4,000g for 5 minutes) and the following protease inhibitors were immediately added: PMSF (1 mmol/L), leupeptin (5 μmol/L), chymostatin (1 μg/mL), and (3-[3-Cholamidopropyl]-dimethylammonio)-2-hydroxy-1-propanesulfonate (0.1 mmol/L). SNs were incubated for 5 hours at 4°C with rabbit anti–hIL-1α or anti–hIL-1β or goat anti–hIL-6. Immune-complexed IL-6 was precipitated with protein A Sepharose CL-4B (Pharmacia) as described. SNs containing both IL-1α and IL-1β immune complexes were incubated at 4°C overnight with magnetic beads, polystyrene microspheres coated with sheep antirabbit IgG (Dynabeads M-200; Dynal Inc, Great Neck, NY). IL-1α and IL-1β complexed to Dynabeads were selected with a magnetic particle concentrator (Dynal). Magnetically separated particles were washed three times with PBS containing 2% BSA (separation medium) and incubated overnight in 0.2 mL separation medium at 37°C to detach both IL-1α and IL-1β. After incubation, the detached IL-1α and IL-1β were collected in the liquid phase by magnetic separation. Purified IL-6, IL-1α, and IL-1β were separated on 16% SDS-PAGE. Gels were dried and then developed by autoradiography. The MWs of developed bands were compared with prestained low MW standards.

**Preparation of F(ab')2 and Fe fragments.** Both nonimmune IgG and ATG were split into their F(ab')2 and Fe fragments by pepsin (Worthington Biochemical, Freehold, NJ) digestion. We used a modification of the method previously described by Kurkela et al. The F(ab')2 fragments were prepared by overnight (pre-determined) digestion of IgG. After this period, most of the Fe portions of the molecules were degraded. The remainder Fe and intact IgG molecules were separated from the F(ab')2 fragments by binding to a protein G-Sepharose (Sigma) column. The F(ab')2 fragments were collected in the effluent and concentrated with a 30-Kd MW...
were prepared by digesting with pepsin for 2 hours. After this period, the percentage of intact molecules was far greater than the amount of Fc molecules. The F(ab')2 fragments were separated from the Fc and IgG molecules by binding to a protein G-Sepharose column. Both Fc and intact IgG molecules were eluted with 0.1 mol/L glycine buffer (pH 2.7). The final protein concentration of the F(ab')2 solution (0.18 mg/mL) was determined by the method described by Bradford using BSA as standard (the cut-off Amicon ultrafiltration cell. The final protein concentration of the Fc solution (0.1 mg/mL) was determined by the method described by Bradford.30

Fc fragments were then separated from the IgG molecules by size-exclusion gel chromatography on a Sephadex G25 (Pharmacia) column. Fc molecules were not degraded as determined by gel electrophoresis on 12.5% SDS-PAGE under nonreducing conditions. The fraction that contained the Fc fragments was concentrated with a 10-Kd MW cut-off Amicon ultrafiltration cell. The final protein concentration of the Fc solution (0.1 mg/mL) was determined by the method described by Bradford.30

RESULTS

Cytokine release by human PB monocytes. In eight separate experiments, two different lots of ATG (844JX and 313BH) were used to stimulate normal human monocytes. The amounts of biologically active IL-1 and IL-6 released were 27-fold and 100-fold, respectively, above the control levels ($P < .05$) (Table 1B). No significant variation among the two lots was noted because the variation observed with a particular lot was always within the standard deviation of the other lot (data not shown). The D10.G4.1 cells used to assay for IL-1 have been previously reported to respond to IL-2.31 Therefore, all SNs with IL-1 levels greater than 20 U/mL were tested for the presence of IL-2 in a proliferative assay with CTLL-2, a clone that has been previously shown to respond only to human IL-2.22 None of the SNs tested stimulated the CTLL-2 cells above background levels.

Cytokine estimations were also performed with an ELISA. SNs from ATG-stimulated (n = 6) and nonimmune horse IgG-stimulated (n = 3) monocytes were tested for IL-1α, IL-1β, and IL-6 (Table 1A). The amounts of each cytokine from ATG-stimulated monocytes were 10- to 15-fold above control (monocytes cultured with medium alone), whereas the levels induced by nonimmune horse IgG were comparable to control levels.

ATG directly stimulates the release of IL-1 and IL-6. Because IL-1 had been reported to stimulate the release of IL-6,33,34 we investigated whether IL-6 was being released directly by ATG or indirectly via IL-1. Monocytes were either cultured in medium or stimulated with ATG, nonimmune IgG, or ATG and NU anti-hIL-1α and anti-hIL-1β. The presence of NU IL-1α and NU IL-1β reduced the free IL-1 to control levels, whereas the IL-6 levels were the same as those of cultures with monocytes stimulated with ATG alone (Table 1). In parallel experiments, monocytes cultured in the presence of equivalent concentrations of anti-IL-1α and -IL-1β resulted in IL-1 levels similar to that obtained in controls (Table 1). Monocytes cultured in the presence of ATG and NU anti-hIL-6 did not affect the amount of IL-1 released. In the presence of NU anti-IL-6, the amount of free IL-6 in the supernatants was reduced by 96%, whereas the amount remaining was threefold above control levels (Table 1). Because we have found that ATG-stimulated monocytes also release GM-CSF (manuscript in preparation), we cultured monocytes in the presence of ATG, NU anti-hGM-CSF, and NU anti-hIL-6. The addition of NU anti-hGM-CSF in the cultures reduced the B9.9 proliferative units to control levels (data not shown).

Release of IL-1 and IL-6 in the presence of ATG and polymyxin B. Endotoxins stimulate human monocytes to synthesize and release both IL-1β and IL-6.36-38 The amount of endotoxin in the ATG preparation (< 2 U/150 mg IgG, determined by the Limulus amebocyte lysate test, Dr Greg Davis [Upjohn], personal communication) was insignificant to our model. Nevertheless, we decided to investigate whether the IL-1 and IL-6 being released were due to contaminating endotoxin. Because polymyxin B binds endotoxin,39 we stimulated monocytes with ATG or nonimmune IgG in the presence or absence of a nontoxic concentration of polymyxin B (Fig 1). The concentration of polymyxin B that was capable of blocking the effects of the optimum concentration of LPS and at the same time maintaining all cells viable was determined to be the nontoxic concentration. In the presence of polymyxin B and LPS, both IL-1 and IL-6 values were reduced to control levels (Fig 1). However, the amount of both IL-1 and IL-6 remained the same with cells stimulated with ATG both in the presence and absence of polymyxin B (Fig 1).

Stimulation of monocytes with F(ab')2 and Fc fragments. Nonimmune IgG and ATG were split into their F(ab')2 and Fc fragments to determine which segment of the molecule was responsible for the stimulatory activity. Monocytes were stimulated with either F(ab')2 or Fc fragments derived from ATG-stimulated monocytes were 10- to 15-fold above control (monocytes cultured with medium alone), whereas the levels induced by nonimmune horse IgG were comparable to control levels.

### Table 1. IL-1 and IL-6 Production by ATG-Stimulated Human Monocytes

<table>
<thead>
<tr>
<th>A. IL-1α</th>
<th>IL-1β</th>
<th>IL-6</th>
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<tbody>
<tr>
<td>Medium (n = 3)</td>
<td>8 ± 3</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>Horse IgG (nonimmune) (n = 3)</td>
<td>15 ± 5</td>
<td>22 ± 3</td>
</tr>
<tr>
<td>ATG (n = 6)</td>
<td>122 ± 14</td>
<td>205 ± 20</td>
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</tbody>
</table>

| B. Biologic Assay (units, mean ± SE) |
|---|---|---|
| IL-1 | IL-6 |
| Medium (n = 8) | 15 ± 4 | 9 ± 1 |
| Horse IgG (nonimmune) (n = 8) | 18 ± 2 | 13 ± 2.5 |
| ATG (n = 8) | 400 ± 30* | 1,380 ± 170* |
| ATG + NU IL-1α + β (n = 5) | 10 ± 2 | 1,436 ± 19 |
| ATG + NU IL-6 (n = 5) | 426 ± 23 | 59 ± 4 |
| NU IL-1α + β (n = 5) | 6 ± 3 | 20 ± 2 |
| NU IL-6 (n = 5) | 8 ± 1 | 17 ± 1 |

*P < .05.
from nonimmune IgG or ATG. Fc cocultured with monocytes did not produce either IL-1 or IL-6 above control levels, whereas the F(\(\text{ab}^\prime\))\(_2\) induced the release of a similar quantity of both interleukins as compared with cells stimulated with the same concentration of the intact ATG (Fig 2).

**Confirmation of biologically active cytokines.** Because most cell lines used in biologic assays have been shown to respond to various cytokines, the presence of IL-1\(\alpha\), IL-1\(\beta\), or IL-6 in the SNs of ATG-stimulated monocytes was confirmed immunologically via Western blots with specific antibodies. Lanes with ATG-stimulated SNs resulted in a band at 17 Kd for membranes blotted with anti–hIL-1\(\alpha\) and anti–hIL-1\(\beta\) (Fig 3A and B, respectively) and 22 Kd for anti–hIL-6–blotted membranes (Fig 3C). Lanes with cells cultured with medium or stimulated with nonimmune IgG showed no detectable bands in the case of immunoblots of IL-1\(\alpha\) and IL-1\(\beta\), and a low level of immunoprecipitates was observed for IL-6. Controls include lanes with purified IL-1\(\alpha\), IL-1\(\beta\), and IL-6 (Fig 3A, B, and C, respectively).

**De novo synthesis.** Transcription of the IL-6 gene has been shown to be initiated by adherence to plastic.\(^{37}\) Because the technique used to separate monocytes involves prolonged adherence to plastic coated with gelatin incubated with autologous plasma, we investigated whether there was a requirement for de novo synthesis for IL-1\(\alpha\), IL-1\(\beta\), or IL-6 in the SNs obtained from cells stimulated with either ATG or nonimmune IgG or cells cultured with medium alone. In all experiments, culture medium included \(^{35}\)S-methionine–supplemented methionine-free medium. SNs obtained from cultures stimulated with ATG and immunoprecipitated with either anti–hIL-1\(\alpha\) or anti–hIL-1\(\beta\) resulted in a band at 17 Kd after autoradiography (Fig 4A), whereas a band at 22 Kd was seen with anti–hIL-
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IL-1α

IL-1β

IL-6

6-precipitated SNs (Fig 4B). Neither IL-1 nor IL-6 was detected with SNs obtained from monocytes cultured with medium or stimulated with nonimmune IgG (Fig 4A and B).

DISCUSSION

The mechanism of action of ATG is thought to be immunosuppressive. This is in part due to its in vivo cytotoxic effect on lymphocytes during ATG therapy, particularly the elimination of an activated lymphocyte population. Hematologic responses seen with high-dose methylprednisolone and more recently with cyclosporine A, two known immunosuppressive agents, suggest that ATG may function primarily, in SAA patients, as an immunosuppressive agent. However, in vitro studies from different laboratories have also placed ATG as an immunostimulatory agent. Our results show that ATG induces the release of both IL-1 and IL-6 by highly purified normal human monocytes (Table 1). This response was not due to the presence of endotoxin contamination because the presence of polymyxin B in coculture with ATG did not alter the values of IL-1 and IL-6 (Fig 1). The inability of polymyxin B to alter the results of ATG cannot be attributed to insufficient quantity because the same concentration of polymyxin B was able to block the effects of the optimum concentration of LPS (Fig 1).

The stimulatory action of ATG on human monocytes was mediated by the F(αβ)2 segment of the molecule and not by the Fc component (Fig 2). This is further supported by the inability of syngeneic nonimmune IgG to stimulate monocytes to release IL-1 and IL-6 (Table 1). In addition, these biologic effects observed were not due to thimerosal, the preservative present in the ATG preparations, because affinity-purified ATG (protein G sepharose) shows the same activity as the intact preparation (results not shown).

Neutralizing units of IL-1 in coculture with ATG did not alter the values of IL-6, implying that the release of IL-6 by ATG was not due to autostimulation by the released IL-1. Also, the release of both IL-1 and IL-6 was not due to nonspecific stimulation of monocytes by opsonization because equivalent Ig concentrations of anti-IL-6, anti-IL-1α, and anti-IL-1β incubated with monocytes did not stimulate the release of IL-6 or IL-1 (Table 1). The presence of NU anti-IL-6 in the ATG-stimulated cultures did not reduce the B9.9 proliferation to background unless anti-GM-CSF was added. This may be due to the ability of the B9.9 cells to respond to GM-CSF present in the cultures, even though others have shown that the B9.9 cells do not respond to GM-CSF, a cytokine released by ATG-stimulated monocytes (manuscript in preparation). It is also possible that GM-CSF present in the SNs may be inducing the release of another growth factor capable of inducing the B9.9 cells to proliferate.

Finally, our results indicate that ATG-induced production of IL-1 and most of the IL-6 requires de novo synthesis. In the latter case, faint bands are detected in the control lanes of the Western blots (Fig 3). The presence of these faint bands may be explained by amplification of back-
ground cytokine levels due to concentration of SNs (×12). Therefore, it seems that the majority of the induced cytokines are due to ATG stimulation and not a consequence of monocyte adhesion to plasma-gelatin–coated plastic. However, the possibility of nonspecific transcription of both the IL-1 and IL-6 genes cannot be excluded because neither the presence of the respective messenger RNA nor the requirement for ATG-mediated transcription were studied. Therefore, at this time, we cannot conclude whether ATG is regulating the release of IL-1 and IL-6 at the transcriptional or translational levels.

Several laboratories have recently reported a decreased production of IL-1 by monocytes from patients with SAA. In a recent study, stromal macrophages from mice exposed to benzene (BZ), a hematotoxin known to cause aplastic anaemia, were unable to process pre–IL-1 to the mature biologically active cytokine. Furthermore, administration of rIL-1 to mice before BZ completely prevented BZ-induced myelotoxicity. These results strongly suggest that decreased HGFs such as IL-1 may be either a cause or a coexistent cause for some cases of SAA. ATG may induce the production of IL-1 in these patients by either stimulating SAA monocytes directly or via other IL-1–producing cells, because it is known that ATG binds to multiple types of cells. ATG, by stimulating the defective monocytes of SAA, may partially restore a possible growth factor imbal-
ance either directly by inducing the release of HGFs by the same monocytes or indirectly by the action of IL-1 and IL-6 on BM accessory cells. A possible indirect mechanism may be the initiation of a cascade effect by the released IL-1 and IL-6 in which other HGFs can be released to further stimulate residual hematopoiesis. In fact, recent studies have shown that both ATG and ALG can induce the release of GM-CSF and IL-3 by PBMCs and T cells,\textsuperscript{12}\textsuperscript{-14} growth factors that may be partially released by IL-1.\textsuperscript{48} Recent clinical trials with HGFs (rhG-CSF,\textsuperscript{49} rhGM-CSF,\textsuperscript{50} and IL-3\textsuperscript{31}) in patients with SAA have been shown to induce transient hematopoietic responses.\textsuperscript{49\textsuperscript{-}51} The return to baseline after discontinuation of the growth factor treatment suggests that hematopoietic suppression can be overridden by stimulatory factors. It is also possible that the transient BM recovery observed with this type of treatment may be due to the replacement of a particular HGF deficient in some SAA patients. The observed production of IL-1 and IL-6 by ATG-stimulated human monocytes may be relevant to the treatment of SAA and further supports the role for ATG in stimulating hematopoiesis.

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Release of interleukin-1 and interleukin-6 from human monocytes by antithymocyte globulin: requirement for de novo synthesis

P Rameshwar and P Gascon