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

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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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MATERIALS AND METHODS

Cell lines. D10.G4.1 and CTLL-2, IL-1- and IL-2-dependent cell lines, respectively, were purchased from the American Type Culture Collection (ATCC; Rockville, MD). The B9.9 murine hybridoma,27 a human IL-6-dependent cell line, was kindly provided by Dr R.F. Nordan (National Institutes of Health, Bethesda, MD).

Cytokines and antibodies. Human recombinant IL-1α (rhIL-1α) and anti–IL-1α, and anti–IL-1β were purchased from Endogen (Boston, MA). Monoclonal murine anti–IL-1α and anti–IL-1β were purchased from Collaborative Biomedical (Bedford, MA). rhIL-6 and murine anti–hGM-CSF were generously provided by Genetics Institute (Cambridge, MA). Goat polyclonal anti–hIL-6 and murine monoclonal anti–hIL-6 were purchased from R&D (Minneapolis, MN) and Collaborative Biomedical, respectively. Biotinylated antimonos IgG was purchased from Vector Laboratories (Burlingame, CA). Anti-Mo2 (CD14) was obtained from ascites derived from the hybridoma, HB-44 (ATCC), grown in mice. Affinity-purified ascites was prepared by binding to a protein G-Sepharose column. The optimum working concentration of the purified antibody was determined by flow cytometry analysis with purified human monocytes (1/200 final dilution of the antibody at 25 mg/mL).

Cell separation. PB was collected from normal subjects after informed consent. Adherent cells were separated using a modification of the method described by Freundlich and Avdalovic.23 Briefly, BMMNCs and plasma were separated by Ficoll8 (Pharmacia, Piscataway, NJ) density gradient centrifugation. Autologous platelet-free plasma was prepared by high-speed centrifugation (1,000g for 15 minutes) and then incubated on endotoxin-free, tissue culture grade gelatin-coated (Sigma) petri dishes (Falcon 3003) for 1 hour. After the period of incubation, plasma was gently washed with warm (room temperature) RPMI 1640 (Cellgro; Mediatech, Herndon, VA) supplemented with 2% fetal calf serum (FCS; HyClone, Logan, UT) (wash medium). BMMNCs (1 × 107/culture dish) were resuspended in calcium and magnesium (Ca2+/Mg2+)-containing RPMI 1640 supplemented with 10% FCS and then incubated for about 1 to 2 hours. Nonadherent cells were removed with warm wash medium and the remaining adherent cells were incubated for 1 hour at cold temperature (4°C) with Ca2+/Mg2+-free tissue culture grade phosphate-buffered saline.
normal human monocytes were plated in 24-well tissue culture dishes (Falcon 3047) at 1 x 10^6 cells/mL in serum-free RPMI 1640 medium. Cells were stimulated for 8 hours with nonimmune horse IgG (10 μg/mL), nonimmune IgG; US Biochemical Co, Cleveland, OH), ATG (10 μg/mL of Upjohn, Kalamazoo, MI), lipoxygenase (LPS), Salmonella typhosa (2 μg/mL; Sigma), polymyxin B (12.5 μg/mL; 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 feeder cells supported the growth of concanavalin A (Con A; Sigma)-stimulated monocytes incubated with medium alone. SNs were tested for the presence of IL-2 in a proliferative assay with the CTLL-2 cell line as described.25 IL-6 activities were determined based on the proliferative response of the B9.9 cells. SNs (0.1 mL) in triplicate were serially diluted in RPMI 1640 (4,00$ for 5 minutes) and then expanded via activation by egg white conalbumin (US Biochemical, Indianapolis, IN) for 45 minutes at a constant current of 400 mA and 50 V. Protein-bound membranes were incubated with PBS containing 2% BSA (US Biological) for 2 hours and then incubated overnight with the following quantity 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 phosphatase-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 x 10^6/mL) were resuspended in methionine-free RPMI 1640 (GIBCO, Grand Island, NY) supplemented with 1 μCi/mL [35S]methionine (1,000 Ci/mmol, ICN, Irvine, CA) and 1 x serum supplement (GMS-A; 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 then tested for the presence of IL-2 in a proliferative assay with the CTLL-2 cell line as described.25 SNs containing both IL-1α and IL-1β immune complexes were incubated at 4°C overnight with magnetizable, polystyrene microparticles coated with sheep antirabbit IgG (Dynabeads M-280; Dynal Inc, Great Neck, NY). IL-1α and IL-1β complexes21 were incubated 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. Gels were dried and then incubated with autoradiography. The MWs of developed bands were compared with prestained low MW standards.

Preparation of F(ab')2 and Fc fragments. Both nonimmune IgG and ATG were split into their F(ab')2 and Fc fragments by pepsin (Worthington Biochemical, Freehold, NJ) digestion. We used a modification of the method previously described by Kurke et al.22 After digestion, the F(ab')2 fragments were purified by protein A-Sepharose (Sigma) chromatography. The F(ab')2 fragments were collected in the eluent and concentrated with a 30-Kd MW.
cut-off Amicon ultrafiltration cell. 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 standard and dye solution were both obtained from BioRad). Purity of the F(ab')2 fragments was confirmed by SDS-PAGE under both reducing and nonreducing conditions. Fc fragments 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 the two latter molecules 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 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.

Statistical analysis. Data were analyzed using the Student's t-test to determine the significance (P value) or differences between experimental and control SNs.

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. 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. 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, 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 those 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-135 and IL-636-38. 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, 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

| Table 1. IL-1 and IL-6 Production by ATG-Stimulated Human Monocytes |
|-----------------|------------------|------------------|
|                 | IL-1α | IL-1β | IL-6 |
| A.               |       |       |      |
| Medium (n = 3)   | 8 ± 3 | 13 ± 3 | 18 ± 3 |
| Horse IgG (nonimmune) (n = 3) | 15 ± 5 | 22 ± 3 | 18 ± 3 |
| ATG (n = 6)      | 122 ± 14 | 205 ± 20 | 240 ± 31 |
| B.               |       |       |      |
| Biologic Assay (units, mean ± SE) |       |       |      |
| IL-1             |       |       |      |
| 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  |       |

Release of IL-1 and IL-6 from human PB monocytes. Monocytes were cultured in serum-free medium alone or stimulated with nonimmune IgG, ATG, ATG and anti–hIL-1α and β, ATG and anti–hIL-6, anti–hIL-1α and β, SNs were tested for IL-1 and IL-6 as described in Materials and Methods. (A) cytokine concentration (ng/mL); (B) biologic activity (units/mL).

*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(ab')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-1α, IL-1β, 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-1α and anti-hIL-1β (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-1α and IL-1β, and a low level of immunoprecipitates was observed for IL-6. Controls include lanes with purified IL-1α, IL-1β, 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. 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-1α, IL-1β, 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 ³⁵S-methionine–supplemented methionine-free medium. SNs obtained from cultures stimulated with ATG and immunoprecipitated with either anti-hIL-1α or anti-hIL-1β 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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**Fig 1.** ATG-induced production of IL-1 and IL-6 by human monocytes is not due to endotoxin contamination. Monocytes were incubated in serum-free medium alone, or stimulated with LPS, LPS and polymyxin B, or ATG in the presence or absence of polymyxin B. Supernatants were tested for both IL-1 and IL-6 as described in Materials and Methods.

**Fig 2.** Effect of the F(ab')₂ and Fc fragments of the ATG molecule on human monocytes. Monocytes were stimulated with the intact nonimmune IgG, ATG, or a similar concentration of F(ab')₂ or Fc fragments of both nonimmune IgG or ATG in serum-free medium. Supernatants were tested for both IL-1 and IL-6 as described in Materials and Methods.
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Fig 3. Western blot analysis of supernatants from monocytes stimulated with ATG or nonimmune IgG. Supernatants were separated on 16% SDS-PAGE (see Materials and Methods). Lane 1, ATG; lane 2, nonimmune IgG; lane 3, medium; lane 4, standard cytokines: 20 ng rHL-1α (A), 5 ng rHL-1β (B), 10 ng rHL-6 (C); lane 5, 5 μL prestoned low MW standard.

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(ab′)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-
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, growth factors that may be partially released by IL-1. Recent clinical trials with HGFs (rhG-CSF, rhGM-CSF, and IL-3) in patients with SAA have been shown to induce transient hematopoietic responses. 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

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