Evidence That Interleukin-6 Does Not Play a Role in the Stimulation of Platelet Production After Induction of Acute Thrombocytopenia

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The induction of thrombocytopenia results in elevated levels of thrombopoietin (TPO), which can be detected in the plasma of experimental animals. Acute, severe thrombocytopenia (platelet count <5% of control) was produced in mice by the administration of either guinea pig or rabbit antimouse platelet antisera. Control mice received equal volumes of normal serum. At various times after the induction of thrombocytopenia (0.5, 1, 2, 3, 4, 6, 12, and 24 hours) citrated plasma was collected, and circulating interleukin-6 (IL-6) levels were measured using the IL-6–dependent murine hybridoma cell line B9. At no time points after induction of thrombocytopenia were plasma IL-6 levels significantly different from control animals that received normal serum. However, injection of heterologous serum did result in slightly elevated plasma IL-6 levels (at 2 and 3 hours) compared with basal levels measured in un.injected animals. This brief increase was not related to the production of thrombocytopenia. Protein fractions from the plasma of thrombocytopenic rabbits were also tested for the presence of IL-6. Preparations that contained TPO, as shown by stimulation of megakaryocyte maturation in vitro, did not contain detectable levels of IL-6. The ability of the B9 assay to detect the elevation of IL-6 levels in murine or rabbit plasma was verified after the administration of bacterial endotoxin, which is known to increase circulating IL-6 concentrations. IL-6 levels were highly elevated in rabbit or mouse serum after the administration of 5 mg/kg or 1 mg/kg of endotoxin, respectively. Anti–IL-6 antisera did not neutralize the in vitro megakaryocyte maturation activity of partially purified TPO from the plasma of thrombocytopenic rabbits. In addition, IgG purified from the same antisera did not neutralize partially purified TPO, as shown after incubation with TPO and subsequent precipitation with agarose-bound protein A. These results show that, unlike TPO, levels of IL-6 do not increase after the induction of acute, severe thrombocytopenia, and strongly suggest that IL-6 does not mediate the thrombopoietic response to acute thrombocytopenia. Although prolonged administration of IL-6 has been shown to induce thrombocytosis, IL-6 and TPO are apparently different and immunologically distinct molecules.

INTERLEUKIN-6 (IL-6) has been shown to stimulate megakaryocyte maturation in vitro and to produce increased peripheral platelet levels that result from an increase in the rate of platelet production in vivo. The administration of IL-6 results in the stimulation of megakaryocytopoiesis in vivo, as shown by an increase in megakaryocyte size and ploidy and by an increase in the number of detectable megakaryocyte colony-forming cells (Meg-CFC) in the bone marrow and spleen. Although the effects of IL-6 on platelet production are more marked than on other blood cell lineages, lack of lineage specificity is indicated by the observation that the administration of IL-6 produced increased numbers of detectable granulocyte-macrophage colony-forming cells (GM-CFC) in the spleen and bone marrow, slightly elevated peripheral leukocyte levels at higher doses, and reticulocytosis in association with erythroid hyperplasia. In addition, after irradiation, total numbers of GM-CFC and erythroid-CFC were elevated by the administration of IL-6 to mice.

The physiologic regulation of platelet production is thought to occur through a humoral mechanism in which decreases in levels of circulating platelets result in the increased production or release of a thrombopoiesis-stimulating activity, designated thrombopoietin (TPO). Elevated levels of TPO can be detected in the plasma or serum of animals 4 to 24 hours after the induction of thrombocytopenia. It is therefore believed that TPO mediates the effects of thrombocytopenia upon thrombopoiesis and megakaryocytopoiesis.

The marked effects of IL-6 on platelet production suggest the possibility that IL-6 could be the physiologic regulator of thrombopoiesis. To test this hypothesis, IL-6 levels in plasma were measured after the induction of acute, severe thrombocytopenia in mice, at times when TPO levels have been shown to be elevated. In addition, IL-6 was measured in preparations of partially purified TPO from the plasma of thrombocytopenic rabbits. Finally, neutralizing antibodies directed against IL-6 were tested for their ability to neutralize TPO. In this report, we present evidence that IL-6 is not elevated after thrombocytopenia, that partially purified TPO does not contain IL-6 activity, and that anti–IL-6 antibodies do not neutralize the in vitro megakaryocyte maturation activity of partially purified TPO. These three lines of evidence strongly suggest that TPO and IL-6 are different molecules.

MATERIALS AND METHODS

Induction of thrombocytopenia. C57BL/6N female mice (15 to 20 g, supplied by Simonsen Laboratories, Gilroy, CA) were rendered thrombocytopenic by intraperitoneal injection of rabbit or guinea pig antimouse platelet antisera. A dose of antisera that produces acute, severe thrombocytopenia (platelet levels below 5% of control) was diluted in 0.9% sterile, pyrogen-free...
Baseline plasma IL-6 levels were determined in a group of five uninjected animals. Induction of thrombocytopenia was verified by measurement of peripheral platelet levels of each animal by cardiac puncture at various times after the induction of thrombocytopenia. Aliquots were removed for determination of platelet counts. For IL-6 determinations, cell-free plasma was then prepared by centrifugation at 3,000 g for 20 minutes. Plasma samples were stored for up to 2 days at 4°C or at −70°C for more extended periods.

Preparation of partially purified TPO. TPO was prepared from the plasma of thrombocytopenic rabbits by sequential lectin chromatography, as described previously. Rabbits were rendered acutely, severely thrombocytopenic by the administration of guinea pig antirabbit platelet antiserum. Plasma was prepared and initially subjected to ammonium sulfate fractionation. The TPO-containing fraction (30% to 40% precipitate) was fractionated by wheat germ agglutinin (WGA) chromatography, followed by concanavalin A (Con A) chromatography. Material fractionated as described has been shown to stimulate thrombopoiesis in mice at a dose of 0.67 μg/g body weight. For antisera neutralization studies, Con A-fractionated proteins were further separated by gel permeation high performance liquid chromatography (GP-HPLC), as previously described.

Liquid culture assay for megakaryocyte maturation. A suspension of murine bone marrow cells was prepared in calcium- and magnesium-free phosphate-buffered saline (CMF-PBS) and treated with 0.5 mmol/L diisopropylfluorophosphate (DFP; Sigma Chemical Co, St Louis, MO) for 20 minutes at room temperature to inactivate endogenous acetylcholinesterase (AChE). Cells were then collected by centrifugation at 250g for 7 minutes, resuspended in 5 mL of CMF-PBS, and layered over 5 mL of 1.077 g/mL Ficoll (Pharmacia LKB Biotechnology, Piscataway, NJ) to remove red blood cells, some mature leukocytes, and other cellular debris. After centrifugation at 600g for 20 minutes, the band of cells at the PBS/Ficoll interface was collected and washed once in Iscove’s modified Dulbecco’s medium (IMDM) supplemented with 1% Nutridoma SP (Boehringer Mannheim Biochemicals, Indianapolis, IN) and penicillin/streptomycin. The final cell preparation was diluted to 100,000 cells per 100 μL in IMDM (supplemented as above).

Cultures were prepared in 96-well tissue culture plates (polystyrene; Corning Laboratories Science Co, Corning, NY) at a final volume of 200 μL (100,000 cells per well) and incubated for 4 days. No serum or growth factors other than test materials were added to the cultures. Total AChE was measured as an indicator of megakaryocyte growth and differentiation in each culture after cell solubilization, using the fluorescence assay described by Burstein.

Quantification of AChE production was performed using a Perkin-Elmer LC-50 fluorometer (Perkin-Elmer Corp, Norwalk, CT) with an automated 96-well plate reader attachment.

Measurement of IL-6 activity. IL-6 activity was measured using the IL-6-dependent murine hybridoma cell line B9. IL-6 levels in serial dilutions of plasma samples and in preparations of TPO were compared with standard curves of purified, recombinant human IL-6 (10 U/μg) from Amgen Biologicals (Thousand Oaks, CA) or purified, recombinant rat (kindly provided by Allelix Biopharmaceuticals, Toronto, Ontario, Canada). In certain experiments (indicated in Results), rabbit serum samples or preparations of TPO partially purified from the plasma of thrombocytopenic rabbits, as described in Materials and Methods, were tested for hepatocyte-stimulating activity (another biologic activity of IL-6) by measuring the induction of α2-macroglobulin in primary cultures of rat hepatocytes, as described.

During these experiments, it was observed that heat-inactivation of murine plasma (30 minutes at 56°C to inactivate complement) destroyed some, but not all, IL-6 activity. In eight comparisons between heat-inactivated and non-heat-inactivated murine plasma, 27% ± 8.1% (SD) of the initial IL-6 activity remained after heating. The addition of murine plasma that had not been heat-inactivated to human IL-6 standards showed that the plasma was not inhibitory in the B9 assay. Therefore, heat-inactivation of murine plasma was not necessary for the measurement of IL-6 levels using the B9 assay. Accordingly, the data presented in Fig 1 are derived from samples that were not heated.

Antiserum neutralization. Antiserum directed against purified recombinant rat IL-6 was prepared in rabbits. Antiserum or normal rabbit serum (NRS) was incubated with TPO or human IL-6 for 1 hour at 37°C before the addition of 100,000 bone marrow cells. The human IL-6 used in these experiments was prepared with a baculovirus vector in SF9 insect cells and purified, as previously described (provided by Cetus Corp, Emeryville, CA). The final concentration of antiserum or normal serum was 10 μL per 200 μL final liquid culture volume. The ability of the antiserum to neutralize rabbit IL-6 was shown by inhibition of IL-6 activity, measured with the B9 assay, in the serum of rabbits after the administration of endotoxin.

In other experiments, IgG purified from the anti-IL-6 antiserum indicated above or from normal rabbit serum was used to confirm
the inability of anti-IL-6 antibodies to neutralize partially purified TPO. GP-HPLC-fractionated TPO or IL-6 was incubated with 100 μg/mL purified IgG overnight at 4°C. Immune complexes were precipitated by centrifugation for 2 minutes (Microfuge B, Beckman Instruments, Palo Alto, CA) and the supernatant was tested for megakaryocyte maturation activity using the liquid culture assay described above.

Statistical analyses. Statistical analyses were performed using the Mann-Whitney U test.

RESULTS

Using the B9 assay described in Materials and Methods, IL-6 levels were determined in the plasma of thrombocytopenic mice at various times after the induction of acute, severe thrombocytopenia and compared with control animals that received normal serum (NS). At 2 to 6 hours after the administration of platelet antiserum (PAS), IL-6 levels were not different from control animals that had received NS (Fig 1). The measurement of IL-6 levels in plasma then was extended to 0.5, 1.0, 12, and 24 hours after acute thrombocytopenia. At no time points were IL-6 levels in the plasma of thrombocytopenic animals different from control animals that received NS.

However, regardless of the presence or absence of thrombocytopenia, IL-6 levels after 2 hours were significantly higher than after 4 hours (P < .02) or 6 hours (P < .01) in mice that had received serum. Furthermore, when compared with basal levels, measured in un.injected animals (12.9 ± 2.6 pg/mL), the concentrations of IL-6 present in plasma 2 or 3 hours after the injection of serum were significantly elevated (P < .01). The data indicate that, independent of production of thrombocytopenia, the administration of heterologous serum resulted in limited but significant elevation of plasma IL-6 levels.

Verification of our ability to detect elevated IL-6 levels in murine plasma was accomplished by the intraperitoneal (IP) administration of endotoxin (1 mg/kg; Escherichia coli lipopolysaccharide 055:B5; Difco Laboratories, Detroit, MI), which is known to produce elevated levels of IL-6 in humans.23 IL-6 levels were measured at 2, 3, 4, and 6 hours after the administration of endotoxin (Fig 1). The administration of endotoxin resulted in a mean plasma IL-6 level of 42.6 ± 8.3 ng/mL, which is approximately 3,300-fold higher than the un injected control. This verified our ability to measure IL-6 in murine plasma using the B9 assay.

Partially purified TPO from the plasma of thrombocytopenic rabbits, prepared by ammonium sulfate precipitation and sequential lectin chromatography steps, as indicated in Materials and Methods, was tested for the presence of IL-6 (Table 1). Two different batches of partially purified TPO that stimulated maturation of megakaryocytes in vitro at a protein concentration of 100 ng/mL (which we have shown correlates with stimulation of thrombopoiesis in vivo)18 had undetectable levels of IL-6 over the concentration range 0.1 ng/mL to 10 μg/mL, measured by either the B9 assay (Table 1) or by induction of α2-macroglobulin in primary cultures of rat hepatocytes22 (data not shown).

To verify that the B9 assay could detect rabbit IL-6, New Zealand White rabbits (1.5 kg) were anesthetized by isofluorane, and endotoxin (5 mg/kg; E. coli 055:B5; Difco) was administered intravenously (IV) in PBS. Serum samples were prepared at 0, 2, and 5 hours. The samples were heat-inactivated (56°C for 30 minutes) and tested for IL-6-like biologic activity in the B9 murine hybridoma assay2 (Table 1). This assay has been shown to detect IL-6-like activity in human, rat, mouse, and dog serum samples and can detect levels of IL-6 well below those required to produce physiologic responses.21,24 Elevation of IL-6 levels in rabbit serum was detected 2 and 5 hours after the administration of endotoxin. The rabbit IL-6 response (Table 1) was not as marked as that shown by mice (Fig 1). However, as indicated in Materials and Methods, heat-inactivation of rabbit sera before the measurement of IL-6 levels may have destroyed some activity. Samples were not remeasured without preheating because our goal of establishing the ability of the assay to detect rabbit IL-6 had been achieved. Rabbit IL-6 also was detected in the same postendotoxin serum samples by the hepatocyte stimulation assay (data not shown).

To confirm the identity of the rabbit-derived activity as IL-6, neutralizing antibodies against recombinant rat or human IL-6 were incubated with the rabbit sera before assay in the B9 system. Both antibodies, which can inhibit rat-, mouse-, and human-derived IL-6, also totally neutralized the biologic activity in rabbit serum that was detected by the B9 assay for IL-6 (data not shown).

Rabbit antirat IL-6 antiserum, previously shown to neutralize rabbit IL-6, was tested for its ability to neutralize the in vitro megakaryocyte maturation activity in fractions of partially purified TPO. Anti-IL-6 was added to liquid cultures of murine bone marrow cells (see Materials and Methods) that contained IL-6 or partially purified TPO at the minimum concentrations required to stimulate

**Table 1. IL-6 Levels in Partially Purified TPO From the Plasma of Thrombocytopenic Rabbits**

<table>
<thead>
<tr>
<th>IL-6 (pg/mL)</th>
<th>Con A-fractionated TPO*</th>
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<tr>
<td>Serum samples after endotoxin administration (h)</td>
<td>Not detectable†</td>
</tr>
<tr>
<td>0</td>
<td>Not detectable</td>
</tr>
<tr>
<td>2</td>
<td>1,100</td>
</tr>
<tr>
<td>5</td>
<td>3,000</td>
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TPO was partially purified from the plasma of thrombocytopenic rabbits (6 hours postinduction of thrombocytopenia) by ammonium sulfate precipitation, WGA chromatography, and Con A chromatography, performed sequentially. IL-6 concentrations were determined using the IL-6-dependent murine hybridoma B9 cell line. Elevated serum IL-6 levels were produced in rabbits by the administration of bacterial endotoxin (5 mg/kg). Partially purified TPO did not contain detectable levels of IL-6. The ability of the B9 assay to detect rabbit IL-6 was verified by detection of elevated levels of IL-6 in rabbit serum after the administration of endotoxin.

*Two separately prepared batches of Con A-fractionated TPO were tested for the presence of IL-6 (no preheating was performed).
†Assay sensitivity to rabbit IL-6 was 7.5 pg/mL based on a rat IL-6 standard.
megakaryocyte maturation (Table 2). Anti–IL-6 antiserum completely neutralized megakaryocyte maturation stimulated by IL-6 (5 ng/mL final concentration), but had no effect on megakaryocyte maturation stimulated by partially purified TPO (50 ng/mL final concentration). In a confirmatory experiment, it was shown that IgG, purified from the same anti–IL-6 antiserum indicated above, was unable to immunoprecipitate (using protein A as described in Materials and Methods) the megakaryocyte maturation activity in GP-HPLC–fractionated TPO (Table 2).

**DISCUSSION**

In this study, we have tested the hypothesis that IL-6 may be the physiologic regulator of thrombopoiesis. This hypothesis is based on the observations that many of the effects of thrombocytopenia upon megakaryocytopenesis and thrombopoiesis can be produced in normal animals by the administration of IL-6. Therefore, acute, severe thrombocytopenia was induced in mice and plasma IL-6 levels were serially measured. Elevated TPO concentrations can be detected as early as 4 hours and as late as 24 hours after acute thrombocytopenia. In contrast, our study showed that, after the induction of thrombocytopenia, there was no increase in plasma IL-6 levels (compared with control animals that received NS) at time points when elevated TPO levels have been described. We verified our ability to detect elevated plasma IL-6 levels by the administration of endotoxin to mice, which produced large increases in IL-6 levels, as previously described in humans.23

As further evidence that IL-6 and TPO are not the same factor, we tested partially purified TPO, obtained from the plasma of thrombocytopenic rabbits, for the presence of IL-6. Our fractionation procedure, which increases the specific activity of TPO by 7,000-fold compared with plasma, would maximize our ability to detect IL-6 if TPO and IL-6 were the same molecule. However, no IL-6 activity was detected in preparations of partially purified TPO. The B9 assay used in this study to measure IL-6 levels has been shown previously to be sensitive to rabbit IL-6 produced in response to endotoxin.23 In addition, we verified the ability of the B9 assay to detect elevated IL-6 levels in rabbit plasma after the administration of endotoxin.

Finally, our data showed that neutralizing antiserum or purified IgG directed against recombinant rat IL-6, and shown also to neutralize rabbit IL-6, was not able to inhibit the megakaryocyte maturation activity of partially purified TPO from the plasma of thrombocytopenic rabbits. The TPO fraction tested was prepared by ammonium sulfate precipitation, lectin affinity chromatography, and GP-HPLC, which results in a purification factor of 14,000× compared with plasma. Use of this fraction maximized the likelihood that a single factor was providing the megakaryocyte maturation activity.

Therefore, based on these three lines of evidence, we have concluded that IL-6 is not identical to TPO, the thrombopoiesis-stimulating activity produced in response to acute thrombocytopenia. However, the current literature is not in agreement on this matter. IL-6 messenger RNA (mRNA) levels in spleen cells are reported to be elevated 1.5 to 2 hours after the administration of antiplatelet serum to produce acute, severe thrombocytopenia in mice.26 Also, a small and brief increase in plasma IL-6 levels was observed after 1.75 hours. Both mRNA and IL-6 levels had returned to control values by 3 hours.26 Our finding that

**Table 2. Effect of Anti–IL-6 Antibody Upon Stimulation of Megakaryocyte Maturation by Partially Purified TPO From the Plasma of Thrombocytopenic Rabbits**

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>% Control (±SE)</th>
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<tr>
<td>IL-6</td>
<td>134 ± 5.3*</td>
</tr>
<tr>
<td>IL-6 + anti–IL-6</td>
<td>99 ± 5.9†</td>
</tr>
<tr>
<td>IL-6 + NRS</td>
<td>128 ± 5.9‡</td>
</tr>
<tr>
<td>TPO</td>
<td>165 ± 5.1§</td>
</tr>
<tr>
<td>TPO + anti–IL-6</td>
<td>146 ± 4.6†</td>
</tr>
<tr>
<td>Purified IgG</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>152 ± 6.5%(</td>
</tr>
<tr>
<td>IL-6 + anti–IL-6 IgG</td>
<td>104 ± 7.4##</td>
</tr>
<tr>
<td>IL-6 + normal IgG</td>
<td>137 ± 6.1###</td>
</tr>
<tr>
<td>TPO</td>
<td>174 ± 16.1###</td>
</tr>
<tr>
<td>TPO + anti–IL-6 IgG</td>
<td>153 ± 10.2###</td>
</tr>
<tr>
<td>TPO + normal IgG</td>
<td>150 ± 14.8###</td>
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Stimulation of megakaryocyte maturation was measured in liquid cultures as described in Materials and Methods. IL-6 or TPO (partially purified by ammonium sulfate precipitation, lectin chromatography, and GP-HPLC) was added at the minimum concentrations required to produce maximal increase in fluorescence (5 ng/mL or 50 ng/mL, respectively). Percent of control, calculated by dividing the absolute mean fluorescence in the presence of test materials by fluorescence produced by a control containing only cells, is presented with standard error (also expressed as a percent of control). Rabbit anti–IL-6 antiserum or NRS was added at a final concentration of 100 μg/mL and incubated with IL-6 or TPO for 1 hour at 37°C before the addition of 100,000 bone marrow cells. The antiserum results presented have been pooled from two separate experiments in which each culture condition was performed in quadruplicate. The inability of anti–IL-6 antibodies to bind to TPO partially purified from rabbit plasma was verified in one additional experiment using IgG purified from the same anti–IL-6 antiserum indicated above. IgG (final concentration, 100 μg/mL) was added to TPO or IL-6 (at minimum concentrations to produce maximal AChE production in culture). Incubation, followed by immunoprecipitation, was as indicated in Materials and Methods. Megakaryocyte maturation activity (TPO or IL-6) remaining in the supernatant was measured using the fluorescence assay. Statistical analysis was performed using the Mann-Whitney U Test.*Significantly different from control (P < .001).†Not significantly different from control; significantly different from IL-6 alone (P < .006).‡Significantly different from control (P = .01); not significantly different from IL-6 alone. §Significantly different from control (P < .0005).∥Significantly different from control (P < .0005); not significantly different from TPO alone. ¶Significantly different from control (P < .005).#Not significantly different from control; significantly different from IL-6 alone (P = .02).**Significantly different from control (P < .005); not significantly different from IL-6 alone. ††Significantly different from control (P < .01).‡‡Significantly different from control (P < .01); not significantly different from TPO alone.
small but significant increases in plasma IL-6 levels can result from the injection of heterologous serum indicates that the previous report must be interpreted cautiously. In addition, this small, transient increase in IL-6 is in marked contrast to TPO levels, which have been reported to be elevated for 4 to 24 hours after the induction of thrombocytopenia.12-14

Further evidence that IL-6 and TPO are not identical can be obtained by comparing the relative effects of IL-6, TPO, and thrombocytopenia. The increase in platelet volume that has been reported after acute thrombocytopenia27,29 or after the administration of partially purified TPO29 or the thrombopoiesis stimulatory activity in human embryonic kidney cell cultures (TGF)29,31 has not been observed after the administration of IL-6 to mice.5,31 In addition, IL-6 and TGF have differing effects upon the frequency of small, acetycholinesterase-positive-positive cells in mice.31 Furthermore, thrombocytopenia does not result in a significant increase in detectable Meg-CFC in the bone marrow of mice,32 whereas the number of Meg-CFC is elevated in the bone marrow after the administration of IL-6.6

Although it has been shown that IL-6 has multiple effects upon thrombopoiesis and megakaryocytes in vivo,7,10 it seems unlikely that a cytokine with the range of biologic activities of IL-6, which include acute phase, inflammatory and immunologic responses, would be the primary regulator of platelet production. Lack of lineage specificity of IL-6, which has been shown to stimulate at least some compartments of both the myeloid and erythroid lineages,5,7,10 also argues against a role for IL-6 in the maintenance of normal platelet levels. However, studies have shown a relationship between IL-6 and the inflammation associated with rheumatoid arthritis or surgery.33-35 Therefore, the observations of the ability of IL-6 to stimulate platelet production suggest that reactive thrombocytosis that occurs in certain inflammatory states may be, in some instances, the result of elevated levels of IL-6. A study of 183 patients with reactive thrombocytosis showed increased serum levels of IL-6 in 83% of the cases, although no correlation between individual platelet counts and IL-6 levels was observed.50 In a similar study of 120 patients, thrombocytosis in the presence of ongoing inflammatory processes was associated with elevated serum IL-6, although IL-6 levels did not correlate with platelet counts; patients with thrombocytosis in the absence of inflammation had normal IL-6 levels.27 Also, in another more limited study of eight trauma patients, no correlation was observed between thrombocytosis and IL-6 levels.38 Therefore, at present, the role of IL-6 in the pathophysiology of thrombocytosis secondary to inflammation remains uncertain.

ACKNOWLEDGMENT
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REFERENCES


