Inhibiting Interleukin-1 and Tumor Necrosis Factor-α Does Not Reduce Induction of Plasminogen Activator Inhibitor Type-1 by Endotoxin in Rats In Vivo

By J.J. Emeis, R. Hoekzema, and A.F. de Vos

In experimental animals and humans, intravenous (IV) injection of endotoxin induces large increases in circulating plasminogen activator inhibitor type-1 (PAI-1), a major inhibitor of blood fibrinolysis. A similar increase is seen after the injection of interleukin-1 (IL-1) or of tumor necrosis factor-α (TNF-α), suggesting that these cytokines mediate the induction, by endotoxin, of PAI-1. To test this hypothesis we pretreated rats, before IV endotoxin, with compounds that inhibit the formation of cytokines (pentoxifylline; dexamethasone), or with compounds that inhibit the action of these cytokines (anti-TNF antiserum for TNF-α; IL-1 receptor antagonist for IL-1). None of these pretreatments affected the induction of PAI-1 synthesis by endotoxin. However, pretreatment did reduce the endotoxin-induced increase in plasma tPA antigen concentration. Thus, the data suggest that, in rats in vivo, TNF-α and IL-1 are not significantly involved in the induction of PAI-1 by endotoxin.

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Materials and Methods

Materials. Endotoxin (lipopolysaccharide [LPS], from Escherichia coli serotype 0128:B12; Sigma, St Louis, MO) was dissolved in sterile saline to a concentration of 10 μg/mL. All other compounds were dissolved in sterile saline containing 1% (wt/vol) sterile, pyrogen-free human serum albumin (Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam, The Netherlands). Recombinant human TNF-α (2.5 × 10^6 U/mg protein; a gift from Dr W. Fiers, Biogent, Gent, Belgium) was diluted to 10 μg/mL. Recombinant human IL-1β (10^6 U/mg; a gift from Dr S. Gillis, Immunex, Seattle, WA) was diluted to 2 μg/mL. Recombinant mouse TNF-α, rabbit-antimouse TNF-α antisera, and an enzyme-linked immunosorbent assay (ELISA) kit for mouse TNF-α were obtained from Genzyme (Boston, MA). Recombinant human IL-1 receptor antagonist (IL-1ra; a gift from Dr D.E. Tracey, The Upjohn Co, Kalamazoo, MI) was diluted to 1 mg/mL. Pentoxifylline (Trental) was from Hoechst AG (Wiesbaden, Germany), and was used as supplied (20 mg/mL). Dexamethasone (Sigma) was first dissolved in ethanol (10 mg/mL), and then diluted to 1 mg/mL in saline. Recombinant human tissue-type plasminogen activator (tPA; Activase) was from Genentech (San Francisco, CA). Recombinant rat JMI-229 tPAZ6 was a gift from Dr J. D. Prior (Porton Developments Ltd, Salisbury, UK). Rabbit-antirat tPA IgG and rat L2 tPA have been described previously. Rabbit-antirat PAI-1 IgG was obtained from American Diagnostica (Greenwich, CT). Biotine, avidin-peroxidase, and tetramethylbenzidine were from Pierce (Rockford, IL).

Animal experimentation. Male Wistar rats (200 to 250 g body weight) were obtained from the Broekman Institute (Someret, The Netherlands). All experiments were performed under Nembutal anesthesia (60 mg/kg, intraperitoneally). Injections were administered into the vein of the penis. Blood was obtained by aortic puncture into precooled syringes, and anticoagulated with 0.13 mol/L trisodium citrate (1 vol to 9 vol of blood). Platelet-poor plasma was immediately prepared at 4°C by centrifugation for 10 minutes at 2,000g, and stored at −20°C. Serum was prepared for 30 minutes at 37°C and 30 minutes at 4°C, followed by centrifugation for 10 minutes at 2,000g.

Animal experiments had been approved by the Animal Experiments Committee of The Netherlands Organization for Applied Scientific Research TNO, and were in accordance with the guidelines on animal experimentation presented to the International Committee of Thrombosis and Haemostasis.

Experimental design. Rats were injected with a compound under study, followed—after an interval of 30 to 60 minutes, as detailed

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Table 1. Effect of Pretreatment With Pentoxifylline or Dexamethasone on the Induction of TNF-α by Endotoxin

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>TNF-α (pg/mL)</th>
<th>Individual Data</th>
<th>Geometric Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>45,640</td>
<td>19,281</td>
<td>1,430</td>
</tr>
<tr>
<td>Pentoxifylline</td>
<td>28,958</td>
<td>11,486</td>
<td>992</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>20,474</td>
<td>770</td>
<td>339</td>
</tr>
<tr>
<td>No LPS controls</td>
<td>45,640</td>
<td>19,281</td>
<td>1,430</td>
</tr>
</tbody>
</table>

Rats (four per group) were pretreated with pentoxifylline (50 mg/kg IV, 1 hour before LPS), or with dexamethasone (2 mg/kg intraperitoneally, 2 hours before LPS), and subsequently injected with LPS (10 μg/kg). Serum for the determination of TNF-α concentrations was obtained 1 hour after LPS injection. For the determination of TNF-α, see Materials and Methods.

Table 2. Effect of Dexamethasone and Corticosterone on the Induction of Plasma PAI Activity by Endotoxin

<table>
<thead>
<tr>
<th>PAI Activity (U/mL)</th>
<th>No. of Rats</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol controls</td>
<td>111 ± 28</td>
</tr>
<tr>
<td>Dexamethasone*</td>
<td>102 ± 24</td>
</tr>
<tr>
<td>Ethanol controls</td>
<td>133 ± 11</td>
</tr>
<tr>
<td>Dexamethasone†</td>
<td>97 ± 24</td>
</tr>
<tr>
<td>Ethanol controls</td>
<td>105 ± 11</td>
</tr>
<tr>
<td>Corticosterone‡</td>
<td>112 ± 16</td>
</tr>
</tbody>
</table>

All data shown are mean ± SD. Rats were pretreated with corticosteroids as indicated, followed by LPS (10 μg/kg IV). Plasma for PAI determinations was obtained 3 hours after LPS. For further details, see Materials and Methods. No significant differences between treatment and control groups were present.

* Dexamethasone 2 mg/kg intraperitoneally, daily for 4 days before LPS.
† Dexamethasone 2 mg/kg intraperitoneally, 2 hours before LPS.
‡ Corticosterone 50 mg/kg intraperitoneally, 2 hours before LPS.
LPS, PAI-1, AND CYTOKINES

Effect of anti-TNF antiserum, IL-1 receptor antagonist (IL-1ra), or both, on PAI induction by LPS. Pretreatment of rats with an antmouse-TNF antiserum (0.1 mL/rat) had no significant effect on the induction of PAI by LPS, compared to pretreatment with control rabbit serum (Table 3). Higher doses of the antiserum (0.15 or 0.3 mL/rat, n = 2 per dose) had no effect either (Table 3). The anti-TNF antiserum did reduce, though, the induction of PAI activity by recombinant human TNF-α (10 μg/kg), or recombinant human IL-1β (1 μg/kg). Rats were killed 3 hours after LPS, TNF or IL-1.

Additive effects of TNF-α and IL-1β. To see whether residual circulating levels of one cytokine would potentiate the other, rats (two per group) were injected with TNF-α (10 μg/kg) with or without simultaneous injection of a small dose of IL-1β (0.1 μg/kg), or with only IL-1β (0.1 μg/kg). No potentiating effect on PAI induction was observed: TNF-α alone gave PAI activities of 18 and 20 U/mL; IL-1β alone 8 and 11 U/mL; TNF-α plus IL-1β 19 and 20 U/mL. Similarly, no potentiation of IL-1β (1 μg/kg) by a small dose of TNF-α (1 μg/kg) was seen: IL-1β alone gave PAI activities of 25 and 25 U/mL; TNF-α alone 12 and 13 U/mL; IL-1β plus TNF-α 30 and 38 U/mL. IL-1β (1 μg/kg) plus TNF-α (10 μg/kg) gave 35 and 42 U/mL. These data suggest that the combined effect of IL-1β and TNF-α is additive rather than synergistic. This makes it unlikely that residual amounts of TNF-α activity (remaining after anti-TNF treatment) would potentiate IL-1β, or that residual IL-1β activity (after IL-1ra treatment) would potentiate TNF-α. The effect of TNF-α on PAI-1 was linearly related to the injected dose of TNF-α over the dose-range 0.5 to 60 μg/kg (n = 9; r = .895; P < .01).

Changes in tPA antigen concentrations. LPS induced a time-dependent increase in tPA antigen (Fig 1), preceding the induction of PAI which commences only after 1 hour. Increases in tPA antigen of a magnitude similar to that induced by LPS were found at 3 hours after the injection of IL-1β and TNF-α (Table 4). The tPA increase after LPS was partly inhibited by pentoxifylline, dexamethasone, anti-TNF antiserum, and IL-1ra (Table 4). Similarly, anti-TNF antiserum and IL-1ra partly inhibited the tPA increase induced by, respectively, TNF-α and IL-1β (Table 4). Inhibition was not complete, presumably because the high PAI levels in the circulation caused the formation of more tPA-PAI complexes, which are cleared from the circulation more slowly than uncomplexed tPA.

### DISCUSSION

This study was designed to test the hypothesis that the induction of PAI-1 by endotoxin is mediated by the cytokines TNF-α and/or IL-1. This hypothesis originated from two sets of observations. Firstly, the observation that endotoxin, a potent inducer of PAI-1, is also a potent inducer in vivo of IL-1 and TNF-α. Secondly, the observation that both cytokines will increase PAI-1 in vivo. IL-1α and β have been shown to induce PAI-1 in rats, whereas TNF-α...
has been shown to induce PAI-1 in mice,33 rats,10,25,34 and humans.35-38 However, the present study provided no support for our hypothesis: all four procedures meant to interfere with PAI-1 synthesis by endotoxin.

Pentoxifylline inhibits the induction, by endotoxin, of TNF-α synthesis by inhibiting the transcription of TNF-α mRNA.39 It is effective in all species tested, including humans,40 chimpanzees,23,41 mouse,42 and rat.43 In this study, it inhibited the induction of TNF-α by 96% (Table 1), but failed to affect the induction of PAI-1 by endotoxin. Dexamethasone, which inhibits the translation of TNF-α mRNA,44 also inhibited the induction of TNF-α (by 98%, Table 1), in agreement with a study in rats which used a similar dosage regimen.45 But, like pentoxifylline, dexamethasone had no effect on PAI-1 induction (Table 2).

In addition, an antimurine-TNF antiserum that also inhibits rat TNF-α had no effect on PAI induction, though it fully inhibited the induction of PAI-1 by human TNF-α (Table 3). All in all, these data showed that blocking the synthesis (pentoxifylline, dexamethasone), or the effect (anti-TNF antiserum) of TNF-α did not affect the induction of increased PAI-1 synthesis by endotoxin.

Similar to TNF-α, IL-1β is induced in rats by endotoxin,46 albeit more slowly, peak levels being obtained only at 5 hours after endotoxin injection,46 i.e., after the peak level of PAI-1 has been reached (at 3 to 4 hours).47 Dexamethasone also inhibits, as for TNF-α, the induction of IL-1 activity47 and of IL-1 mRNA,48 via effects on translation and secretion.49 Pentoxifylline is not known to reduce the induction of IL-1.50 To block the effects of IL-1, we used an IL-1 receptor antagonist51-52 that effectively inhibits IL-1β-induced effects in a variety of species,51 including rats.53,54 However, like dexamethasone, IL-1ra had no effect on PAI-1 induction by endotoxin in this study, though it inhibited the induction of PAI by human IL-1β (Table 3).

Endotoxin not only induces increased plasma levels of PAI-1, but also of tPA, induction of tPA preceding that of PAI-1, as has been observed both in humans27 and in chimpanzees.23 Increased tPA antigen levels have also been described in humans after treatment with TNF-α.55-58 In the present study similar increases were noted after treatment of rats with endotoxin (Fig 1), TNF-α, and IL-1β (Table 4), an observation not made in rats before. The increase in tPA antigen after the injection of TNF-α was reduced by anti-TNF antiserum, and the increase after IL-1β by IL-1ra (Table 4). The increase in tPA antigen after endotoxin injection was reduced, though to a lesser extent, by pretreatment with pentoxifylline, dexamethasone, anti-TNF antiserum, and IL-1ra. This suggests that pretreatment with anti-TNF antiserum and IL-1ra was not only effective against injected (human) TNF-α or IL-1β, but also against endogenous TNF-α and IL-1. That tPA was not reduced to normal control levels is presumably due to the fact, mentioned above, that the induction of PAI-1 still occurred in all animals, resulting in increased circulating levels of tPA-PAI complexes that are detected by our tPA ELISA assay. Also, it is likely that tPA release is induced59 after endotoxin injection by other endotoxin-induced compounds such as platelet-activating factor, catecholamines, vasopressin, etc.

Because synergistic effects between TNF-α and IL-1 have been reported (eg, ref 56), and because in our experiments low residual levels of cytokines activity are likely to be present during treatment with antiserum or with receptor antagonist, we investigated whether synergistic effects on PAI synthesis could be detected. As described above, no such synergy between TNF-α and IL-1β was found, using various combinations of the two cytokines, but rather additive effects. Moreover, because the PAI response to TNF-α was linear over the TNF concentration range 0.5 to 60 µg/kg, we consider it unlikely that residual amounts of TNF (or IL-1) would significantly have affected the PAI concentrations.

In a previous publication34 we have shown that a variety of autacoids that are induced or released by endotoxin in vivo (eg, cyclo-oxygenase and lipoxygenase products, platelet-activating factor, catecholamines, histamine, cyclic nucleotides, opioids, vasopressin, thrombin) are not involved in the induction of PAI-1 by endotoxin. It has also been shown that IL-6 is unable to induce PAI-1 in rats in vivo,16,57 as is the case in vitro.25,58 Our present observations suggest that IL-1 and TNF-α do not mediate the effect of endotoxin on PAI-1 either. Thus, the mechanism by which endotoxin induces increased synthesis of PAI-1 in vivo remains unexplained. This situation is the more unfortunate because it was recently suggested23 that TNF-α mediates, in chimpanzees, the endotoxin-induced increase in tPA that precedes the increase in PAI-1 activity, an observation in agreement with our data. It follows that inhibiting TNF-α will, at least

| Table 4. Effect of Pretreatment on Endotoxin-Induced tPA Antigen Levels |
|-----------------------------|-----------------|----------------|
|                             | tPA Antigen     | No. of Rats   |
|                             | (ng/mL)         |               |
| 1. Saline-treated control rats | 2.4 ± 0.2      | 4              |
| 2. Saline, then LPS         | 8.1 ± 2.5       | 10             |
| 3. Normal rabbit serum, then LPS | 8.4 ± 3.0     | 4              |
| 4. Pentoxifylline, then LPS | 6.2 ± 1.5       | 4              |
| 5. Dexamethasone, then LPS  | 6.3 ± 1.2       | 4              |
| 6. Anti-TNF antiserum, then LPS | 6.3 ± 1.3     | 4              |
| 7. IL-1ra, then LPS         | 5.8 ± 1.9       | 4              |
| 8. Control rabbit serum, then TNF | 5.7 ± 0.3     | 4              |
| 9. Anti-TNF antiserum, then TNF | 3.2 ± 0.1     | 4              |
| 10. Saline, then IL-1       | 6.2 ± 1.0       | 3              |
| 11. IL-1ra, then IL-1       | 3.6 ± 0.3       | 3              |

All data shown are mean ± SD. Groups 2-7 were not significantly different by one-way ANOVA. When groups 2 and 3 and groups 4-7 were combined, the two new groups (non-pretreated v pretreated) were significantly different by Student’s t-test (P < .01). Group 8 was significantly different from group 9, and group 15 from group 11, by Student’s t-test (P < .05). Rats were pretreated with saline, control rabbit serum (0.1 mL/rat), pentoxifylline (50 mg/kg IV), dexamethasone (2 mg/kg intraperitoneally), anti-TNF antiserum (0.1 mL/rat), or IL-1ra (1 mg/kg), followed by either LPS (10 µg/kg), recombinant human TNF-α (10 µg/kg), or recombinant human IL-1β (1 µg/kg). Rats were killed 3 hours after LPS, TNF, or IL-1. For further details, see Materials and Methods.
in rats, inhibit the profibrinolytic, tPA-mediated, effect of endotoxin without interfering with its antifibrinolytic, PAI-1-mediated effect. In combination, these effects might then shift the fibrinolytic balance in blood even further to an inhibitory state, worsening the procoagulant effects of endotoxin. It would be of interest to study whether this situation, as described here for rats, also applies to primates and humans.

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