Effect of Human Recombinant Cytokines on the Induction of Macrophage Procoagulant Activity

By Iris Schwager and Thomas W. Jungi

A panel of human recombinant cytokines was tested for induction of procoagulant activity (PCA) in human monocyte-derived macrophages. Nonadherent culture conditions were used, and PCA was determined with whole cells rather than cell lysates. It was assured by Limulus amebocyte lysate assay that tested cytokines displayed low levels of endotoxin activity within the range of biologic activity. Additional evidence to rule out an endotoxin effect was provided by heat-inactivation experiments. Interferon-γ (IFN-γ), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) were strong macrophage PCA inducers. The low level of PCA induced by IL-2, granulocyte-macrophage colony-stimulating factor (GM-CSF), M-CSF, IL-4, IL-6, IL-10, and IFN-α could not be distinguished from that induced by traces of endotoxin contaminating the preparations. Transforming growth factor-β decreased constitutively expressed PCA within 24 hours of exposure. PCA induced by IFN-γ, IL-1β, and TNF-α depended largely on tissue factor expression, as evidenced by experiments with factor X-deficient plasma and antithrombin factor antibodies. In macrophages subcultured in adherence, IL-1β was a strong PCA inducer, whereas IFN-γ and TNF-α promoted little PCA increase. This observation and different kinetics of PCA induction suggested that mechanisms of PCA induction are distinct for the three cytokines. Thus, we showed that well-characterized cytokines critically involved in the promotion of cell-mediated antimicrobial defense/delayed-type hypersensitivity and considered for clinical application promote local fibrin deposition by a direct effect on macrophages.

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MONOCYTES AND MACROPHAGES are unique, among leukocytes, in their capacity to express enhanced levels of procoagulant activity (PCA) upon appropriate stimulation. PCA is assumed to be of pathogenetic significance in a variety of clinical disorders, including gram-negative sepsis and delayed-type hypersensitivity reactions. An enhanced level of PCA expression was found to be the most important host factor leading to death in experimental infection with murine hepatitis virus. Enhanced mononuclear phagocyte-associated PCA is a hallmark of many disease conditions and may serve as a prognostic factor. PCA is mediated to a large extent by cell surface-associated tissue factor, although several other factors of the extrinsic coagulation pathway were reported to contribute to macrophage PCA.

At least two major pathways of stimulation lead to the induction of elevated levels of cell surface-associated PCA. First, PCA is induced by lipopolysaccharide (LPS) of gram-negative bacteria and possibly by other microbial (particularly bacterial) constituents. Enhanced mononuclear phagocyte-associated PCA is a hallmark of many disease conditions and may serve as a prognostic factor. PCA is mediated to a large extent by cell surface-associated tissue factor, although several other factors of the extrinsic coagulation pathway were reported to contribute to macrophage PCA.

In this study, we have determined the effect of various human recombinant cytokines on induction of macrophage PCA under comparable assay conditions that have been optimized for PCA induction by IFN-γ as a prototype cytokine. The study points to IFN-γ, IL-1β, and TNF-α as being major macrophage-inducing cytokines. Transforming growth factor-β, in contrast, decreased macrophage PCA. The effects of all other tested cytokines could not be distinguished from that of contaminating endotoxin.

MATERIALS AND METHODS

Reagents. The sources of the cytokines used in this study, and their bioactivities, are listed in Table 1. Media and additives for cell culture were from Seromed (Munich, Germany) or from Gibco-Life Technologies (GIBCO; Basel, Switzerland). Fresh human AB serum and platelet-poor plasma were provided by the Blood Transfusion Service (Berne, Switzerland). Human serum albumin (HSA) was from Behringwerke (Marburg, Germany). LPS from Escherichia coli O55:B5 and O111:B4 and factor X-deficient plasma were bought from Sigma Chemical Co (St Louis, MO). LPS from E coli O55:B5 was obtained from Sken (Allschwill, Switzerland) as part of the LAL kit. Goat-antihuman tissue factor was from American Diagnostica (Greenwich, CT).

Isolation and cultivation of mononuclear phagocytes. Mononuclear phagocytes were isolated fromuffy coats obtained from healthy volunteer blood donors by isopycnic centrifugation on Ficoll-Hypaque (Seromed). Exposure of cells to endotoxin was avoided by monitoring all media and buffer solutions to which cells were exposed for endotoxin, using a sensitive LAL assay (see be-
low). Reagents and composite solutions promoting accelerated LAL coagulation were excluded. Mononuclear cells were adjusted to 10^6 monocytes per milliliter in modified RPMI 1640 containing 2% homologous heat-inactivated (30 minutes at 56°C) serum. Modified RPMI 1640 contained an additional 2 mmol/L glutamine, penicillin (100 IU/mL), streptomycin (100 μg/mL), vitamins for minimum essential medium (GIBCO; 0.4% vol/vol), nonessential amino acids (GIBCO; 1% vol/vol), sodium pyruvate (1 mmol/L), and was buffered with HEPES (10 mmol/L) to pH 7.4. Cells were placed into tissue culture flasks (75 cm^2; Falcon, Basel, Switzerland). After 1 hour at 37°C in a humidified CO_2 (5%) incubator, adherent monocytes were freed of lymphocytes by repeated rinsing, and were cultured overnight in the same medium fortified by another 5% of heat-inactivated human serum. Then cells were dislodged by rinsing with chilled phosphate-buffered saline and recovered by centrifugation. Harvested cells were greater than 92% monocytes, as judged by staining with Turk's solution and for nonspecific esterase activity. Cells were cultured at 0.5 × 10^6/mL in the previous medium fortified by another 5% of heat-inactivated serum in bags made from teflon foil (FEP 100A; Dupont de Nemours, Geneva, Switzerland) in a humidified CO_2 incubator at 37°C.

At day 7 or 8 after cell isolation, cells were harvested, washed with saline, and resuspended at the concentration required for the PCA assay. At this time, the yield averaged to 50% of the cells originally placed into the teflon bag, the viability was greater than 90% (usually between 95% and 99%), and the population consisted of approximately 95% macrophages as determined by morphology, staining for nonspecific esterase and by flow cytometry, using a FACScan (Becton Dickinson, San Jose, CA). Fluorescence-labeled antibodies characterizing macrophages included anti-CD14 (DAKO, Glostrup, Denmark) and OKM5 (Ortho, Raritan, NJ). Antibodies characterizing other leukocyte subsets were from Becton Dickinson. Occasionally, some free cell nuclei that stained with propidium iodide, but not with trypan blue or with any leukocyte surface-marker-specific antibodies, were also observed.

**Stimulation by cytokines.** At predetermined intervals before harvesting of macrophages, teflon bags were opened for the addition of cytokines at various concentrations, and reslated. Negative or positive control cultures consisted of macrophages that did not receive cytokines, or that were stimulated with a protein source. Cytokines, encephalomyocarditis virus; CPE, cytopathic effect; VSV, vesicular stomatitis virus; MT, murine thymocyte costimulation; EBV, Epstein-Barr virus; BMM, bone marrow-derived macrophage; P, tested by the producer or supplier; E, tested by an external expert; H, tested in our laboratory.

### Table 1. Bioactivity of Cytokines Used in This Study

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Supplier/Producer</th>
<th>Specific Activity/ED_{50}</th>
<th>Test System</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>CBP</td>
<td>24 × 10^4 U/μg</td>
<td>Inhibition of EMCV</td>
</tr>
<tr>
<td>IFN-α</td>
<td>Roche</td>
<td>2 × 10^4 U/μg</td>
<td>Propagation in WISH cells</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Cetus</td>
<td>3 × 10^4 U/μg</td>
<td>CPE induced by VSV</td>
</tr>
<tr>
<td></td>
<td>Bachem</td>
<td>2,600 U/μg</td>
<td>L529 cell cytotoxicity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ED_{50}: 0.2 ng/mL</td>
<td>L529 cell cytotoxicity</td>
</tr>
<tr>
<td>IL-1β</td>
<td>NIBSCO</td>
<td>10^4 U/μg</td>
<td>MTC</td>
</tr>
<tr>
<td>IL-2</td>
<td>GIBCO</td>
<td>5 × 10^4 U/μg</td>
<td>CTL7 cell proliferation assay</td>
</tr>
<tr>
<td></td>
<td>CBP</td>
<td>7.3 × 10^4 U/μg</td>
<td>CTL7 cell proliferation assay</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ED_{50}: 150 U/mL</td>
<td>OVA7 cell proliferation assay</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>5 × 10^2 U/μg</td>
<td>CTL7 cell proliferation assay</td>
</tr>
<tr>
<td></td>
<td>Cetus</td>
<td>18 × 10^2 U/μg</td>
<td>CTL7 cell proliferation assay</td>
</tr>
<tr>
<td>IL-4</td>
<td>Sandoz</td>
<td>5 × 10^5 U/μg</td>
<td>MTC</td>
</tr>
<tr>
<td>IL-6</td>
<td>NIBSCO 89/514</td>
<td>5 × 10^4 U/μg</td>
<td>Stimulation of EBV-transformed B lines</td>
</tr>
<tr>
<td></td>
<td>ED_{50}: 0.2 U/μg</td>
<td>10 × 10^5 U/μg</td>
<td>Stimulation of EBV-transformed B lines</td>
</tr>
<tr>
<td></td>
<td>NIBSCO 89/548</td>
<td>5 × 10^4 U/μg</td>
<td>Stimulation of EBV-transformed B lines</td>
</tr>
<tr>
<td>TGF-β</td>
<td>CC</td>
<td>ED_{50}: 0.6 μg/ml</td>
<td>Inhibition of MTC</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Cetus</td>
<td>5 × 10^5 U/μg</td>
<td>Mouse BMM proliferation assay</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Sandoz</td>
<td>1.1 × 10^4 U/μg</td>
<td>M07E cell proliferation assay</td>
</tr>
</tbody>
</table>

**Abbreviations:** Suppliers of cytokines: CBP, Collaborative Biomedical Products (Bedford, MA); Roche, Hoffmann-La Roche (Basel, Switzerland); Cetus, EuroCetus (Amsterdam, The Netherlands); Bachem (Bubendorf, Switzerland); NIBSCO, National Institute for Biological Standards and Control (Potters Bar, Hertfordshire, UK); GIBCO, Gibco-Life Technologies (Basel, Switzerland); CC, Crystal Chem (Chicago, IL); Sandoz (Basel, Switzerland).
PCA assay. PCA was determined by a turbidimetric, kinetic recalcification time assay.\textsuperscript{34} Washed macrophages (0.75 to 3 × 10\(^4\) in 100 \(\mu\)L saline) were dispensed into a prewarmed (37°C) microtiter plate also containing 100 \(\mu\)L of pooled platelet-poor plasma. After 20 minutes, these wells and calibration wells containing various concentrations of thromboplastin (Thromborel S; Behringwerke, Marburg, Germany) were given 100 \(\mu\)L CaCl\(_2\) (25 mmol/L) by a multichannel pipette, and the plate was placed immediately thereafter in a thermostated (37°C) enzyme-linked immunosorbent assay (ELISA) reader. The increase in turbidity was registered over 20 minutes at 365-nm wavelength. Recalcification times, deduced from maximal slopes of the sigmoid turbidity curves, were converted to thromboplastin units (TU), with 1 TU corresponding to the recalcification time obtained with a 10\(^4\)-fold dilution of thromboplastin. Recalcification time was determined for thromboplastin between 1 and 10\(^4\) TU, 1 TU and 10\(^4\) TU corresponding to a recalcification time of 300 to 500 seconds and of 20 to 25 seconds, respectively. In most experiments (eg, Figs 2 to 4), three cell concentrations (3 × 10\(^4\), 1.5 × 10\(^4\), and 0.75 × 10\(^4\) cells per well, each assayed in triplicate) were used, allowing the calculation of specific PCA (TU per 10\(^4\) cells) on the basis of the linear regression slope. In some experiments (eg, Figs 1 and 5) specific activity was determined with 3 × 10\(^4\) cells per well only. PCA of stimulated cells was also expressed as a stimulation index, ie, PCA of stimulated cells:PCA of nonstimulated control cells cultured under otherwise identical conditions.

In some assays (eg, Fig 6), PCA was determined in microtiter plates containing monolayer cultures of macrophages. Here, culture medium was replaced by 100 \(\mu\)L prewarmed (37°C) saline, and the plate was thermostated at 37°C. Then, 100 \(\mu\)L plasma was added, followed by the addition of calcium chloride and reading 20 minutes later.

Cytokine assays. TNF was determined by the L929 cell cytotoxicity assay as described.\textsuperscript{39} In our hands, 50% of L929 cells were lysed within 18 hours by 5 to 8 NIBSCO U/mL (0.5 to 0.8 U/well). IL-1\(\beta\) activity was measured in a thymocyte costimulatory assay, using cultures of thymocytes from C3H/HeJ mice supplemented with PHA (1.25 \(\mu\)g/mL).\textsuperscript{30,40} The thymocyte costimulatory assay was also used to determine activity of recombinant human (rhu) TGF-\(\beta\), which dose-dependently inhibits thymidine incorporation induced by optimal concentrations of IL-1\(\beta\).\textsuperscript{39} Macrophage colony-stimulating activity was determined by a proliferation assay, using murine bone marrow-derived macrophages generated by culturing cells collected from the tibiae of C3H/HeJ mice for 6 days in teflon bag cultures as described.\textsuperscript{41} IL-6 and IL-2 activity was kindly determined by Dr Karl Frei (University of Zürich), using 7TD1 cells\textsuperscript{52} and OVA7 cells,\textsuperscript{43} respectively, in a proliferation assay. The activity of IL-4 was documented (1) by induction of IgE production in B-cell cultures supplemented with EL-4 cells and T-cell supernatants,\textsuperscript{44} and (2) by the downregulation of macrophage CD14 over a 24-hour culture period, as evidenced by flow cytometry. The bioactivity of IFN-\(\gamma\) was asserted by the reduction of IgG-mediated erythrophagocytosis in macrophages, as described earlier.\textsuperscript{45}

LAL assay. A kinetic, turbidimetric variant of the LAL assay was used to monitor for the presence of endotoxin contaminants in cytokine preparations and in all reagents to which monocytes or macrophages were exposed. The assay was performed essentially as described.\textsuperscript{44} Briefly, 50 \(\mu\)L of a sample to be tested was added to microtiter plate wells containing 50 \(\mu\)L of LAL (Haemachem, St Louis, IL) and the increase in turbidity was monitored immediately thereafter in a kinetic ELISA reader thermostated at 37°C. Endotoxin from \textit{E. coli} O55:B5 was used as an endotoxin standard. The sensitivity of the assay was approximately 2 pg/mL for serum-free solutions. Serum-containing media could be assayed at a 20-fold dilution at the highest, which reduced the detection sensitivity accordingly.

RESULTS

Responsiveness of suspended monocyte-derived macrophages to LPS. Cultured monocytes that had differentiated to macrophages expressed a PCA activity of 2.8 ± 2.0 TU/10\(^3\) cells (n = 75), which was considerably higher than that of fresh monocytes (<0.1 TU/10\(^3\) cells). Macrophages exposed to LPS (1 \(\mu\)g/mL) for 6 hours in suspension and then tested for PCA, expressed 40.3 ± 24.6 TU/10\(^3\) cells (n = 32); the stimulation index for optimally LPS-stimulated cells (1 \(\mu\)g/mL for 6 hours) was 14.4 ± 7.5 (n = 32). The elevated PCA of macrophages appeared to be constitutive and not caused by inadvertent stimulation by subdetectable traces of endotoxin, because supernatants of control cells did not contain detectable TNF-\(\alpha\) activity (Fig 1). Moreover, varying concentrations of LPS added to suspension cultures significantly enhanced PCA and TNF production down to a concentration of 10 to 20 fg/mL (Fig 1 and data not shown).

Bioactivity of cytokines. Most cytokines were tested for bioactivity either in our laboratory, or by a neutral external institution (Table 1). IL-1\(\beta\) (GIBCO) and TNF-\(\alpha\) (Bachem) had no detectable activity up to 40 ng/mL and 2 \(\mu\)g/mL, respectively, as determined twice in bioassays. All others displayed bioactivity in the expected concentration range. From all cytokines tested, the degree of endotoxin contamination was determined by a sensitive turbidimetric LAL assay. The two cytokines with the highest LAL activity when compared with their bioactivity (IL-2 from GIBCO and IFN-\(\alpha\) from Collaborative Biomedical Products) were excluded from further studies. Most cytokines had low, but detectable pyrogenic activity, allowing an assessment of PCA activity in the cytokine activity range yet below an endotoxin concentration significantly influencing the results (Table 2). IL-1\(\beta\) from GIBCO did not contain detectable LAL activity.

Comparison of different culture conditions for determini-
Cytokines and Macrophage Procoagulants

An optimal IFN-γ concentration was determined by bioassays (see Tables 1 and 2). Purified monocytes that had matured to macrophages in nonadherent (teflon) containers were tested for PCA expression following stimulation by IFN-γ under distinct culture conditions. Cells were either stimulated in nonadherent cultures, or were harvested, washed, and subcultured in tubes that were rotated for 24 hours in medium containing either HSA or AB serum. Plates: Harvested, washed cells were subcultured in microtiter plates for 24 hours in medium containing either HSA or AB serum. Results represent means of two experiments, each performed in triplicate.

Effect of various cytokines on induction of PCA in macrophages. Based on the previous experiments, macrophages cultured in teflon bags for 7 to 9 days in the presence of homologous serum (15%) were exposed to various cytokines at varying concentrations and were assayed for PCA expression at various intervals after cytokine stimulation. The selected cytokine concentrations were aimed at displaying an expected bioactive cytokine concentration. LAL activity is expressed in pg/mL of endotoxin E. coli 055:B5.

Table 2. Endotoxin Activity of Cytokines as Determined by LAL Assay

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Supplier/Producer [lot]</th>
<th>LAL Activity per Tested Dose (pg/mL)</th>
<th>LAL Activity of Maximal Concentration Used in PCA Assay (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>CBP 11.7 pg/50 × 10^4 U</td>
<td>0.12</td>
<td>0.12</td>
</tr>
<tr>
<td>IFN-α</td>
<td>Roche 6.6 pg/50 × 10^4 U</td>
<td>0.13</td>
<td>0.13</td>
</tr>
<tr>
<td>IFN-α</td>
<td>CBP 278.7 pg/10 × 10^4 U</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Cetus 8.6 pg/75.4 × 10^4 U</td>
<td>0.034</td>
<td>0.034</td>
</tr>
<tr>
<td>IL-1β</td>
<td>NIBSCO 28.9 pg/2 × 10^4 U</td>
<td>1.34</td>
<td>1.34</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Gibco 5.9 pg/80 × 10^4 U</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>IL-2β</td>
<td>Gibco 624 pg/20 × 10^4 U</td>
<td>9.36</td>
<td>9.36</td>
</tr>
<tr>
<td>IL-2β</td>
<td>Cetus 89.7 pg/10 × 10^4 U</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IL-3</td>
<td>CC 7.2 pg/2.5 μg</td>
<td>0.029</td>
<td>0.029</td>
</tr>
<tr>
<td>IL-4</td>
<td>CBP 4.4 pg/10 × 10^4 U</td>
<td>0.13</td>
<td>0.13</td>
</tr>
<tr>
<td>IL-6</td>
<td>NIBSCO 6.3 pg/10 × 10^4 U</td>
<td>0.19</td>
<td>0.19</td>
</tr>
<tr>
<td>TGF-β</td>
<td>CC 8.8 pg/1 μg</td>
<td>0.068</td>
<td>0.068</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Cetus 7.5 pg/10 × 10^4 U</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Sandoz 5.4 pg/23 × 10^4 U</td>
<td>0.023</td>
<td>0.023</td>
</tr>
</tbody>
</table>

Boldface type denotes cytokines with a LAL activity > 0.2 pg/mL for an expected bioactive cytokine concentration. LAL activity is expressed in pg/mL of endotoxin E. coli 055:B5.

Abbreviation: ND, not done.

Expression was maximal around 24 hours (Fig 3). Based on a larger set of experiments (n = 10), an average stimulation index of 11.4 was obtained, which corresponded to a shortening of recalcification time from 306 ± 72 seconds to 98 ± 24 seconds. TNF-α (Cetus) consistently induced dose-dependent PCA, albeit to a lower degree than IFN-γ; TNF-α-induced PCA peaked after 6 hours and required relatively large cytokine concentrations (Fig 3). PCA induction of similar kinetics was obtained with IL-1β (Fig 3). The dose-

![Fig 2. Specific PCA of IFN-γ-stimulated (M: 500 U/mL for 24 hours) and control (C) macrophages cultured under different conditions. Teflon bags: Suspension cultures in teflon bags were exposed to IFN-γ. Tubes: Teflon bag cultured cells were harvested, washed, and subcultured in tubes that were rotated for 24 hours in medium containing either HSA or AB serum. Plates: Harvested, washed cells were subcultured in microtiter plates for 24 hours in medium containing either HSA or AB serum. Results represent means of two experiments, each performed in triplicate.](www.bloodjournal.org)

![Fig 3. PCA induced by IFN-γ, IL-1β, or by TNF-α in macrophages cultured in teflon bags. Results represent means ± 4 SD, each determined in triplicate.](www.bloodjournal.org)
response patterns showed lot-specific variations for IL-1 (Fig 3 and data not shown). The high potency of IL-1β (NIBSCO) was explained by its high degree of endotoxin contamination (Table 2), the low activity of IL-1β GIBCO by its lack of bioactivity (Table 1). IL-1β (CBP), which had similar bioactivity as the NIBSCO preparation yet a lower degree of endotoxin contamination, showed strong PCA-inducing power at high concentrations (Fig 3); the peak was also obtained after 6 hours. IFN-γ, IL-2 (Cetus), IL-6, and IL-10 induced little PCA, the highest activity being obtained after 6 hours of exposure (not shown). IL-2 from GIBCO had PCA-inducing activity at relatively high concentrations (not shown), an effect probably caused by its high level of endotoxin contamination (Table 2). M-CSF–induced PCA was moderate and varied between donors. It did not show the rapid decline after 6 hours observed for most other cytokines (not shown). IL-4 (CBP) lacked any detectable PCA-inducing power up to 30 ng/mL (Fig 4 and data not shown), although it had the expected bioactivity. The bioassays used were a B-cell stimulation system, and IL-4–mediated downregulation of macrophage CD14 within 24 or 48 hours, as evidenced by flow cytometry (data not shown). TGF-β (Crystal Chem) not only lacked PCA-inducing power, it reduced “constitutive” PCA of d8 macrophages to a virtually undetectable level within 48 hours (Fig 4).

Possible effects of contaminations of microbial origin. In the present culture system, macrophage PCA was induced by LPS at concentrations greater than 100-fold lower than the lowest concentration detectable in the LAL assay (Fig 1). Therefore, the heat lability of cytokines was used as an independent criterion to document a cytokine-mediated rather than an endotoxin-mediated effect. Heat inactivation was performed as recommended, and was found to preserve PCA-inducing activity of purified LPS, but to completely destroy TNF-α and IL-1β bioactivity as shown in cytotoxicity and thymocyte costimulatory assays, respectively (not shown). The reduction in PCA-inducing activity of cytokines by heat-inactivation is shown in Fig 5, which confirms that among the tested cytokines, only IFN-γ, IL-1β, and TNF-α have significant PCA-inducing capacity for macrophages. All other cytokine preparations had similar PCA-inducing activity after heating, suggesting that their moderate PCA-inducing effect was caused by trace contaminants of these preparations.

Evidence for distinct cytokine-mediated PCA induction pathways. The distinct kinetics of PCA induction (Fig 3) suggest that monokine-mediated PCA induction is mediated by a pathway distinct from that of IFN-γ–induced PCA. A similar observation was made in experiments with macrophages subcultured in microtiter plates. Such cells showed a weak response to IFN-γ and to TNF-α, but a strong response upon 6-hour stimulation with IL-1β, regardless of whether medium contained autologous serum (15%) or HSA (1%) (Fig 6 and data not shown). Although IL-1β–induced PCA in microtiter-cultured macrophages varied considerably, the higher PCA of IL-1β–exposed cells as opposed to cells subjected to all other treatment was consistent.

**DISCUSSION**

It is well established that products of activated T cells induce PCA in mononuclear phagocytes, but the mediators have been incompletely characterized. This report represents the first study in which a broad panel of recombinant human cytokines has been tested for induction of PCA in human macrophages. Other advantages over earlier studies are (1) use of cells cultured in suspension, thus excluding adherence-related effects possibly superseding cytokine activity; (2) use of phenotypically stable macrophages instead of monocytes prone to "spontaneously" express high PCA; (3) independent proof of bioactivity for the tested cytokines; (4) inclusion of heat inactivation controls to rule out toxic contaminants (Fig 6 and data not shown). The high PCA-inducing activity of purified LPS, but to completely destroy TNF-α and IL-1β bioactivity as shown in cytotoxicity and thymocyte costimulatory assays, respectively (not shown). The reduction in PCA-inducing activity of cytokines by heat-inactivation is shown in Fig 5, which confirms that among the tested cytokines, only IFN-γ, IL-1β, and TNF-α have significant PCA-inducing capacity for macrophages. All other cytokine preparations had similar PCA-inducing activity after heating, suggesting that their moderate PCA-inducing effect was caused by trace contaminants of these preparations.

**Fig 4.** PCA macrophages exposed to IL-4 (10 ng/mL) or TGF-β (1 ng/mL for varying intervals in suspension [telfon bag] cultures. Results represent means ± SD, each determined in triplicate.

**Fig 5.** PCA induced by various native (■) and heat-inactivated (○) recombinant cytokines. Optimal concentrations were used as determined in separate experiments (Fig 3 and data not shown). IFN-γ and TGF-β were applied for 24 hours, all other cytokines for 6 hours. LPS: 0.4 pg/mL LPS E. coli 055:B5. *Statistically significant difference (P < .01) between native and heated preparation.
IFN-γ-stimulated cultures. We conclude that IFN-γ induces PCA without involvement of IL-1 and TNF-α. Moreover, the strong PCA induction in the absence of conspicuous IL-1 and TNF-α production suggests that these distinct markers of macrophage activation are independently regulated, although being induced, in part, by the same activating agent(s).

Apart from IFN-γ, a mediator derived from Th1 type CD4 cells,49 or from CD8 cells,50-52 no other T-cell-derived cytokine with PCA-inducing activity could be discerned. This appears to include even IL-2, which has been reported to induce PCA in monocytes.27,29 In our hands, the effect of IL-2 could not be distinguished from that of contaminating endotoxin. Similarly negative results include granulocyte-macrophage CSF (GM-CSF), IL-3, IL-4, and IL-10. TNF-β was not tested, but is likely to be positive, in view of its shared receptors or shared activity spectrum with TNF-α.22,53

Among cytokines produced by mononuclear phagocytes themselves, TNF-α and IL-1β were strong PCA inducers, and the observed effects were, at least in part, not related to contaminations with low concentrations of endotoxin, as shown by heat inactivation experiments (Fig 5). The PCA-inducing concentrations for both mediators were an order of magnitude higher than that required for the conventional bioassays using murine cells. The results with TNF-α confirm those of earlier studies suggesting that this cytokine induces PCA in mononuclear phagocytes.29,30 Likewise, IL-1 has earlier been reported to induce PCA in monocytes.26-28 Notably, these two monokines induce PCA not only in mononuclear phagocytes but also in endothelial cells.54-56 PCA induced by monokines, particularly IL-6 and the hematopoietic growth factors M-CSF and GM-CSF, was low and could not be distinguished from that of traces of endotoxin contaminating the cytokine preparations. Whether these cytokines, which are almost inactive by themselves, are able to modulate LPS-induced PCA is under current investigation. Of particular interest is that TGF-β not only lacked PCA-inducing activity, but was able to reduce, but not fully, LPS-induced PCA. This activity was not shared by IL-4 and IL-10, which also downregulate macrophage functions such as the expression of surface membrane markers and the production of cytokines.57-65 However, IL-4 was recently reported to downregulate tissue factor expression by endothelial cells and monocytes after their exposure to LPS, IL-1β, or TNF-α.66

A potential role of contaminating endotoxin in contributing to cytokine-induced PCA deserves a comment in view of the exquisite sensitivity of macrophages to LPS (eg, Fig 1). Sensitivity exceeds that of the LAL test by 2 orders of magnitude when cells are stimulated in suspension (this study) but is lower when cells are stimulated in adherence (T.W.J., unpublished observation). The observation that monocyte-derived macrophages are not stimulated by subdetectable traces of endotoxin-contaminating media and culture vessel is supported by the reproducible finding that the addition of 10 ng/mL of LPS to the culture for 6 hours significantly elevates PCA and induces TNF bioactivity. A suspension system was required for this PCA study because the effect of some of the cytokines was superseded by an
adherence-induced effect (Figs 1 and 6). The most appropriate way to distinguish between a cytokine effect and an effect caused by trace contaminants is heat inactivation, because this abrogates reproducibly cytokine activity but not the PCA-inducing power of bacterial contaminants. Using this criterion, only IFN-γ, IL-1β, and TNF-α exert PCA-inducing power on their own. A survey of earlier reports suggesting that IL-2 and M-CSF also induce PCA in mononuclear cells27,28,31 shows that a role of trace contaminants was not ruled out with the same scrutiny. Likewise, other cytokines with putative inflammatory properties (GM-CSF, IL-6) also appear to be inactive in this system. Moreover, our study unexpectedly showed large differences with regard to the level of endotoxin contamination of commercial cytokines, including preparations used as international standards. Mononuclear phagocyte-expressed PCA was shown to be caused by enhanced expression of tissue factor, although a number of other factors of the extrinsic coagulation pathway may contribute.1,13-17 Some of these monocyte constituents are activation-dependent but tissue-factor independent.15 Using factor X-deficient plasma exerting no accelerated coagulation by the addition of tissue factor, macrophages exposed to IFN-γ or LPS shortened the coagulation time of factor X-deficient plasma more than unstimulated control cells,22 which is consistent with the notion that a portion of PCA is tissue-factor independent. This earlier finding was confirmed for LPS-induced PCA, but could be substantiated neither for IL-1β-induced nor for TNF-α-induced PCA (T.W.J., unpublished observation, 1992): (1) Using factor X-deficient plasma, monokine-stimulated cells exerted PCA levels indistinguishable from cell-free wells containing amounts of fluid-phase tissue factor equivalent to those expressed by stimulated cells. (2) Cytokine-induced PCA was completely neutralized by a polyclonal antitissue factor antiserum. Although these observations are open to several interpretations, we conclude that IL-1β and TNF-α induce the expression of tissue factor in macrophages as they do in endothelial cells.54,56 To what extent cytokine-stimulated macrophages enhance coagulation caused by the orchestrated and surface-focused assembly of the prothrombinase complex15,16 in addition to tissue-factor expression is open.

The low PCA-inducing power of IFN-α could not be distinguished with certainty from an endotoxin-mediated effect. When macrophages were exposed to IFN-α over extended periods of time (48 hours), PCA decreased to lower levels than in untreated control cells, thus being reminiscent of the PCA inhibitory activity earlier described.33 Whether this is caused by a direct IFN-α effect or by an indirect regulatory mechanism is not known. Macrophages exposed to viral antigens or infected with viruses generate IFN-α. A recent study of our laboratory suggested that canine brain macrophages infected in vitro with dog distemper virus elicit PCA without involvement of T cells.35 If human macrophages react in an analogous manner as did canine glial cells, one may assume that virus-induced PCA is not caused by an IFN effect.

In conclusion, we show that among cytokines, the ones characterizing a cell-mediated immune reaction associated with macrophage activation are the only ones capable of inducing strong macrophage procoagulant-inducing activity. This is consistent with fibrin being associated with delayed inflammatory lesions, particularly with macrophages from such lesions.26,66,69 In addition, these cytokines are more often than not hallmarks of autoimmune or alloimmune processes,70,71 some of which have been shown to be associated with enhanced procoagulant activity of mononuclear cells.8

The proof of macrophage PCA-inducing activity for IFN-γ, IL-1β, and TNF-α does not exclude a role for "macrophage procoagulant inducing factor," a hitherto little characterized cytokine.23 Moreover, because relatively high concentrations of IL-1β and TNF-α are required for inducing macrophage PCA, an investigation on cytokine synergism using the present in vitro system appears worthwhile. Our study shows that at least in humans, some well-characterized cytokines available in recombinant form and being of clinical interest exert powerful macrophage procoagulant-inducing activity and must be considered as potentially thrombogenic agents in vivo, acting on both endothelial cells and mononuclear phagocytes. Moreover, because cytokines such as TNF-α and IL-1β exert endocrine effects, a locally constrained bacterial infection may mediate more generalized hemorrhagic complications by the induction of monokines.

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