Interleukin-1 Induces Tumor Necrosis Factor (TNF) in Human Peripheral Blood Mononuclear Cells In Vitro and a Circulating TNF-like Activity in Rabbits

Takashi Ikejima, Seijiro Okusawa, Pietro Ghezzi, Jos W. M. van der Meer, and Charles A. Dinarello

Recombinant human interleukin-1 (IL-1), injected into rabbits, induces the synthesis of endogenous IL-1. Also, IL-1 induces its own gene expression and synthesis in human peripheral blood mononuclear cells (PBMC). In this study, tumor necrosis factor-α (TNF-α) production by PBMC of 40 individuals stimulated with IL-1α or IL-1β was determined by specific radioimmunoassay (RIA). After 3 h of PBMC incubation with IL-1, TNF-α mRNA was detected. IL-1α stimulated both IL-1β and TNF-α, but there was no correlation in the amount of TNF-α or IL-1β synthesized in the PBMC of 29 individuals. IL-1-stimulated adherent cells produced ~50% more TNF-α than did unfractionated PBMC. Coincubation with interferon-γ (IFN-γ) did not change the amount of IL-1-induced TNF-α, whereas in the same culture IFN-γ inhibited (70%) IL-1-induced IL-1 production. Endogenous pyrogen and TNF-like activity were detected in the sera of rabbits 3.5 h after injection of either IL-1α or IL-1β. These studies demonstrate that IL-1 induced TNF-α production in vivo and in vitro.

Previously, we have demonstrated that TNF-α induces circulating IL-1 in rabbits and synthesis from human peripheral blood mononuclear cells (PBMC) in vitro [12]. The same has been observed using human endothelial cells [26]. We recently showed that the expression of both IL-1α and IL-1β genes in human PBMC [27], human endothelial cells [28], and smooth muscle cells [29] is stimulated by IL-1 itself. In this study we test the possibility that IL-1 induces TNF-α in human PBMC and that IL-1 induces circulating TNF-like activity in the rabbit. Because IL-1-induced IL-1 production is inhibited by interferon-γ (IFN-γ) [30], we examined the effect of IFN-γ on IL-1-induced TNF-α production.

Materials and Methods

IL-1, TNF-α, and IFN-γ. Recombinant human (rh) IL-1α and IL-1β (gifts of Dr. Alan Shaw, Glaxo Institute for Molecular Biology, Geneva, Switzerland) had specific activities of 6.1 and 5.3 × 10^7 units/mg, respectively, as measured with thymocytes in a costimulation assay. IL-1 preparations contained <100 pg of lipopolysaccharide (LPS)/mg of protein as measured by the Limulus amebocyte lysate test (Cape Cod Associates, Woods Hole, MA), which has a limit of detection of 15 pg/ml LPS. The source of rhTNF-α has been previously described [12]. IFN-γ, produced in Escherichia coli, was the same as used in previous studies [30]. LPS from E. coli was purchased from Sigma Chemical (St. Louis).

Specific radioimmunoassays for human TNF-α and IL-1β. Methods for radioimmunoassay (RIA) for human TNF-α [31] and IL-1β [32] have been reported. The sensitivities of these RIAs were 40 and 80 pg/ml, respectively.

Human PBMC. Blood from healthy male volunteers was drawn into heparinized syringes. PBMC, separated by centrifugation over ficoll-hypaque (Pharmacia, Piscataway, NJ; Winthrop Laboratories, New York), were suspended at 5 × 10^6/ml in RPMI 1640 medium (M. A. Bioproducts, Walkersville, MD) that had been subjected to
ultrafiltration to remove endotoxins as previously described [33]. Heat-inactivated autologous human serum (2%) was added, and 100 μl of this suspension was placed in flat-bottomed 96-well microtiter plates containing an equal volume of IL-1 diluted in RPMI 1640 medium.

In some experiments, IFN-γ (1-1000 units/ml) was added to the cell culture. In other experiments, microtiter plates were coated with autologous sera at room temperature overnight. After removing the sera, 100 μl of PBMC suspension was added and incubated at 37°C for 1.5 h in a humidified 3% CO₂ atmosphere. Nonadherent cells were removed by washing three times in RPMI 1640 medium, and 200 μl of RPMI 1640 medium containing 1% autologous serum and IL-1 (α or β) was added to each well. More than 90% of adherent cells were positive for α-naphthyl acetate esterase activity as detected by the cytologic demonstration kit (Sigma). These cells were incubated for various time periods. Culture supernatants or lysates prepared by three freeze-thaw cycles [32] were assayed for TNF-α and IL-1β.

**Rabbit antihuman antibodies.** Rabbits were initially immunized with rHL-1 (α or β) by multiple intradermal injections in complete Freund's adjuvant. Six weeks later, the animals were boosted by four weekly intradermal injections of IL-1 in incomplete Freund's adjuvant, followed by four weekly intravenous injections. Serum was precipitated in 40% saturated ammonium sulfate, and these immunoglobulin preparations neutralized IL-1 activity as measured in the D10.G4.1 cell bioassay [27].

**Isolation of human RNA and Northern blot analysis.** Human PBMC were suspended in cold ultrafiltered RPMI 1640 medium with 1% human AB serum at 2.5 × 10⁶ cells/ml in 50-ml polypropylene centrifuge tubes (Falcon, Oxnard, CA). Cells were stimulated with 100 ng/ml IL-1α, and tubes containing 30 ml of the cell suspension were gently rotated at 0.25 rpm inside a bottomless roller bottle at 37°C for 3 h. Cells were collected after centrifugation at 500 g, and the cell pellet was lysed in guanidine isothiocyanate. Total RNA was recovered after centrifugation through CsCl. After ethanol precipitation, the RNA content was measured spectrophotometrically using an absorbance of 260:280 nm.

RNA samples (15 μg each) were subjected to electrophoresis in 1.2% agarose (Bethesda Research Laboratories, Rockville, MD) containing 2.2 M formaldehyde. After examining the ethidium-stained gel directly under ultraviolet light irradiation (260–360 nm) at the end of the electrophoresis, the RNA was transferred to nylon membranes (Amersham, Arlington Heights, IL) and fixed by ultraviolet irradiation. Membranes were probed with 3²P-labeled 1.1-kilobase (kb) fragment (SsrI to PstI) of full-length cDNA clone of the human IL-1β precursor [5] or the human TNF-α cDNA fragment. The latter probe (Du Pont Pharmaceuticals, Wilmington, DE) was a 470-base pair (bp) fragment (bp 381-854) of the full-length cDNA. 3²P-labeled probes were prepared using a random primed DNA labeling kit (Boehringer Mannheim, Mannheim, FRG).

The specific activity of the resulting DNA was ~1 × 10⁶ counts per minute (cpm)/μg. Since mRNAs coding for human TNF-α and IL-1β were detected at the same loci (1.6 kb) in Northern blot analyses, hybridization with the TNF probe was done first, followed by the hybridization with the IL-1β probe. Strong stripping solution containing 2.5% formamide, 0.5% standard saline citrate, 0.05% Denhardt's solution, 5 × 10⁻⁴ M EDTA, and 0.5% sodium dodecyl sulfate was used for stripping the radioactive cDNA probe for TNF-α.

**Bioassay for TNF-like activity in the rabbit.** TNF activity was measured by its cytotoxicity for the tumorigenic murine fibroblast L929 (ATCC CCL1: American Type Culture Collection, Rockville, MD) [34]. Briefly, L929 cells were seeded in flat-bottomed 96-well microtiter plates (Becton Dickinson, Oxnard, CA) at a density of 5.5 × 10⁵ cells/well in RPMI 1640 medium containing 5% fetal calf serum (FCS; Hyclone Laboratory, Logan, UT), 2 mM t-glutamine, and 100 units/ml penicillin and 100 μg/ml streptomycin. After overnight incubation at (37°C) the medium was removed and known amounts of rhTNF-α and samples, each containing actinomycin D (2 μg/well; Sigma), were added. Plates were then further incubated for 20 h, after which the supernatant was removed and the cells were stained in 0.1% crystal violet prepared in 100% methanol for 20 min.

Absorbance was measured at 600 nm by Dynatech MR 600 Microelisa Auto Reader (Dynatech, Alexandria, VA). The mean absorbance of eight duplicates was calculated for each sample or control. Cytotoxicity was calculated as follows: % cytotoxicity = [1 − (absorbance of sample/absorbance of control)] × 100. An unpaired t test was used to show the significance of cytotoxicity on L929 cells with the rabbit sera.

**Results**

**Detection of TNF-α in IL-1-stimulated PBMC.** Cells from three donors were incubated with IL-1 (α or β) for 48 h, and total TNF-α production was measured by RIA. IL-1 (α or β) at 100 ng/ml induced similar amounts of TNF-α. The time course for TNF-α production (figure 1) shows that peak production occurred at 12 h and appeared stable for the following 36 h. We next studied PBMC from four donors incubated with IL-1 (α or β) over a broad dose range (1-100 ng/ml) for 24 h; results (figure 2) show that TNF-α production was maximal after stimulation with 100 ng/ml IL-1 (α or β).

We examined TNF-α production by PBMC stimulated with this dose of IL-1 in a larger population of donors (n = 20). TNF-α production was above basal levels (unstimulated) after incubation with either IL-1α or -1β in 18 donors (figure 3). We compared the production of TNF-α from these same cells stimulated with LPS. As figure 3 also shows, 1 ng/ml
LPS produced a similar amount of TNF-α (mean level, 2260 ± 140 pg/ml) from these 20 donors as did 100 ng/ml IL-1α or IL-1β.

Effect of antihuman IL-1 on LPS-induced TNF-α production. As reported previously [27], 0.01 mg/ml of these antibodies neutralize the biologic effects of 100 pg/ml IL-1 (α or β). Because response to LPS exceeds 100 pg/ml IL-1, we chose a higher concentration of immunoglobulin (G + M) (100×) to attempt to inhibit the TNF response to LPS. TNF-α synthesis was not affected by the presence of both anti-IL-1α and anti-IL-1β in the culture, suggesting that LPS induction of TNF-α occurs independent of the autocrine effects of IL-1 (α or β) (table 1).

Production of TNF-α in IL-1-stimulated adherent cells. We also examined whether TNF-α induced by IL-1 was synthesized exclusively by monocytes and whether lymphocytes played a role in this induction. IL-1 (α and β) was added to adherent cells, and the TNF-α response was compared with that for PBMC culture. As shown in figure 4, TNF-α production by the adherent cells at 12 or 24 h was higher than that by PBMC, especially when the stimulant was IL-1α (50% higher).
Table 1. Effect of combined treatment with rabbit antihuman IL-1α and antihuman IL-1β immunoglobulin on TNF-α production in lipopolysaccharide (LPS)-stimulated human peripheral blood mononuclear cells (PBMC).

<table>
<thead>
<tr>
<th>Donor no.</th>
<th>Normal immunoglobulin</th>
<th>anti-IL-1α</th>
<th>anti-IL-1β</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.83</td>
<td>5.80</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>1.24</td>
<td>1.17</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>4.45</td>
<td>4.93</td>
<td></td>
</tr>
</tbody>
</table>

NOTE. PBMC were incubated in the presence of 1 mg/ml rabbit immunoglobulin (G + M) and were then stimulated with LPS (100 ng/ml). Total cytokine production was measured by radioimmunoassay. Normal immunoglobulin was prepared from unimmunized rabbits. LPS-stimulated PBMC from donors 1, 2, and 3 produced 5.66, 2.72, and 4.43 ng/ml IL-1α, respectively, in the presence of 1 mg/ml normal immunoglobulin.

Effect of IFN-γ on IL-1β and TNF-α production induced by IL-1α. In previous studies we demonstrated that IL-1 induced IL-1 in PBMC [27] and that IFN-γ inhibited this response [30]. In this study, we examined whether IFN-γ had a further effect on IL-1-induced TNF-α production. As figure 5 shows, coincubation of cells with various concentrations (1–1000 units/ml) of IFN-γ had no consistent effect on the production of IL-1α-stimulated TNF-α, whereas IL-1-induced IL-1 production was suppressed by >70%.

We also compared the effect of IFN-γ on IL-1α- and IL-1β-induced TNF-α production in PBMC from 20 human donors (figure 6). Again, IFN-γ had no consistent effect on IL-1-induced TNF. IFN-γ itself did not induce TNF production in these donors, in contrast to previous studies. We attribute this to the removal of endotoxin from the RPMI medium by ultrafiltration [33]. In addition, the failure of IFN-γ to enhance TNF production supports the fact that IL-1-induced TNF-α is not due to contamination by LPS.

No correlation between TNF-α and IL-1β production from human PBMC after stimulation with IL-1α. Production of TNF-α and IL-1β from PBMC was induced by 100 ng/ml IL-1α in another group of 29 donors (figure 7). TNF-α production was detected in 22 donors (limit of detection, 40 pg/ml; range, 100 pg–4.0 ng/ml; mean, 0.45 ± 0.96 ng/ml). On the other hand, IL-1β production was detected in 24 donors (range, 80 pg–4.2 ng/ml; mean, 0.81 ± 0.90 ng/ml). It is apparent (figure 7) that no correlation exists between TNF and IL-1 production. These studies support previous observations that TNF-α and IL-1 (α or β) production from PBMC is not linked when LPS is used as a stimulus [35].

IL-1α induces IL-1β and TNF-α mRNA. As shown in figure 8A, we could detect mRNA coding for TNF-α after 3 h. Unstimulated PBMC also produced small but detectable IL-1β mRNA that increased after incubation with IL-1α. Emission at 590 nm from the ethidium-stained gel under shortwave ultraviolet irradiation demonstrated that the ribosomal bands were intact and had about the same intensity in both stimulated and unstimulated PBMC samples (figure 8B). Low levels of TNF-α mRNA may be related to the induction stimulus
Figure 6. Effect of IFN-γ on IL-1-stimulated TNF production in PBMC from 20 individuals. PBMC were cultured for 24 h with 100 ng/ml each IL-1α or rhIL-1β. Cultures were in the presence or absence of IFN-γ (100 units/ml).

Figure 7. Comparison of TNF-α production and IL-1β production in IL-1α-treated (100 ng/ml) PBMC from 29 donors. Total TNF-α and IL-1β were determined by RIA after a 24-h incubation. Dotted lines indicate detection limits of the RIA.

Figure 8. A. IL-1α–induced TNF-α and IL-1β mRNA from PBMC. RNA was extracted from cells after 3 h of incubation with (+) or without (−) 100 ng/ml IL-1α. The blot was hybridized with a labeled probe for TNF-α, autoradiographed, and then stripped. The blot was hybridized again with labeled probe for IL-1β. Samples 1 and 2 were from two different donors. B. Ethidium bromide–stained agarose gels corresponding to donor 1 and 2.

provided by IL-1 or the short length of cDNA probe for TNF-α (470 bp).

Induction of endogenous pyrogen activity during IL-1-induced fever. At concentrations of 7 μg/kg, IL-1α (figure 9A) or IL-1β (figure 9B) induced biphasic fevers when injected intravenously into rabbits. The first peak occurred 40–50 min and a second peak 180–220 min after IL-1 injection. The plasma from febrile animals 3–3.5 h after IL-1 (α and β) injection was passively transferred to new rabbits, producing brief, monophasic fevers in the recipients (figure 9A, B, insets). These monophasic fevers represent typical endogenous pyrogen fever patterns [1–4]. By contrast, plasma from saline-injected rabbits produced no change in rectal temperatures (figure 9A, inset).

Appearance of TNF-like activity in the serum of IL-1-injected rabbits. Rabbit serum collected during the second febrile peak induced by IL-1 was cytotoxic to L929 cells (figure 10). This effect was not due to the serum because the same amount of control preinjected serum alone induced little cytotoxicity. Even normal rabbit serum (10%) added to various concentrations of rhTNF-α did not change the sensitivity to the cytotoxic effect of TNF-α (data not shown).
Figure 9. Febrile responses of rabbits injected intravenously with 7 μg/kg IL-1α (A) and -1β (B). Each point represents the mean of three rabbits. CTRL = rabbit receiving saline. At the times indicated by arrows, febrile rabbits were bled and the plasma (10 ml) was injected into recipient rabbits (insets). Inset in A also shows the response of the recipients to the control plasma prepared from rabbits injected with saline.

Figure 10. Cytotoxic activity on L929 cells of sera obtained from two rabbits 3–3.5 h after injection of 7 μg/kg IL-1α and -1β. L929 cells were treated overnight with dilutions of serum in the presence of actinomycin D. CTRL = serum taken before injection of IL-1. Results represent mean ± SD of triplicate cells.

Figure 11. Time course for the appearance of cytotoxic activity in rabbit sera after injection of IL-1α or -1β (9 μg/kg each). Sera were assayed at a 25% concentration for TNF-like activity using cytotoxicity of L929 cells. Results represent mean ± SE of eight wells of the L929 cells. * = significance of cytotoxicity between IL-1α- and IL-1β-induced sera after 5 h (P < .001).

We next studied the time of appearance of cytotoxic activity in the circulation of IL-1-injected rabbits. Three hours after IL-1 injection, serum from these animals induced 80%–90% cytotoxicity on L929 cells (figure 11). Interestingly, the cytotoxicity of the serum from the IL-1β-injected rabbits decreased in the circulation by 5 h, while most of the IL-1α-induced cytotoxic activity was still elevated after 5 h (figure 11).

Discussion

Numerous studies have demonstrated that endotoxin induces the production of IL-1 and TNF in macrophages and monocytes and that these cytokines are probably responsible for
the multiple acute-phase responses associated with sepsis and bacterial toxemias [36, 37]. An antibody, highly specific against murine TNF-α, has been shown to protect BALB/c mice against the lethal effects of E. coli endotoxin [38]. Similarly, a goat anti-TNF antibody has been reported to inhibit TNF production induced by endotoxin and to protect rabbits from endotoxin shock [39].

Both in vivo and in vitro, endotoxin-stimulated IL-1 and TNF may reach local or systemic levels that themselves may affect the production and activity of these cytokines. In this regard it has been reported that ≤1 μg/kg of TNF-α can induce monophasic fevers in rabbits; if the dose of TNF-α is increased 10-fold, IL-1-like activity is detected in plasma 3.5 h after TNF injection [12]. This observation was confirmed in vitro by the ability of TNF to induce IL-1 in human PBMC [12] and cultured endothelial cells [26]. Philip and Epstein [40] also reported that TNF alone and IFN-α and IFN-γ can enhance IL-1 production.

IL-1 induces IL-1 gene expression and synthesis in human PBMC, smooth muscle cells, and endothelial cells. Each form of IL-1 induces transcription of mRNA for either form within 1 h [27–29]. In this study we show another effect of IL-1: the induction of TNF. TNF induction by IL-1 (α or β) in cultured human PBMC was demonstrated by Northern blot analysis of TNF-α mRNA and by measurement of TNF immunoreactive protein. Accumulation of TNF-α mRNA might be due to the activation of transcription or increased stability of the mRNA.

Our results are based on immunoreactive TNF-α. The difference between the bioactivity and immunoreactivity of TNF-α has been reported recently [41–43]. Low levels of cytotoxic bioactivity of TNF, compared with that as measured by RIA, may be due to inactive forms, inhibitors, or unstable forms of receptor-ligand binding. High levels of cytotoxic activity may be due to lymphotoxin. In our previous studies [31], however, a correlation was found in PBMC cultures between the levels of TNF-α as measured by RIA and TNF activity as detected by a cytotoxic effect on L929 cells. The correlation coefficient was 0.89 over a wide detection range (100 pg–100 ng/ml). However, we cannot rule out that IL-1 also induces lymphotoxin in PBMC.

We next considered the possibility that because LPS stimulates IL-1 production, part of the TNF responses to LPS might be via IL-1. When we measured the amount of immunoreactive IL-1 (α and β) [32, 35] induced by LPS stimulation of PBMC, levels of either form were consistently in the range of 3–10 ng/ml. However, TNF-α production by PBMC stimulated with these concentrations of IL-1 (α or β) ranged from 100 to 250 pg/ml (figure 2), far below the amount of TNF-α (2260 ± 140 pg/ml) synthesized in response to LPS.

In addition, coincubation of PBMC with antibodies directed against both forms of IL-1 did not affect TNF-α production induced by 100 ng/ml LPS. At the concentration used (1 mg/ml), these antibodies previously have been shown to neutralize 10 ng/ml of IL-1 (α or β) [27] and should have been sufficient to neutralize the amount of IL-1α (table 1) induced by the highest concentration of LPS studied here. We conclude from these data that the levels of IL-1 induced by LPS are not high enough to account for the concomitant synthesis of TNF-α. These results make it highly unlikely that TNF synthesis in response to LPS is mediated by an autocrine effect of IL-1.

Relatively greater amounts of TNF-α were synthesized by IL-1-stimulated adherent cells compared with PBMC, indicating that this TNF-α response is due mainly to esterase positive cells, in most cases monocytes. The greater production of TNF-α in the adherent cells might be attributed to an increase in IL-1 binding to the adherent cells, thereby possibly reducing the binding of IL-1 to lymphocytes, or an elimination of an inhibitory role by lymphocytes on monocyte TNF synthesis. Because IFN-γ has been reported to have both stimulatory and inhibitory effects on cytokine-induced cytokine production [12, 30, 43], we studied its effects on IL-1-induced TNF-α production. However, IFN-γ reduces LPS- and IL-1-induced PGE2 release from human cells [44]. Furthermore, IL-1-induced IL-1 production is significantly reduced by coincubation with IFN-γ [30], whereas IFN-γ consistently increases the IL-1 in response to LPS and other microbial agents. In the present study, we did not observe a consistent effect of IFN-γ on IL-1-induced TNF-α production.

The separate effects of IFN-γ on IL-1-induced IL-1 production versus TNF-α production suggest that IL-1 synthesis is regulated differently from that of TNF-α. This observation is supported by our data showing no apparent linkage of IL-1β and TNF-α production by PBMC of 29 donors. Burchett et al. [45] reported that the production of TNF-α and IL-1 by LPS-stimulated monocytes was differentially regulated by IFN-γ. These observations are also supported by the known differences that exist in the regulation of gene expression and protein synthesis for IL-1 and TNF-α.

The biologic rationale for the mutual induction of IL-1 and TNF-α is still unclear. However, because IL-1 has been shown to induce hypotension [46] and the regression of some tumors in vivo [47], the ability of IL-1 to induce circulating TNF-like activity as well as IL-1 may provide a mechanism for this effect. Recently, it was reported that prolonged daily injection of IL-1 into monkeys induced a circulatory TNF-like activity that was thought to suppress hematopoiesis [48]. The synergism of IL-1 and TNF-α has been reported on antiproliferative activity on the A375 melanoma cell line [49], augmentation of PGE2 production from human lung fibroblasts [50], and cytotoxicity of chondrosarcoma, fibrosarcoma, and carcinoma lines [51]. TNF-α also potentiates the effects of IL-1 on rat pancreatic islets of Langerhans insulin production [52], on rat bone resorption [53], in a model of IL-1-induced local Shwartzman reaction [54], and in the induction of a shock-like state in rabbits [46]. Thus, the induction of one or more cytokines (e.g., TNF-α) by another
cytokine (e.g., IL-1) appears more efficient in inducing cytotoxicity and inflammation than synthesizing larger amount of either monokine alone.

Acknowledgments

We thank Kathy Kimball for help in the preparation of the cytotoxicity system, Scott F. Orencole for technical assistance, Ralf Schindler for preparing human TNF probes, and Heide Wen and Xixin Zhang for RIA.

References

4. Hanson DF, Murphy PA. Demonstration of interleukin 1 activity in apparently homogenous specimens of the pl 5 form of rabbit endogenous pyrogen. Infect Immun 1984;45:483-490


