Urate Crystals Stimulate Production of Tumor Necrosis Factor Alpha from Human Blood Monocytes and Synovial Cells
Cytokine mRNA and Protein Kinetics, and Cellular Distribution

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Abstract

Crystals of monosodium urate (MSU) provide a dose-dependent stimulus for the production by human blood monocytes of tumor necrosis factor (TNF), a cytokine with proinflammatory properties; TNF activity was inhibited selectively by monoclonal antibody to TNF alpha. Biologically active cell-associated TNF activity peaked at 3 h and was exceeded at 6 h by extracellular activity, which peaked at 12–18 h. Comparable kinetics were observed with immunoreactive TNF alpha. TNF alpha mRNA accumulation in monocytes stimulated with MSU crystals appeared as a single peak at 2–4 h, kinetics compatible with rapid production of a short half-life transcript. In contrast, crystals of calcium pyrophosphate or of hydroxyapatite did not stimulate significant production of TNF or of message.

Fresh tophaceous material from a patient with gout contained significant levels of TNF alpha and cells cultured from the tophus produced TNF alpha in vitro. In rheumatoid synovial cells, spontaneous release of TNF alpha was increased by in vitro exposure to MSU crystals.

Taken together with earlier work, these results support an expanded view of gouty inflammation in which the crystal-stimulated production of cytokines provides a crucial link between crystal deposition and many of the clinical and pathological features of both acute and chronic gouty arthritis. (J. Clin. Invest. 1991. 87:1375–1381.) Key words: gout • inflammation • tumor necrosis factor • arthritis • gene expression

Introduction

Acute gouty arthritis is thought to be an inflammatory response to microcrystals of monosodium urate (MSU) that precipitate in joint tissues from supersaturated body fluids or are shed from preexisting articular deposits (for review, see 1). Until the last decade, urate crystals were thought to interact primarily with PMN, the cells within which they are found in acute gouty synovial fluid. A proposed sequence consisted of the ingestion of crystals by PMN, generation of PMN-derived inflammatory mediators including chemotactic factors, and infiltration of more PMN, producing a vicious cycle of propagation and amplification of the inflammatory response. Urate crystals themselves were shown in vitro to stimulate a number of inflammatory mediators (2).

Useful as far as it went, this scheme could account neither for the initiation of gouty inflammation, free crystals also being present in tissues between inflammatory attacks, nor for associated systemic features (fever, leukocytosis, generation of acute-phase reactants), which appeared, as of the early 1980s (3, 4), no longer attributable to PMN. These considerations led us initially to examine the release of “endogenous pyrogen” (EP; the original designation for fever-producing cytokines) from monocytes and macrophages exposed to MSU (5, 6). We found that the activation process for EP production was dose dependent, did not require phagocytosis of the crystals, was independent of serum factors or of mediation by other blood leukocytes, and could not be attributed to contamination of the crystal by bacterial endotoxin (LPS) (5). We then examined in detail IL-1 (7), a pyrogenic cytokine with multiple biological actions, finding high levels of IL1 beta released.

Tumor necrosis factor (TNF)/cachectin, the subject of this report, is a 17-kD inducible peptide (8) produced mainly by activated macrophages, with biological actions very similar to those of IL-1 (9), although it is the product of a different gene (10) and acts via a distinct set of cell surface receptors (11, 12). Systemic properties of TNF include induction of fever (13), acute phase response (14), and metabolic (15) and hemodynamic changes (16, 17). Of relevance to the pathogenesis of inflammatory arthritis and joint destruction, TNF alpha stimulates cartilage (18) and bone (19) resorption, activates synovial cells to produce proinflammatory mediators (20), and increases expression of MHC (21) and adhesion (22) molecules. It also induces the production of other macrophase-derived cytokines, such as IL-1 (13), IL-6 (23), and IL-8 (24). TNF alpha has been found in symptomatic joints of arthritic patients and has been implicated in acute and chronic arthritic diseases (25–27).

Taken together with earlier studies, the results of this work support an expanded view of gouty inflammation (6) that can account for many clinical and pathological facts of gout that were unapproachable a decade ago.

Methods

Materials. All reagents and culture media had undetectable levels of endotoxin as assessed by Limulus lysate gelation (E-Toxate; Sigma Chemical Co., St. Louis, MO) lower detection limit: 0.025 IU/ml). Human peripheral blood cells were cultured in RPMI 1640 medium

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1. Abbreviations used in this paper: CPPD, calcium pyrophosphate dihydrate; EP, endogenous pyrogen; HA, hydroxyapatite; hr, human recombinant; MSU, monosodium urate; TNF, tumor necrosis factor.

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Tumor Necrosis Factor a Production by Urate Crystal–Stimulated Cells
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gaard preservative-free criteria) concentration of (Gibco) unless with (Gibco Ltd., Paisley, UK) to obtain stored of cultured (68x629) were crystals (tested by (6). Crystal removal by here have in suspension a 0.05-5 (alpha L929 cells (a DNA. Sweden), on kinetic formaldehyde for form 

sectioned preparations for formaldehyde in 0.9 detergent, which was lysted by detergent, and nuclei were pelleted by centrifugation. Cytoplasmic preparations were extracted with phenol/chloroform and resuspended in 0.9 M NaCl/90 mM sodium citrate/7.4% formaldehyde for 15 min at 60°C. Cytoplasmic preparations were transferred onto nylon membranes (Hybond N; Amersham International, Amersham, UK) using a slot blot apparatus (Minifold II; Schleicher & Schuell, Dassel, FRG). Membranes were exposed to UV and prehydrized for 8-20 h (50% formamide, 240 mM NaCl, 30 mM sodium citrate, 40 mM sodium phosphate, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% BSA). Hybridization was carried out in fresh prehybridization solution to which denatured sheared calf thymus and 32P-labeled probe had been added. After 18-24 h at 37°C, membranes were washed four times (15 min) in 0.1% SDS and decreasing salt concentration (from 0.3 M NaCl, 30 mM sodium citrate, to 15 mM NaCl, 1.5 mM sodium citrate). Blots were visualized by exposure of x-ray films (Aga-Gevaert, Dunstable, UK) at ~80°C with intensifying screens for 4-4 d. To obtain quantitative data, we scanned autoradiograms at OD350 in a densitometer (CS9900; Shimadzu Instruments, Kyoto, Japan) (calibration at ×16, integral of peak area = scanning densitometry [SD] units).

For Northern analysis, total cellular RNA was isolated using the guanidinium/phenol method (34). Briefly, cellular proteins were disintegrated in 4M guanidine isothiocyanate, followed by an extraction with phenol, and equilibration in ice-cold Tris/acetate buffer, pH 7.4. After two cycles of chloroform/isoamyl-alcohol (24:1) extractions, total RNA in the final aqueous phase was precipitated in 50% isopropanol at 4°C. The pellets obtained were washed in 70% ethanol and resuspended in 10 mL 3-[N-morpholino]-2-hydroxypropanesulfonic acid (MOPS) buffer.

Samples were size-fractionated in agarose/formaldehyde gels (1.2% Seakem agarose, 6.7% formaldehyde, 20 mL MOPS, 5 mM sodium acetate, 1 mM EDTA), and transferred overnight to nylon membranes by capillary blotting. All subsequent steps were as previously indicated for total cytoplasmic RNA preparations. TNF in taphus. Fresh topaceous material was obtained by needle aspiration from an interphalangeal joint. The mass was immediately fragmented and resuspended in 0.5 mL serum-free RPMI 1640 medium, and then pelleted by centrifugation. Supernatant was aspirated and tested for immunoreactive TNF alpha. The pellet was resuspended in fresh medium and fractionated on a Ficoll density gradient. Cells (80% polymorphonuclear, 15% lymphocytes, 5% monocytes by morphological criteria) were cultured for 18 h in RPMI 1640 (10^7 cells/mL) and supernatants tested for TNF alpha.

**Results**

**Induction of TNF biological activity by crystals.** Human blood adherent mononuclear cells were incubated in the presence or absence of MSU, HA, or CPPD crystals for 18 h. Supernatants were collected and tested for TNF biological activity (Fig. 1). MSU-stimulated cells and, to a lesser extent, cells treated with HA crystals released significant amounts of TNF-like activity. Stimulation by HA crystals was not a constant observation and appeared to be related to the individual donor. Cells incubated in medium only or with CPPD crystals failed to produce any significant extracellular activity. TNF induction by MSU crystals was dose dependent, and in each single series of experiments maximal stimulation occurred at 1.2 mg MSU/mL. To test the specificity of these biological activities, we tested supernatants in the presence of anti-human TNF alpha monoclonal antibody (TNF-E, kindly provided by Dr. G. Adolf). Results in Table I show selective inhibition of L929 cytotoxicity, indicating that the cytotoxicity observed was mediated by TNF alpha.

**TNF alpha production in synovial cells.** Freshly isolated human RA synovial fluid mononuclear cells (MNC) spontaneously released immunoreactive TNF alpha in a 20-h incubation (Fig. 2 A). This is likely to reflect activation in vivo rather than inadvertent activation during separation procedures, since autologous blood MNC produced no TNF activity without prior stimulation (data not shown). The synovial cells could be further stimulated to release TNF by incubation with...
0.4 mg/ml MSU crystals, while HA failed to induce further significant release of TNF alpha.

Kinetics of production and cellular distribution of TNF alpha in MSU crystal-stimulated cells. Cellular fractions representing extracellular and cell-associated compartments were prepared (described in Methods) from crystal-stimulated adherent mononuclear cells. Fractions were collected and tested in duplicate in a monospecific TNF alpha RIA (lower detection limit, 50 pg/ml). (B) TNF alpha in tophus. I, supernatant from freshly aspirated tophus resuspended in 0.5 ml serum-free RPMI 1640. II, supernatant (18-h culture) of cells isolated from gradient centrifugation of tophaceous material. III, supernatant (18 h) from unstimulated human adherent mononuclear cells. Results are expressed as TNF alpha/10⁷ cells/ml and are the mean of two determinations.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample with MAB anti-TNF alpha (1.5 µg/ml)</th>
<th>Sample with control MAB (1 µg/ml)</th>
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</thead>
<tbody>
<tr>
<td>MSU-induced supernatant 0.4 mg/ml</td>
<td>17.61 ± 0.34</td>
<td>&lt;4</td>
</tr>
<tr>
<td>MSU-induced supernatant 0.8 mg/ml</td>
<td>36.92 ± 2.67</td>
<td>&lt;4</td>
</tr>
<tr>
<td>MSU-induced supernatant 1.2 mg/ml</td>
<td>27.36 ± 1.08</td>
<td>&lt;4</td>
</tr>
<tr>
<td>LPS-induced supernatant 100 ng/ml hrTNF beta</td>
<td>1469.90 ± 136.97</td>
<td>&lt;4</td>
</tr>
<tr>
<td>1 ng/ml hrTNF alpha</td>
<td>24.08 ± 3.34</td>
<td>21.68 ± 2.85</td>
</tr>
<tr>
<td>1 ng/ml hrTNF alpha</td>
<td>22.13 ± 2.45</td>
<td>&lt;4</td>
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L929 cell cytotoxicity by supernatants of stimulated adherent mononuclear cells. Supernatants were tested for TNF biological activity in the presence or absence of specific anti-human TNF alpha monoclonal antibody, or irrelevant mouse anti-human monoclonal (anti-human DR). Results are expressed in half-maximal units per milliliter (±SEM).
in 3. Kinetics of cells were tested HA, or density centrifugation; the mean was calculated early protein in detectable levels of adherence; or fraction was TNF that material contained 220 TNF that reduced TNF alpha/107 TNF from obtained in this experiment.

In this study we found that MSU crystals provide a dose-dependent stimulus for TNF production in human monocytes (Fig. 1). At supraoptimal concentrations of MSU crystals it is likely that reduced TNF alpha production was a consequence of the known cytolitic effects of these crystals. Cytotoxicity was completely inhibited by anti-TNF alpha MAb (Table I), suggesting that TNF beta was not produced in these cultures. HA crystals in some experiments induced release of low levels of TNF bioactivity but this finding was inconsistent and in time-course

Figure 3. Kinetics of production and cellular distribution of TNF alpha in MSU crystal-stimulated cells. Human adherent mononuclear cells (10⁶/ml) were cultured for up to 18 h in the presence of MSU, HA, or CPPD crystals. (A) Extracellular and cell-associated fractions were tested for TNF activity, expressed in cytotoxicity units. Each fraction was tested in duplicate wells on the horizontal axis: A, cell lyse after density centrifugation; B, fractions from cells at the end of plastic adherence; C, immediately after stimulation (time zero).

Discussion

In this study we found that MSU crystals provide a dose-dependent stimulus for TNF production in human monocytes (Fig. 1). At supraoptimal concentrations of MSU crystals it is likely that reduced TNF alpha production was a consequence of the

Figure 4. Crystal-induced TNF alpha mRNA accumulation. Total RNA from adherent mononuclear cells (4 h, 10⁶/ml) was fractionated in a 1.5% agarose/formaldehyde gel (15.0 µg/lane) and transferred to membranes. Hybridization to the 32P-labelled TNF alpha probe was monitored by autoradiography. Cells had been cultured in control medium (lane A), or stimulated with LPS, 100 ng/ml (lane B), or crystals at 0.4 mg/ml: MSU (lane C), HA (lane D), and CPPD (lane E). Relative positions of RNA size markers, in kilobase, are shown at left.

Figure 5. Kinetics of crystal-induced TNF alpha mRNA accumulation. Cytoplasmic RNA preparations were transferred and hybridized to a 32P TNF alpha cDNA probe. Shown are results from preparations of 4 × 10⁶ cells RNA/slot, which had been cultured in the absence of stimulus (CM), or stimulated with lipopolysaccharide (LPS, 100 ng/ml), or crystals at 0.4 mg/ml (MSU, HA, CPPD). Quantitative data were obtained by scanning at OD₅₅₀ in a Shimadzu CS9000 densitometer, using a ×16 calibration. The area of the peaks was used as a measure of TNF alpha mRNA within each set of cultures (scanning densitometry [SD] units). Hybridization with control probe (p7B6) showed homogeneous loading (data not shown).
experiments (below) was not confirmed. CPPD crystals showed no TNF-inducing activity.

We also found spontaneous production of TNF alpha by RA synovial fluid adherent mononuclear cells (Fig. 2 A). TNF production could be further stimulated by addition of MSU to the cells, while HA crystals had no effect. Ex vivo evidence for the relevance of TNF alpha in gouty arthritis was found in fresh tophaceous material from an interphalangeal joint (Fig. 2 B). It contained significant amounts of immunoreactive TNF alpha, and cells obtained from the tophus continued to release this cytokine over 18 h incubation (120 pg TNF alpha/10^7 cells/ml).

The failure of HA and CPPD to induce significant TNF synthesis was confirmed in time-course experiments where neither crystal type induced either cell-associated TNF biological activity (Fig. 3) or mRNA accumulation (Figs. 5 and 6). These two crystals also do not induce significant synthesis of IL-1 in our hands (7).

In contrast, LPS-free crystals of MSU induced time-dependent accumulation of TNF alpha in both cell-associated and extracellular compartments, with detectable levels at 3 h in cell lysates, followed by extracellular translocation of biologically active TNF alpha, found mainly in the supernatants at 9–18 h (extracellular TNF alpha, 2.24 ng/ml; 90.5% of the total).

In these cultures, immunoreactive TNF alpha generally followed the pattern of distribution of TNF bioactivity. However, discrepancies were noticed at late stages of incubation, perhaps indicating the presence of cytotoxicity unrelated to TNF alpha and/or production of biological inhibitors (26). It is of interest that we found no evidence of TNF accumulation in the cell-as-

Figure 6. Kinetics of crystal-induced TNF alpha mRNA accumulation and of concomitant extracellular immunoreactive TNF alpha. Levels of TNF alpha mRNA quantified as described in the legend to Fig. 5 are indicated by closed symbols. Open symbols represent levels of extracellular TNF alpha protein assessed by RIA in the same cultures (lower detection limit, 100 pg/ml). Adherent cells were cultured for the indicated lengths of time, in the presence of different stimuli: (A) LPS, 100 ng/ml; (B) MSU, 0.4 mg/ml; (C) medium control; (D) HA or CPPD, at 0.4 mg/ml.
associated fractions, suggesting that the reported form of membrane-associated TNF alpha (35) could only exist as a transient event in our conditions.

TNF alpha mRNA accumulation appears as a single peak at 2-4 h (Fig. 6). In studies using phorbol stimulation (36) a similar monophasic response was observed but with maximum levels at a later time (12-16 h). The mRNA kinetics are compatible with rapid production of a short half-life transcript. Unstimulated cells accumulated low levels of TNF alpha mRNA at late stages of incubation but this was not followed by translocation of immunoreactive protein. This may represent untranslated mRNA as has been reported for TNF alpha in macrophages from C3H/HeJ mice (37). The kinetics of production of biologically active TNF alpha are remarkably similar to those of IL-1 alpha and IL-1 beta (38), and would argue against sequential induction of one cytokine by another. The peak level of total TNF produced in response to MSU crystals (2.44 ng/ml) was lower than those of IL-1 in the same experiment (IL-1 alpha, 9.9 ng/ml; IL-1 beta, 10.9 ng/ml). The dose-response relation of MSU concentration to total production of TNF alpha, IL-1 alpha, or IL-1 beta was similar in all three cases, with optimal stimulation occurring between 0.8 and 1.2 mg/ml MSU crystals (38).

TNF and IL-1 have independent proinflammatory and immunopotentiating activities and each could therefore contribute to the response to MSU crystal deposition. Both TNF and IL-1 are powerful stimuli of IL-6 (23) and IL-8 (24) production, which could (respectively) account for the pronounced acute phase response and neutrophil chemotaxis associated with MSU crystal deposition. Both IL-6 and IL-8 have been found in supernatants after stimulation of monocytes with urate crystals (39, 40).

The likely clinical correlates of these considerations, formulated for “EP/IL-1” (6), but expandable to include TNF alpha and the other cytokines that it and IL-1 engender, can be summarized as follows: (a) Initiation of acute gouty arthritis. Urate crystals, brought into contact with mononuclear phagocytes within the joint, can generate cytokines chemotactic for PMN. When the latter cells encounter ingestible particles (urate crystals) they can propagate and amplify the inflammatory response (see Introduction), producing acute gout. As crystals may be present when inflammation is not, they would seem to be the fuel for the inflammatory engine, not the spark; hence acute gout is properly “crystal associated” rather than “crystal induced” (1). Although adsorbed material might affect the phlogistic potential of crystals (2), they still have to be “told” when to change their coats; i.e., there must be a prior series of controls. Candidates for the inflammatory sparks that introduce crystals to resident mononuclear phagocytes would include local trauma, gross or micro-, whose effects in normal individuals (those without crystal “fuel”) would quickly fade or remain subclinical. (b) Acute polyarticular gout. Cytokines generated in gouty joints can disseminate to other areas of the body, as evidenced by systemic manifestations such as fever (hypothalasus), leukocytosis (bone marrow), and acute phase reactants (liver). They are therefore prime suspects as the messengers that result in the typical intense inflammation that one sees in overlying skin, and that sometimes lead other joints to become inflamed, as if by “contagion.” A logical corollary to the latter point is that the inciting inflammatory cytokines may originate in extrarticular sites of trauma, inflammation, or infection, thus explaining why both acute gouty arthritis and pseudogout are often superimposed upon trauma or infection elsewhere, and why polyarticular gout has a predilection for elderly, debilitated, often febrile individuals. Cytokines arriving in a joint charged with CPPD crystals may be especially important in initiating pseudogout, as those crystals seem not to induce production either of TNF alpha, as shown here, or of IL-1 (7). (c) Tophi and chronic tophaceous gout. Tophi contain IL-1-producing cells (mononuclear phagocytes) in close proximity to IL-1 inducers (urate crystals), surrounded by cytokine-inducible material (fibrous tissue). Sometimes tophi reside in characteristic sclerotic-rimmed punched-out areas formerly occupied by material (cartilage and bone) whose resorption is also inducible by cytokines. In chronic tophaceous gout, through persistent stimulation of mononuclear phagocytes, rampant tophaceous material in and about joints may engender chronic inflammatory processes similar to those seen in rheumatoid arthritis, which this condition resembles clinically.

Thus, in view of the biological actions of TNF and IL-1, their reported synergy in several human systems (41-43) and the possibility of mutual induction (13, 44), we believe that stimulation of IL-1 and TNF production by MSU crystals may be a crucial link between crystal deposition and both the acute and chronic inflammatory reactions of gouty arthritis.

Acknowledgments

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