

Endotoxin Induction of Tumor Necrosis Factor Is Enhanced by Acid-labile Interferon- α in Acquired Immunodeficiency Syndrome

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Abstract

High levels of an acid-labile IFN- α have been demonstrated in the sera of patients with symptomatic HIV infection. IFNs have been shown to enhance the cytotoxic and antiproliferative actions of tumor necrosis factor (TNF), which is a potent mediator of inflammation and sepsis. We show that the acid-labile IFN- α present in AIDS sera can induce TNF synthesis and sensitize blood monocytes (BM) to endotoxin stimulation resulting in further synthesis of TNF in vitro. TNF production by BM from patients with HIV infections and normal controls was measured by a cytotoxicity assay on L929 cells using human TNF α as a standard. BM from AIDS patients spontaneously produce high levels of TNF and are hypersensitive to endotoxin stimulation, resulting in enhanced synthesis of TNF. In determining the mechanism involved, we demonstrated that treatment of normal BM with AIDS sera results in induction of TNF. Neutralization of the acid-labile IFN- α in AIDS sera with polyclonal anti-IFN- α antibodies results in diminution of TNF induction. In addition, pretreatment of normal BM with AIDS sera, IFN- α , or IFN- γ renders the cells hypersensitive to endotoxin. Consequently, activation of the TNF system by the acid-labile IFN- α contributes to some of the physiological disturbances, such as the wasting syndrome, and to the pathophysiology of sepsis in AIDS patients.

Introduction

High levels of an unusual acid-labile IFN- α have been documented in sera of patients with HIV infection (1, 2) and are implicated in the immunopathogenesis of AIDS. Persistently elevated levels of IFN and its inducible enzyme, 2',5'-linked oligoadenylate synthetase, in patients with HIV infection is indicative of disease progression from AIDS-related complex to AIDS (3, 4). Recently we have demonstrated downregulation of IFN- α receptor expression on blood mononuclear cells from AIDS patients by the acid-labile IFN- α and consequent hyporesponsiveness of the cells to IFN- α treatment in vitro (5). Although the regulatory significance of this acid-labile IFN- α subtype in the pathogenesis of AIDS remains to be determined, IFNs have been shown to enhance the cytotoxic, tu-

morcidal, antiviral, and antiproliferative activities of tumor necrosis factor (TNF)¹ in vitro (6–9).

On the physiological level TNF mediates inflammation and sepsis, induces cachexia, and suppresses hematopoiesis (10, 11). Recent reports indicate that cytokines play an essential role in the induction of synthesis of TNF by monocytes and macrophages during inflammation (12–14). Therefore, abnormal expression of cytokines in disease processes such as HIV infections may result in increased TNF synthesis.

Activation of macrophages and monocytes involves a number of cellular processes. IFN- γ sensitizes monocytes and renders them responsive to signals such as endotoxin (LPS) and foreign antigens (12–14). The combined actions of IFN- γ and LPS result in enhancement of cytotoxic and tumoricidal activities mediated by matured macrophages and monocytes. Synergistic interaction between IFNs and LPS has been observed for the synthesis of IL-1 in human monocytes (15). A recent report also indicates that LPS and IFN- γ act synergistically on human fibroblasts in the induction of factor B, which is one of the key proteins involved in the activation of the alternate complement pathway in inflammation (16).

In light of these synergistic interactions of LPS and IFN we investigated the induction of TNF synthesis by the cytokine and the bacterial endotoxin. We also determined the role of the acid-labile IFN- α present in AIDS sera in the enhancement of TNF synthesis and the sensitization of monocytes to LPS stimulation.

Methods

Patients. The patients in this study were homosexual males aged 25–45 yr who had clinically defined HIV infections (groups III and IVc) according to surveillance criteria established by the Centers for Disease Control (17). The patients in group III had persistent lymphadenopathy without any constitutional disease ($n = 6$). In contrast, the patients in group IVc ($n = 9$) had recovered from opportunistic infections including disseminated cytomegalovirus, pneumocystis carinii pneumonia, or disseminated Herpes simplex. At the time of the study they were clinically stable without evidence of active opportunistic infections. All patients had positive IgG antibodies to HIV by ELISA assays. They were followed at the Toronto General Hospital AIDS clinic. The control subjects ($n = 14$) consisted of healthy laboratory personnel, medical students, and residents.

Cells and serum samples. PBMC were isolated from heparinized blood samples using Ficoll-Hypaque density gradient centrifugation (5, 18). The cells were incubated in a tissue culture dish (Falcon Plastics, Cockeysville, MD) at 37°C for 30 min for blood monocyte (BM) separation (5). The procedure was repeated twice to enrich the monocytic population. BM isolated were stained with a monoclonal anti-Mo2 (CD14) antibody (Mo2-RD1/KC56-FITC; Coulter Electronics Inc., Hialeah, FL) and subsequently studied by a cell analyzer (EPICS

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1. *Abbreviations used in this paper:* BM, blood monocyte; TNF, tumor necrosis factor.

profile analyzer; Coulter Electronics Inc.) to determine the purity of the cells. Serum samples obtained simultaneously were stored at -70°C until the time of assay for IFN levels or used in TNF induction experiments.

Determination of IFN titers. The levels of IFN- α in the sera were determined by titrating for antiviral activity on T98G (glioblastoma) cells against National Institute of Allergy and Infectious Diseases reference standard G-023-901-527 (5, 18). In brief, 5×10^4 T98G cells were seeded into each well of 96-well microtiter plates and incubated with twofold serial dilutions of patients' serum samples for 16 h and then challenged with encephalomyocardial virus (0.1 plaque forming units per cell). Virus-induced cytopathic effects were assessed by microscopic examination and by staining the cells with 0.1% crystal violet in 5% ethanol solutions. The IFN titer was defined as the reciprocal of the highest dilution of serum samples capable of protecting 50% of the cells from viral-induced cytopathic effects. All titers were expressed as international units/milliliter.

Acid lability of the IFN in the sera was determined by treating the serum samples with 0.2 M hydrochloric acid to pH 2 for 6 h at 25°C and subsequently neutralized to pH 7.4 with 0.2 M sodium hydroxide.

Effect of anti-IFN- α and - γ antibodies. Sheep polyclonal anti-human IFN- α and - γ antibodies (IFN Sciences Inc., New Brunswick, NJ) were used to test the immunological specificity of the IFN subtype in the sera. Serum samples (100 μl) from AIDS patients were pretreated with 2,000 neutralizing units of anti-human IFN- α or - γ antibodies. The resultant mixtures were used for the treatment of BM for TNF synthesis.

Induction of TNF synthesis by sera from patients with HIV infection. Normal BM (0.5×10^6 , $n = 14$) in 0.375 ml of RPMI medium, supplemented with 10% FCS, were treated with 0.125 ml of serum samples from normal controls ($n = 12$), HIV group III ($n = 6$), and IVc ($n = 9$) patients. The IFN titers in the sera were determined as described above. After incubation at 37°C for 18 h culture supernatants were assayed in triplicate for TNF-mediated cytotoxicity (19–21).

Sensitization of monocytes to LPS induction of TNF. Normal BM (0.5×10^6 , $n = 10$) in 0.5 ml of RPMI medium were treated with sera from normal controls ($n = 10$), IFN- α_2 (Schering Corp., Kenilworth, NJ), IFN- γ (Biogen Inc., Cambridge, MA), TNF α (Genentech Inc., South San Francisco, CA), or individual AIDS patients' sera ($n = 4$) containing the acid-labile IFN- α . The final concentration of the normal and AIDS sera in the incubation medium was 25% of that of the original, whereas the concentration of the acid-labile IFN- α in the AIDS sera was 512 U/ml. After incubation at 37°C for 18 h the cells were washed and stimulated with 10 $\mu\text{g}/\text{ml}$ of LPS (*Escherichia coli* extract, Sigma catalogue No. L2630; Sigma Chemical Co., St. Louis, MO) for 18 h for TNF induction.

Interaction of IFN- α or - γ with LPS on TNF synthesis. Normal BM (0.5×10^6) in 0.5 ml of RPMI 1640 medium were treated with varying concentrations of LPS, IFN- α_2 , or IFN- γ . To evaluate the interaction of IFN- α or - γ with LPS on the induction of TNF synthesis, BM were treated with various concentrations of IFN- α or - γ with 1.0 μg of LPS. After incubation at 37°C for 18 h the culture supernatants were assayed for TNF activities.

TNF assay. TNF titers in supernatants from BM cultures were quantitated in 96-well microtiter plates by a cytotoxicity assay using L929 (mouse fibrosarcoma) cells. In brief, 5×10^4 cells were seeded into each well and incubated for 24 h at 37°C with twofold serial dilutions of the supernatants in α -MEM medium containing 10% fetal bovine serum and 1 $\mu\text{g}/\text{ml}$ of actinomycin D (catalogue No. 102008; Boehringer Mannheim Biochemicals, Indianapolis, IN). TNF-induced cytopathic effects were assessed by microscopic examination and by staining the cells with 0.1% crystal violet in 5% ethanol solutions. A unit of cytotoxicity was defined as the concentration at which 50% of the L cells were showing cytopathic effects after 24 h. In each assay purified recombinant human TNF α (5×10^7 U/mg; Genentech Inc.) was used as a standard. To determine the specificity of the cytotoxic factors, monoclonal anti-TNF α antibodies (Genentech Inc.) were used to neutralize the cytotoxic effects of the culture supernatant.

Statistics. The results were analyzed for statistical significance with the nonpaired *t* test.

Results

IFN titers in the sera of patients with HIV infections. To demonstrate the presence of the acid-labile IFN- α in the serum samples, IFN assays were performed on T98G cells. The IFN levels in the sera of patients with HIV groups III and IVc infections were determined to be 11.2 ± 12 ($n = 6$; range, 0–32) and 350 ± 160 ($n = 9$; range, 64–512) U/ml, respectively. All patients with group IVc infection had elevated levels of the acid-labile IFN- α , whereas only four of six patients with group III infection had detectable IFN in their serum. The IFN subtype in the patients' sera could be neutralized by polyclonal anti-IFN- α antibodies or inactivated by acid treatment (pH 2), consistent with previous reports (1, 2, 5). Preincubation of the samples with 2,000 U/ml of the anti-IFN- α antibodies before antiviral assays resulted in complete neutralization or at least a 16-fold (95%) decrease in IFN activity. In normal controls ($n = 14$) assayed concurrently, no IFN was detectable in the serum.

Synthesis of TNF by BM from AIDS patients. The purity of BM used in the experiments was determined to be in the range of 85–95% by staining with anti-Mo2 (CD14) antibodies and analysis by the EPICS profile analyzer. To investigate the activation of the TNF system in AIDS we measured the synthesis of TNF by BM in patients with HIV infections. Supernatants of BM cultures from AIDS patients showed high levels of TNF (18.7 ± 7.0 U/ml, $n = 7$, $P < 0.005$) compared with those of the normal controls (1.1 ± 0.96 U/ml, $n = 14$; Fig. 1). The cytotoxic factor in the supernatant was determined to be TNF α by neutralization experiments using a monoclonal anti-TNF α antibody (Genentech, Inc.). When the cells were treated with 10 $\mu\text{g}/\text{ml}$ LPS there was an increase in TNF synthesis by the AIDS cells to a level of 48.5 ± 16 U/ml ($n = 7$, $P < 0.02$) compared with that of the normal cells (16 ± 5 U/ml, $n = 7$; Fig. 1). Therefore, it appears that BM from AIDS patients are spontaneously producing high levels of TNF and are hypersensitive to LPS stimulation.

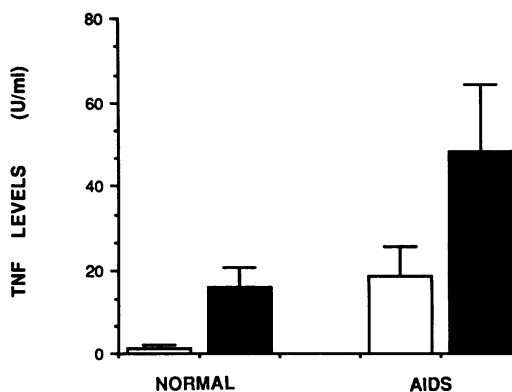


Figure 1. Synthesis of TNF by BM from patients with HIV infections. BM (0.5×10^6) from normal controls ($n = 14$) and patients with group IVc HIV infections ($n = 7$) were incubated at 37°C for 16 h in the presence (solid bars) or absence (open bars) of LPS (10 $\mu\text{g}/\text{ml}$). Triplicate samples were assayed for TNF-mediated cytotoxicity (see Methods).

Induction of TNF synthesis by the acid-labile IFN- α . To investigate the effect of the acid-labile IFN- α on the regulation of TNF synthesis, normal BM were treated with sera from patients with group III and IVc HIV infections. When normal cells were treated with AIDS sera (group IVc, $n = 9$) containing the acid-labile IFN- α (Fig. 2 *d*), there was induction of TNF synthesis (37.3 ± 13 U/ml, $P < 0.005$) compared with untreated controls (1.1 ± 0.96 , $n = 14$; Fig. 2 *a*). In contrast, sera from patients with group III infection ($n = 6$) containing low levels of the acid-labile IFN- α did not induce TNF synthesis significantly (Fig. 2 *c*). When parallel experiments were performed on normal cells treated with normal sera, no induction of TNF synthesis was observed ($n = 12$; Fig. 2 *b*).

To determine the agent in the AIDS sera responsible for the induction of TNF synthesis, the serum samples were treated with sheep polyclonal anti-IFN- α antibodies. This treatment neutralized the antiviral activity of the sera mediated by the acid-labile IFN- α with a concomitant 80% decrease in TNF induction by the reactants ($n = 5$, Fig. 2 *e*). Polyclonal anti-IFN- γ antibodies did not have significant effects on the TNF induction ability of the AIDS sera (results not shown). The results suggest that the agent in the AIDS sera mainly responsible for the TNF induction is the acid-labile IFN- α .

Sensitization of monocytes to LPS induction of TNF. To investigate whether IFN was responsible for the hypersensitivity to LPS of blood monocytes from AIDS patients, normal BM were treated with IFN- α , IFN- γ , TNF, and AIDS sera containing the acid-labile IFN- α . After incubations at 37°C for 16 h the cells were washed and stimulated with 10 μ g/ml of LPS. The results (Fig. 3) show that pretreatment with IFN- α or - γ or AIDS sera renders the normal BM hypersensitive to LPS stimulation, resulting in further synthesis of TNF in vitro. The respective levels of TNF in the supernatants were significantly increased to 59 ± 10 U/ml ($n = 7$, $P < 0.05$), 149 ± 43 U/ml ($n = 6$, $P < 0.001$), and 128 ± 24 U/ml ($n = 4$, $P < 0.001$) compared with cells with normal sera pretreatment (35 ± 10.5 , $n = 10$). In contrast, pretreatment of cells with TNF did not enhance the responsiveness to LPS stimulation ($n = 7$). AIDS sera, when preneutralized with anti-IFN- α antibodies, did not

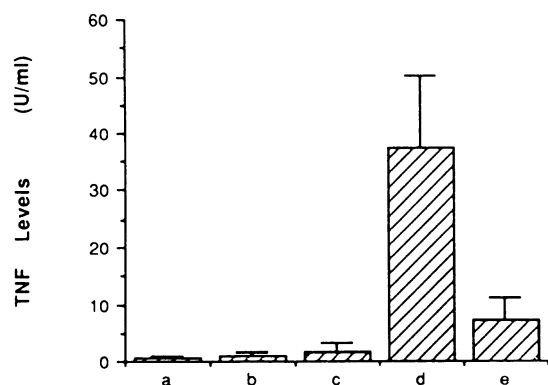


Figure 2. Induction of TNF synthesis by the acid-labile IFN- α . BM (0.5×10^6) from normal individuals were incubated at 37°C for 16 h with: (a) controls, no treatment ($n = 14$); (b) normal sera ($n = 12$); (c) sera from patients with HIV group III infection ($n = 6$); (d) sera from patients with HIV group IVc infection ($n = 9$); (e) sera from patients with HIV group IVc infection, preneutralized with anti-IFN- α antibodies ($n = 5$). Triplicate samples of the culture supernatant were assayed for TNF levels (see Methods).

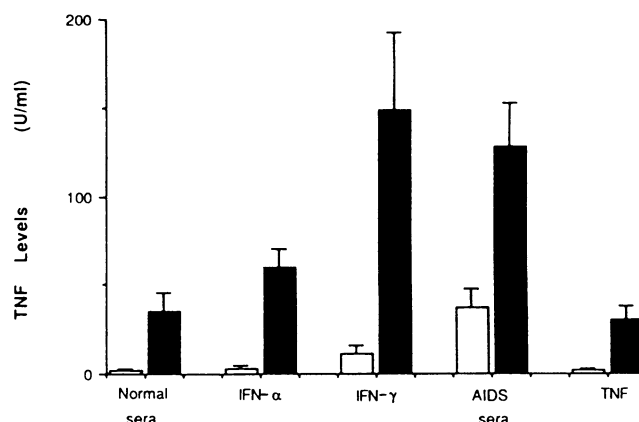


Figure 3. Sensitization of monocytes to LPS induction of TNF. BM (0.5×10^6) from normal individuals were treated at 37°C for 16 h with: (a) normal sera ($n = 10$); (b) IFN- α , 100 U/ml ($n = 7$); (c) IFN- γ , 100 U/ml ($n = 6$); (d) sera from patients with HIV group IVc infection ($n = 4$); (e) TNF, 10 U/ml ($n = 7$). After washings the cells were incubated at 37°C for 16 h in the presence (solid bars) or absence (open bars) of LPS (10 μ g/ml). Triplicate samples of the culture medium were assayed for TNF levels.

sensitize normal BM to LPS stimulation (results not shown). It therefore appears that the acid-labile IFN- α present in AIDS sera is responsible for the hypersensitivity of BM to LPS stimulation.

Synergistic interaction of IFN- α or - γ with LPS on TNF synthesis. Since pretreatment of normal BM with IFN results in hypersensitivity to LPS, we determined the interaction of IFN- α or - γ with LPS on the induction of TNF synthesis (Fig. 4). Normal BM responded to LPS with a concentration-dependent induction of TNF (Fig. 4 *A*). When the cells were treated with IFN- α or - γ in combination with LPS, there was a synergistic induction of TNF synthesis (Fig. 4 *A*). For example, at low concentrations of LPS (1.0 μ g/ml) in the absence of IFN- α or - γ the level of TNF in the medium was 20 ± 6.0 U/ml ($n = 5$). Simultaneous treatment of the cells with either IFN- α or - γ at this LPS concentration resulted in increased synthesis of TNF to 64 ± 20 and 152 ± 47 U/ml, respectively ($n = 5$). At high concentrations of LPS, such as 100 μ g/ml, the extent of IFN- α or - γ enhancement of LPS induction of TNF was less remarkable (Fig. 4 *A*).

Similarly, the extent of IFN enhancement of LPS induction of TNF synthesis depends on the concentration of IFN- α or - γ in the incubation medium. IFN- α by itself has minimal effects on the induction of TNF synthesis (Fig. 4 *B*). However, normal BM, when treated simultaneously with LPS (1.0 μ g/ml) and various concentrations of IFN- α or - γ , showed significant induction of TNF synthesis (Fig. 4, *B* and *C*). It appears that IFN- γ is a more potent enhancer of TNF synthesis with levels of TNF up to 560 ± 260 U/ml ($n = 5$) in the incubation medium (Fig. 4 *C*). When cells were treated with LPS, IFN- α , and IFN- γ simultaneously we did not observe further synergistic induction of TNF as compared with the LPS and IFN- γ treatment (results not shown).

Discussion

TNF α is a soluble polypeptide produced by activated monocytes and macrophages upon exposure to bacterial or proto-

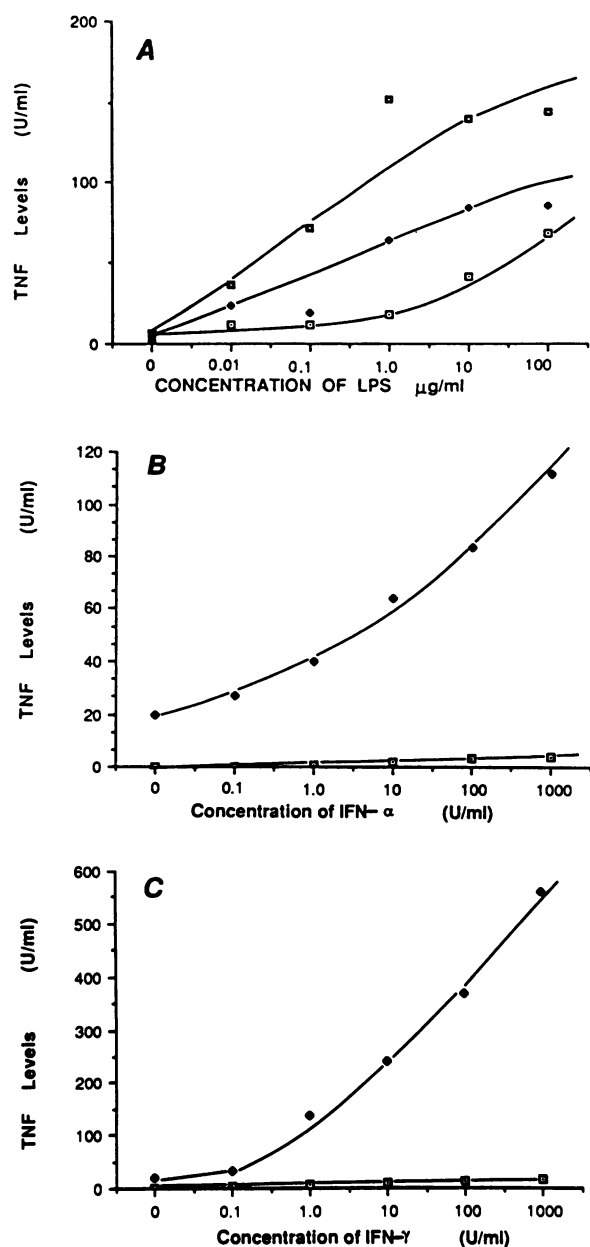


Figure 4. Synergistic interaction of LPS with IFN- α and - γ . Normal BM (0.5×10^6) were treated with: (A) Indicated concentrations of LPS with or without IFN- α or - γ (100 U/ml). □, Controls, no IFN; ◆, IFN- α ; ◻, IFN- γ . (B) Indicated concentrations of IFN- α with or without LPS (1.0 μ g/ml). □, Controls, no LPS; ◆, LPS. (C) Indicated concentrations of IFN- γ with or without LPS (1.0 μ g/ml). □, Controls, no LPS; ◆, LPS. After incubations at 37°C for 16 h triplicate samples of the culture supernatants were assayed for TNF levels. Each point represents the mean level of TNF in five experiments; SD were < 25% about the mean.

zoal products (10, 21–23). In addition to its role in the elimination of pathogens, TNF is thought to contribute to cytotoxicity and cytostasis in other immune processes including tumor destruction and autoallergic reactions (10, 11). Recent reports indicate that TNF is a potent mediator of inflammation and cachexia (10, 11, 23). Synthesis of TNF by blood monocytes

and macrophages is regulated by cytokines including IFNs, foreign antigens, and bacterial products such as LPS (11–14).

Since high levels of the acid-labile IFN- α present in the sera of patients with HIV infections have been implicated in the pathogenesis of AIDS (1–5), we investigated whether this IFN- α subtype is capable of activating the TNF system by inducing the synthesis of TNF. In this paper we show that blood monocytes from AIDS patients spontaneously produce high levels of TNF and are hypersensitive to LPS stimulation, resulting in further induction of TNF synthesis in vitro (Fig. 1).

We have determined the agent in AIDS sera responsible for induction of TNF synthesis by BM is the acid-labile IFN- α . Normal BM when treated with AIDS sera containing high levels of the acid-labile IFN- α produce high levels of TNF (Fig. 2 d). Similar treatment of normal BM in the presence of anti-IFN- α antibodies results in an 80% reduction in TNF synthesis (Fig. 2 e). Further, sera from normal controls and patients with HIV group III infection (containing low levels of the acid-labile IFN- α) do not induce TNF synthesis (Fig. 2, b and c). These results strongly suggest that the agent in AIDS sera responsible for the majority of the TNF induction is the acid-labile IFN- α . However, it is possible that the IFN subtype serves as a cofactor with other as yet unidentified agents, as the serum factors responsible for the balance of the TNF induction (Fig. 2 e) remain to be determined.

In determining the mechanism of hypersensitivity of the AIDS cells to LPS stimulation, we have shown that the same phenomenon can be reproduced in normal BM if the cells are pretreated with IFN- α , IFN- γ , or AIDS sera containing the acid-labile IFN- α (Fig. 3). Neutralization of the AIDS sera with anti-IFN- α antibodies abrogates this hypersensitivity while anti-IFN- γ antibodies do not (results not shown). Therefore, sensitization of BM from AIDS patients to LPS stimulation is probably due to high levels of the acid-labile IFN- α in their sera.

Synergistic interactions of LPS and IFN on the induction of soluble proteins involved in inflammation, specifically IL-1 and factor B of the alternate complement pathway, have previously been reported (15, 16). Synergism has also been studied for tumor cell killing in macrophages where IFN- γ enhances the sensitivity of the macrophages to LPS stimulation, resulting in increased cytotoxic and tumoricidal activities (13, 14). In evaluating the interaction of IFN- α and - γ with LPS we have shown that IFN- α or - γ enhances the induction of TNF by LPS at IFN concentrations as low as 1 U/ml in vitro (Fig. 4). This low level of IFN is entirely within the physiological range. As discussed above, the levels of the acid-labile IFN- α present in sera from AIDS patients are as high as 350 ± 160 U/ml (see Results). It is therefore conceivable that high levels of the IFN- α subtype in these patients provide suitable conditions for the LPS induction of TNF synthesis in vivo during sepsis.

Experimental animals including mice, rats, and rabbits, when injected with gram-negative bacteria or LPS, produce high levels of TNF (23, 24). The released cytokine induces severe metabolic acidosis, hypotension, and hemorrhagic necrosis of organs including adrenal gland, kidney, and lung (23–25). On the cellular level, it is known that TNF causes neutrophil degranulation, production of superoxide anions and hydrogen peroxide (26, 27), induction of procoagulant

activity, and suppression of thrombomodulin synthesis in endothelial cells (28). These pathophysiological processes are ameliorated if the animals are pretreated with a neutralizing MAb against TNF (29, 30). Thus, TNF has been shown to be a potent mediator of septic shock.

Recent reports indicate that gram-negative bacterial sepsis in AIDS patients is frequently associated with hypotension, respiratory failure, and high case fatality rate (31, 32). In these patients it is possible that LPS interacts synergistically with the acid-labile IFN- α present in the plasma to produce high levels of TNF in vivo. Consequently, the pathophysiological processes described above are induced by the cytokine, resulting in clinical deterioration of the patients during sepsis.

Details of the cellular mechanisms underlying the spontaneous production of TNF and the hypersensitivity to LPS stimulation in AIDS patients remain to be investigated. Our observation suggests that high levels of the acid-labile IFN- α subtype in AIDS sera contribute significantly to the enhancement of TNF synthesis. This enhancement of TNF synthesis could also occur through sensitization of the cells to repetitive exposure to viral, bacterial, or other foreign antigens in vivo. The role of HIV gene products, such as the envelope proteins, in contributing to the activation phenomenon must also be considered.

In conclusion, activation of the TNF system by the acid-labile IFN- α may contribute to physiologic disturbances such as the wasting syndrome observed in AIDS. Furthermore, the acid-labile IFN- α may play a key role in the pathophysiological changes, in particular the enhancement of TNF synthesis, involved in septic shock in AIDS patients.

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