Pleiotropic responses to interferon-gamma in humans

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Chapter 5

Interferon-gamma in healthy subjects; selective modulation of inflammatory mediators

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Abstract

Cytokines are important mediators involved in the pathogenesis of sepsis. Administration of cytokines, like tumor necrosis factor (TNF)-α and interleukin (IL)-6 to animals and humans mimics disturbances on acute phase protein levels, leukocyte activation parameters and coagulation and fibrinolysis as seen in sepsis. Clinical data and studies in animals suggest that interferon (IFN)-γ is another mediator in the host inflammatory response, which could be of importance in the pathophysiology of sepsis. The role of IFN-γ in human host inflammatory responses, however, has not been studied. To evaluate the role of IFN-γ on the human inflammatory response, we studied the acute effects of recombinant human IFN-γ (rhIFN-γ, s.c. 100 $\mu g/m^2$) administration on a selection of host inflammatory mediators: the cytokine/chemokine cascade system, acute phase proteins, humoral and membrane activation markers of the innate cellular immunity and coagulation/fibrinolysis parameters. IFN-γ increased plasma levels of IL-6, IL-8 and IFN-gamma inducible protein-10 (IP-10) ($p<0.05$), but did not affect plasma levels of other cytokines (IL-2, IL-4, IL-10, TNF-α, IL-12p40/p70). Plasma concentrations of C-reactive protein and secretory phopholipase A2 both increased ($p<0.05$). Plasma levels of the leukocyte activation marker elastase-alpha1-antitrypsin complexes increased after IFN-γ administration ($p<0.05$), IFN-γ increased the percentage of high affinity Fcγ- receptor (FcγRI) positive neutrophils ($p<0.05$), but did not affect the mean fluorescence intensity of FcγRI on neutrophils. There was a modest procoagulant and profibrinolitic effect of IFN-γ, as evidenced by increased plasma levels of prothrombin fragment F1+F2, tissue-plasminogen activator and plasmin-alpha2-anti-plasmin complexes ($p<0.05$). We conclude that IFN-γ selectively affects host inflammatory mediators in humans.

Introduction

Sepsis is a clinical syndrome with a high mortality, induced by an excessive host inflammatory response to invading microorganisms and their products. Severe sepsis may result in serious hemodynamic, metabolic, coagulatory and fibrinolytic derangement's (1-3). Among important mediators involved in the host inflammatory response are subsets of leukocytes, complement and coagulation/ fibrinolysis cascade systems and cytokines (1,4,5).

Cytokines that are enhanced in sepsis are tumor necrosis factor-α (TNF-α), Interleukin-1β (IL-1β), IL-6, IL-10 and interferon-gamma (IFN-γ) (4,6-8). Evidence for the pivotal role of cytokines in the host inflammatory response are derived from human and animal studies in which endotoxin, cytokines or neutralising antibodies to cytokines or receptors were administrated (4).

IFN-γ is a pleitropic proinflammatory cytokine which is produced by several cell types, including activated lymphocytes, natural killer cells and macrophages (9). Animal studies are contradictory about the role of IFN-γ in sepsis. Pretreatment of endotoxin-challenged mice with homologous IFN-γ increased mortality (10). Antibodies to IFN-γ protected against LPS-induced lethality in mice and chimpanzees, even when antibodies were administered 2 hours after the challenge (10-12). These data indicate a proinflammatory role of IFN-γ. However, IFN-γ receptor deficient (IFN-γR$^{-/-}$) mice suffering from sepsis originating from a local infection, showed a decreased survival as compared to TNF-αR$^{-/-}$ mice and controls, indicating a protective role of IFN-γ levels in surviving sepsis (13). Hence, the role of IFN-γ in human host inflammatory responses is not fully understood.

To evaluate the role of IFN-γ in the human inflammatory response, we measured in healthy subjects, in a saline-controlled crossover study, the acute effects of recombinant human IFN-γ (rhIFN-γ, s.c. 100 μg/m^2) administration on a selection of host inflammatory mediators: the cytokine/chemokine cascade system, the acute phase proteins, humoral and membrane activation markers of the innate cellular immunity and coagulation/fibrinolysis parameters.

Subjects and Methods

Subjects

Six healthy male volunteers (age 22 ± 1 yr, mean ± standard error [SE]) participated in the study. They were all in good health, had not experienced any febrile disease in the month prior to the study and did not use any medication. The study was approved by the Research Committee and the Medical Ethical Committee of the Academic Medical Center, Amsterdam. All subjects gave written informed consent.

Study design

Each subject was studied twice, at least four weeks elapsed between the two study-periods. On one occasion the subjects received recombinant human interferon-gamma (rhIFN-γ) (Immukine, Boehringer Ingelheim GmbH, Ingelheim/Rhein, Germany), on the other occasion saline (control study). The order in which rhIFN-γ or saline was given was determined by balanced assignment. Before the start of the study, a 19-Gauge catheter was inserted into a hand vein. The catheter was kept patent by infusion of saline solution. During both studies the subjects were confined to bed. Just before 9.00 A.M. (t=0) blood samples for baseline values were collected. At t=0, 100 $\mu g/m^2$ rhIFN-γ or a similar volume of saline was injected subcutaneously. At 30 min and 1, 2, 4, 6, 8, 10, 12 and 24 hours after injection of rhIFN-γ or saline, blood was drawn for the measurement of leukocytes and differential counts, plasma cytokines, leukocyte activation markers, acute phase proteins and coagulation/fibrinolysis parameters. Additionally, at t=48 hours after injection of IFN-γ, blood was drawn for determination of plasma cytokine and acute phase protein levels.

Assays

Blood drawn for determination of plasma parameters was collected in tubes provided with adequate additives and centrifuged at 4 ^{0}C for 10 minutes at 1550 g. Supernatants were immediately stored in aliquots at –80 ^{0}C. All samples were thawed only once. Serial plasma samples of each individual subject were tested in the same run in duplicate. IFN-γ plasma levels were measured using an in-house sandwich ELISA with a detection limit of 31 pg/ml (14). IL-2, IL-4, IL-6 IL-10, TNF-α, IL-12p40, IL-12p70, C-reactive protein (CRP) and secretory phopholipase A2 (sPLA2) plasma levels were measured using sandwich ELISA's (CLB, Amsterdam, The Netherlands). IL-8 and interferon-gamma inducible protein-10

(IP-10) were also measured by ELISA (Biosource, Etten-Leur, The Nether-lands; and R&D Systems, Abington, UK, respectively). Plasma con-centrations of neutrophilic elastase complexed to α_1-antitrypsin (elastase-α1Atc, referred to as elastase) and lactoferrin were measured by specific radioimmunoassays (RIA) (15). Coagulation and fibrinolysis parameters in plasma were detected by ELISA; thrombin-antithrombin III (TAT) complexes (Behringwerke AG, Marburg, Germany), prothrombin fragment F1+F2 (Behringwerke AG, Marburg, Germany), plasmin-α2-antiplasmin (PAP) (16), tissue-plasminogen activator (t-PA; Asserachrom t-PA, Diag-nostica Stago,

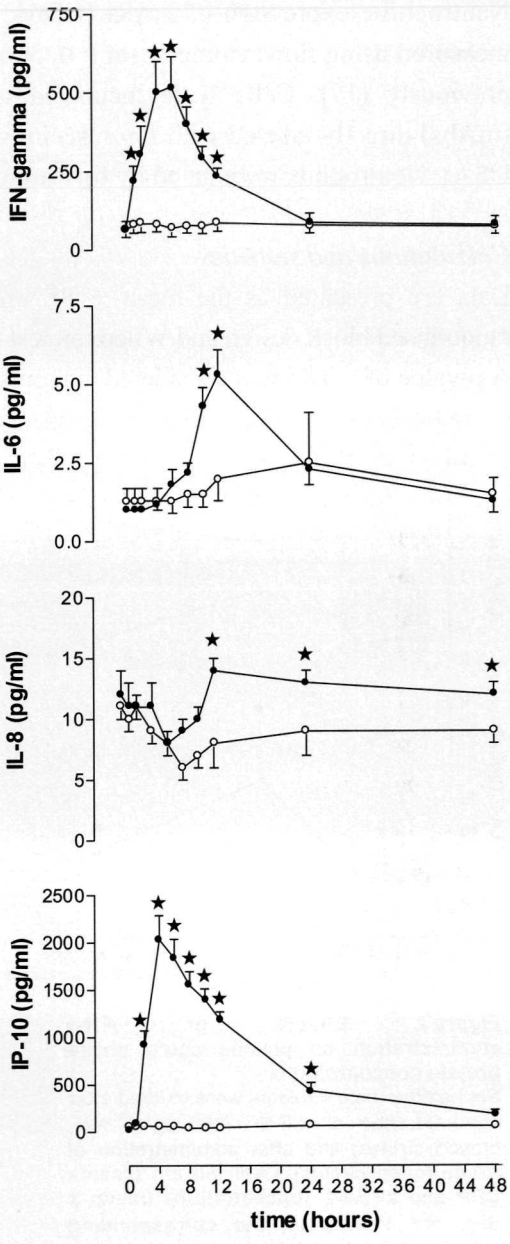

Figure 1 **Effects of IFN-γ administration on cytokines and chemokines in plasma:**
Six healthy male subjects were studied after administration of rhIFN-γ (100 μg/m^2 s.c.; closed circles) and after administration of saline (open circles). Depicted are plasma IFN-γ, IL-6, IL-8 and IP-10 concentrations (mean ± SE). ★= P<0.05 vs the corresponding value on the control day.

Asnieres-sur-Seine, France) and plasminogen activator-inhibitor type 1 (PAI-1; TintElize PAI-1, Biopool, Umea, Sweden). Neutrophil counts were determined by flow cytometry (Technicon H1 system, Technicon Instruments, Tarrytown, USA).

Neutrophilic expression of FcγRI (CD64, high affinity Fc receptor for IgG) was measured using flowcytometry (at t=0, 30 min, and 4, 8 and 24 hours) as described previously (17). Cells were incubated with anti-CD64 monoclonal antibodies (mAbs) directly labeled with fluorescein isothiocyanate (Medarex, Annandale, NJ, USA). Neutrophils were gated by forward and side scatter parameters.

Calculations and statistics

Data are presented as the mean ± SE and analysed by analysis of variance for randomised block design and Wilcoxon test to compare data at individual time points. A p-value of < 0.05 was considered to represent statistical significance.

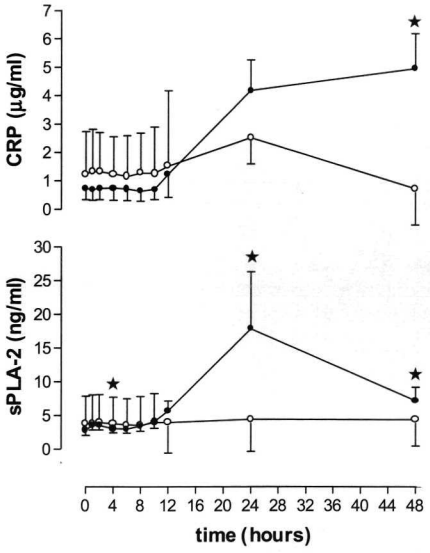

Figure 2 **Effects of IFN-γ administration on plasma acute phase protein concentrations:**
Six healthy male subjects were studied after administration of rhIFN-γ (100 µg/m^2 s.c.; closed circles) and after administration of saline (open circles). Depicted are plasma CRP and sPLA-2 concentrations (mean ± SE). ★= P<0.05 vs the corresponding value on the control day.

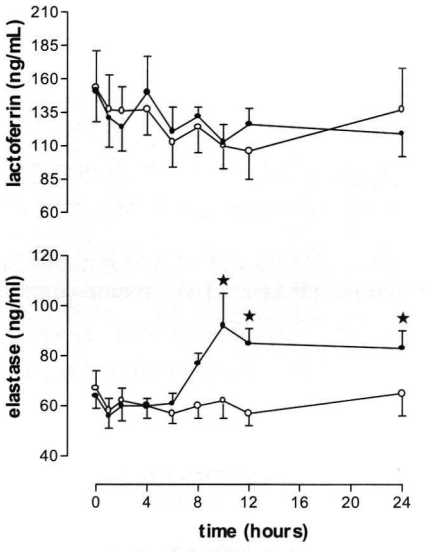

Figure 3 **Effects of IFN-γ administration on plasma neutrophil activation marker concentrations:**
Six healthy male subjects were studied after administration of rhIFN-γ (100 µg/m^2 s.c.; closed circles) and after administration of saline (open circles). Depicted are plasma concentrations of lactoferrin and elastase (mean ± SE). ★= P<0.05 vs the corresponding value on the control day.

Results

Baseline levels of all parameters did not differ between the control and intervention study.

Clinical effects of IFN-γ

IFN-γ caused an increase in temperature from 36.2 ± 0.2 to 36.9 ± 0.1 ^{0}C ($p<0.05$ versus control). Blood pressure was not different between the control and intervention studies, whereas the pulse rate increased significantly after IFN-γ (18). IFN-γ administration did not cause chills, nausea or other signs of acute illness.

Cytokines and chemokines (fig.1)

Cytokines: During the control study, IFN-γ levels remained around the detection limit of the assay (31 pg/ml). In the intervention study, IFN-γ levels increased gradually to 518 ± 96 pg/ml after 6 hours ($p<0.05$ vs control). The IFN-γ plasma levels at 24 and 48 hours after IFN-γ injection were not different from pretreatment values. Upon IFN-γ administration plasma levels of IL-6 gradually increased to reach peak levels at 12 hours after the injection ($p<0.05$ vs control). In contrast, IFN-γ administration had no effect on plasma levels of IL-2, IL-4, IL-10, TNF-α, IL-12p40 and IL-12p70.

Chemokines: No changes in chemokine levels (IL-8, IP-10) were detected upon injection of saline. IL-8 levels increased 12 hours after IFN-γ ($p<0.05$ vs control) to return gradually to baseline thereafter. IP-10 levels were increased already 1 hour after IFN-γ to reach peak values at t=4 hours ($p<0.05$ vs control).

Acute phase proteins (fig. 2)

No changes in APP levels (CRP, sPLA2) were detected during the control study. Plasma CRP level started to increase 24 hours after IFN-γ administration and was significantly elevated at t=48 hours ($p<0.05$ vs control study). sPLA2 reached a peak level at 24 hours after IFN-γ and had not yet returned to baseline levels at t=48 hours ($p<0.05$ vs control study).

Granulocyte and monocyte activation (figs. 3 and 4)

No changes in leukocyte activation markers (elastase and lactoferrin) were detected during the control study. Levels of elastase were elevated 8 hours after IFN-γ

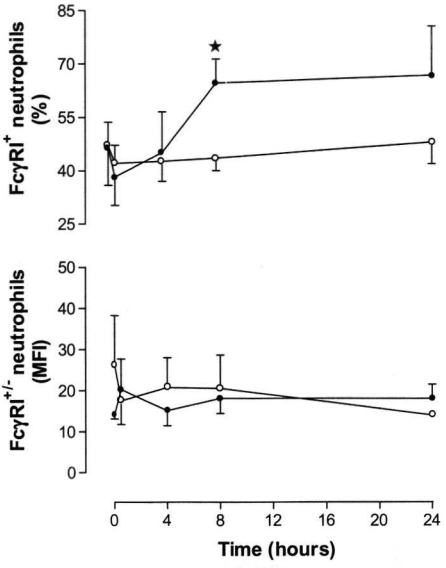

Figure 4 **Effects of IFN-γ administration on FCγRI (CD64) expression on neutrophils:**
Six healthy male subjects were studied after administration of rhIFN-γ (100 μg/m^2 s.c.; closed circles) and after administration of saline (open circles).
Upper panel: **Percentage of peripheral blood neutrophils expressing FcγRI (mean ± SE). ★ = P<0.05 vs the corresponding value on the control day.**
Lower panel: **MFI of FcγRI on the total peripheral blood neutrophil population (mean ± SE). Differences between the control and intervention studies were not significant (MFI = mean fluoresence intensity).**

($p<0.05$ vs control study). No effect of IFN-γ on plasma lactoferrin levels was observed.

No changes in expression of CD64 (MFI or percentage of positive cells) were detected during the control study. After IFN-γ administration, the percentage of $CD64^+$ neutrophils increased ($p<0.05$ vs control), whereas no effect was measured on the MFI of CD64.

Coagulation and fibrinolysis (fig 5)

Coagulation. No changes in parameters of stimulation and inhibition of plasminogen activation were detected during the control study. IFN-γ induced a significant increase of prothrombin fragment F1+F2 levels at t=12 hours ($p<0.05$ vs control). Simultaneously, TAT-complexes tended to increase although the changes in levels did not reach significance.

Fibrinolysis During the control study no changes were detected. IFN-γ induced transient increases in plasma t-PA antigen and PAP complexes (t-PA and PAP; $P<0.05$ vs controls). No effect was observed on PAI-1 levels.

Discussion

Our data show that IFN-γ administration to healthy subjects induces profound effects on circulating chemokine levels, neutrophil activation, acute phase protein release and coagulation/fibrinolysis parameters, with only mild clinical signs of inflammation and -except for a moderate IL-6 response- no effects on plasma cytokine levels. Administration of TNF-α, IL-6 or IFN-α in comparable molar amounts, however, is known to induce much stronger effects on bloodpressure and temperature and even causes nausea (19-21). Our observations are relevant for human pathophysiology, since mean IFN-γ serum levels during the present study (~105 pg/ml) are in range with those reported in septic shock patients with increased IFN-γ levels (33-630 pg/ml) (8). The present study can not be compared with data from literature since effects of IFN-γ on the host inflammatory response have not been investigated in humans, except for two studies by Gluzko and Schiff on fibrinolysis and granulocyte activation, respectively (22,23).

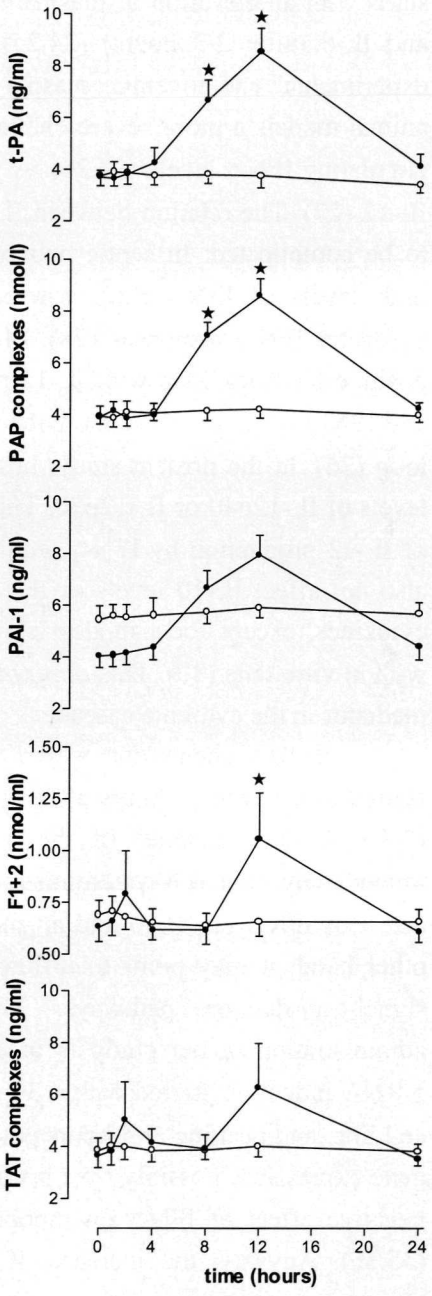

Figure 5 **Effects of IFN-γ administration on fibrinolysis and coagulation parameters:**
Six healthy male subjects were studied after administration of rhIFN-γ (100 μg/m^2 s.c.; closed circles) and after administration of saline (open circles). Depicted are plasma concentrations of t-PA, PAP complexes, PAI-1, prothrombin fragment F1+F2 and TAT-complexes (mean ± SE). ★= P<0.05 vs the corresponding value on the control day.

Induction of experimental endotoxemia in humans and non-human primates, induces activation of cytokine cascade within one to two hours (4). This response starts with an elevation of plasma TNF-α, subsequently followed by rises of IL-1 and IL-6 (after 2-3 hours) (24,25), and IL-12p40/70 (3 hours) (26). In human experimental endotoxemia, plasma levels of IFN-γ do not increase, whereas in animal models a more severe challenge is consistently followed by an increase in circulating IFN-γ levels (24,26). A potent inducer of IFN-γ in vitro and in vivo is IL-12 (27). The relation between IL-12 levels and IFN-γ in vivo, however, seems to be complicated. In septic baboons high IL-12 levels did not correspond with high levels of IFN-γ (26), whereas in mouse models neutralization of IL-12 abrogates IFN-γ responses (28). Moreover, IFN-γ is thought to be involved in a positive feedback loop with IL-12 production (26,29). The course of IL-12p40/70 and IFN-γ induction in septic baboons is consistent with such a positive feedback loop (26). In the present study, however, IFN-γ alone did not induce measurable levels of IL-12p40 or IL-12p70. Thus, apparently, the positive feedback regulation of IL-12 production by IFN-γ requires participation of other cytokines. IFN-γ did also not affect IL-10 levels or the production of other pro- or anti-inflammatory cytokines, except for a modest elevation of plasma IL-6, which is in agreement with in vitro data (30). This observation argues for a role of IFN-γ as an end-effect mediator in the cytokine cascade.

IL-8, a chemokine which is associated with neutrophil trafficking (31), started to increase 12 hours after IFN-γ administration. However, plasma levels of IP-10, another member of the CXC chemokine subfamily, increased almost immediately after IFN-γ administration. These differences may be due to a direct effect of IFN-γ on IP-10 and an indirect effect of IFN-γ on IL-8 release. On the other hand, it may point to different post-receptor mechanisms, i.e. to different signal-transduction pathways. The rapid increase of IP-10 upon IFN-γ administration in our study is in agreement with in vitro data, in which IP-10 mRNA induction started within 30 minutes past IFN-γ stimulation of a lymphoma cell line, and peaking at 5 hours post IFN-γ (32). IFN-γ upregulates monocyte IL-8 gene expression, possibly by a posttranscriptional mechanism (33,34). However, a negative effect of IFN-γ on monocyte IL-8 production has also been described (35,36). Anyway, the increase of IL-6, IL-8 and IP-10 stresses the important

immunoregulatory properties of IFN-γ in the context of an inflammatory response in humans.

Two major APP's, CRP and sPLA2, increased following IFN-γ administration. The mechanism for this increase is not completely clear. IFN-γ can directly induce APP production by hepatoma cell-line Hep G2 in vitro (37). On the other hand, we can not exclude that IL-6 is involved in the production of APP's. A third stimulator of APP secretion are corticosteroids. In our study, however, no correlation was found between the increase in cortisol and in APP's. That the initial elevation of sPLA2 was followed soon by an increase of circulating CRP fits with the concept that APP's interact with injured cells (38,39).

During degranulation neutrophilic granulocytes release elastase and lactoferrin, which are considered as markers for neutrophil activation (40,41). IFN-γ selectively induced the release of elastase, but not of lactoferrin. This discrepancy is remarkable, since the trigger needed to release elastase is considered to be stronger than the trigger needed for lactoferrin release (15). However, predominant or exclusive increases of elastase have also been observed in clinical situations (15,42). Another parameter for the activation of neutrophilic granulocytes is the expression of FcγRI (CD64, high affinity Fc receptor for IgG). The percentage of neutrophils expressing FcγRI increased after IFN-γ administration, whereas no effect was measured on the MFI of FcγRI on neutrophils. The latter finding is in contrast to the study by Schiff et al. (23), who administered IFN-γ (50 $\mu g/m^2$/day) to healthy subjects for two days, and noticed an increased MFI of FCγRI expression on neutrophils. We have no clear explanation for these different results, except that Schiff injected less IFN-γ. It remains to be established to what extent the observed effects of IFN-γ on neutrophils reflected direct or indirect interaction, via mediators like corticosteroids, for IFN-γ induces a small, but significant, rise in plasma cortisol levels (18,43,44). In addition, mediators such as IL-8 and IP-10, both affecting leukocyte functions, may also have contributed to the effects of IFN-γ on neutrophils. An effect via IL-8, however, seems less likely since the course of plasma levels suggested that the IL-8 secretion occurred at a time that elastase already was elevated. A direct effect of IFN-γ on neutrophil kinetics and activation is another possibility, since IFN-γ can affect neutrophil activation and adherence to endothelial cells in vitro (45,46).

Cytokines are important mediators in the imbalance between coagulant and anticoagulant mechanisms in sepsis and endotoxemia (5). In vivo studies in humans and non-human primates revealed specific effects of individual cytokines on coagulation and fibrinolysis activation (47-51). IFN-γ induced a modest fibrinolytic activity, as measured by enhanced levels of t-PA. This increased PA activity was not blunted by increased PAI-1-activity, ultimately resulting in enhanced plasmin generation, as evidenced by increased levels of PAP complexes. In addition to these pro-fibrinolytic effects, IFN-γ also caused activation of coagulation, as reflected by a modest increase in F1+2 levels and a (non-significant) increase in TAT-complexes. A previous study, in which a lower dose of IFN-γ was given on four consecutive days, reported a similar activation of fibrinolysis but a simultaneous inhibition of plasminogen activation due to enhanced levels of PAI-1 (22). The differential effects of IFN-γ on PAI-1 levels may well be due to the differences in treatment regimens. Effects on fibrinolytic activity may be mediated by a direct effect of IFN-γ on endothelial cells. In vitro studies report modulation of fibrinolysis by IFN-γ on different cell lines, although results are contradictory (52,53). Effects of IFN-γ on coagulation parameters, however, are most likely to be mediated by IL-6, which explains the relative late elevation of F1+2 levels (49).

In conclusion, the present study demonstrates a number of pro-inflammatory effects of IFN-γ in healthy individuals. Synergism of IFN-γ with other mediators of inflammation, like TNF-α, may increase these effects (11).

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