Interleukin-2 induces activation of coagulation and fibrinolysis: resemblance to the changes seen during experimental endotoxaemia

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Summary. The administration of Interleukin-2 (IL-2) causes the release or generation of other cytokines such as tumour necrosis factor (TNF) which, by disturbing the anticoagulant properties of the endothelium, may induce a procoagulant state in patients receiving this drug.

We therefore evaluated the effects of IL-2 on coagulation and fibrinolysis in 14 patients receiving 12 or 18 × 10⁶ IU/m²/d of IL-2 given as a 15 min infusion for 5 d. Blood samples were drawn at short intervals after the first IL-2 infusion. The parameters were analysed by way of analysis for repeated measures (F tests rather than t tests).

During the first day, thrombin-antithrombin (TAT) complexes started to increase 2 h after the IL-2 infusion, reaching peak levels at 4 h (n=14; 11.2±6.4 μg/l vs 49.8±49.2 μg/l, P<0.01). Plasmin α2 antiplasmin (PAP) complexes showed a similar pattern rising from a mean baseline value of 17.5±7.6 nmol/l to 66.8±47.7 nmol/l at 4 h (P<0.01). In four patients the peak of PAP preceded that of TAT. Tissue plasminogen activator (tPA) rose from a mean baseline value of 4.9±3.7 μg/l to 26.3±13.5 μg/l at 4 h (P<0.01). Plasminogen-activator-inhibitor-1 (PAI-1) levels increased from 59±35 μg/l to 113±39 μg/l at 6 h (P<0.01). tPA PAI-1 complexes increased from 0.15±0.07 to 0.69±0.21 nmol/l at 6 h (P<0.01).

Our study indicates that IL-2 activates the coagulation and fibrinolytic systems in vivo. The changes resemble the perturbations observed after endotoxin/TNF administration. These abnormalities may play a role in the side-effects induced by IL-2 therapy.

Interleukin-2 (IL-2) is a cytokine secreted by activated T cells, which is undergoing evaluation as an anti-tumour agent. IL-2 has been shown to produce partial and complete remissions in 15–30% of the patients with metastatic renal cell cancer or melanoma (Rosenberg et al. 1989; Eberlein et al. 1988; West, 1989; Dutcher et al. 1989; Fischer et al. 1989; Oliver, 1988). The administration of IL-2 is, however, accompanied by substantial side-effects, which include the vascular leak syndrome, renal and hepatic dysfunction and haemodynamic perturbations resembling those changes seen in septic shock (Rosenberg et al. 1989; Eberlein et al. 1988; West, 1989; Dutcher et al. 1989; Fischer et al. 1989; Oliver, 1988; Ognibene et al. 1988). Since IL-2 itself does not directly affect endothelial cells (Rosenstein et al. 1986) these phenomena must be mediated by the in vivo generation of other cytokines (Balkwill & Burke, 1989), such as tumour necrosis factor (TNF) and Interleukin-1 (Chong et al. 1989; Gemlo et al. 1988; Mier et al. 1988). The role of TNF as an important mediator of IL-2 induced toxicity is illustrated by several observations. Mier et al. (1990) have shown that the concurrent administration of dexamethasone with IL-2 prevented the in vivo generation of TNF, resulting in amelioration of the vascular leak syndrome and the degree of hypotension observed after IL-2 infusion. In addition, passive immunization against TNF can partially abrogate IL-2 toxicity in animals (Fraker et al. 1989).

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When endothelial cells are perturbed by noxious stimuli such as TNF, a variety of changes occur, including synthesis of tissue factor (TF) (Nawroth et al. 1986; Nawroth & Stern, 1957; Bevilacqua et al. 1984, 1986), plasminogen-activator inhibitor (PAI-1) (Gramse et al. 1986; Emeis & Kooistra, 1986; van Hinsbergh et al. 1988; Schleef et al. 1988) and the loss of thrombomodulin (Nawroth et al. 1986) all of which promote a procoagulant state. In addition, platelet activating factor (PAF) is released, leading to the aggregation and adhesion of platelets, vasoconstriction and increased vascular permeability (Bussolino et al. 1986). Administration of a single injection of TNF to healthy volunteers elicits a rapid and sustained activation of the common pathway of coagulation, probably induced via the extrinsic route (v.d. Poll et al. 1990), indicating that TNF can also induce a pro-coagulant state in vivo.

Considering the fact that TNF is an important mediator by which IL-2 can induce its toxicities in vivo, we supposed that administration of IL-2 might also be accompanied by disturbances in coagulation and fibrinolysis. Indeed, we have recently observed a coagulopathic state in patients receiving IL-2 (Strack van Schijndel et al. 1989). Although the development of thrombocytopenia during IL-2 treatment (Ettinghausen et al. 1987) has been regularly described, there are fewer published data relating to the changes in the coagulation and fibrinolytic systems of patients receiving IL-2.

To evaluate the activation of coagulation and fibrinolysis we studied the sequential changes of both systems in 14 patients undergoing IL-2 therapy, utilizing several assays for their activation products. Our results indicate that both systems become activated during treatment with IL-2 and that the observed activation pattern shows a striking resemblance to the changes seen during experimental endotoxaemia.

**PATIENTS AND METHODS**

**Patients.** Fourteen patients with metastatic malignant melanoma or renal cell carcinoma undergoing IL-2 therapy in the department of medical oncology were studied. All patients gave informed consent and the protocols were approved by the ethical and scientific committees of the Free University Hospital.

The group consisted of 11 males and three females, who took part in a phase II study of a combination treatment with Interferon-gamma (IFN-γ) followed by IL-2. The diagnosis was malignant melanoma in six and metastatic renal cell cancer in eight patients. The Karnofsky performance status was between 70% and 100% in all patients and their median age was 54 years (range 37–66 years).

Before treatment, all patients had normal platelet numbers and no coagulation abnormalities as detected by routine assays (prothrombin time, cephalin time). The treatment was given in the medium care section of the medical oncology ward. After pretreatment with IFN-γ given by subcutaneous or intramuscular injection at a dose of 100 μg/m²/d for 5 consecutive days, the patients received on the 5 subsequent days IL-2 at a dose of either 12 or 18 IU/m²/d as a bolus infusion over 15 min. All patients received 500 mg acetaminophen four to six times per day during IFN-γ therapy and 25 mg or 50 mg indomethacin three times daily during treatment with IL-2 in order to ameliorate fever, chills, headache and myalgia.

**Drugs.** Recombinant IFN-γ was provided free of charge by Boehringer-Ingelheim BV (Alkmaar, The Netherlands). It had a specific activity of >1 × 10⁷ IU/mg protein based on antiviral activity. Recombinant human IL-2 was provided partly free of charge by Eurocetus BV (Amsterdam, The Netherlands). The IL-2 had a specific activity of 18 × 10⁶ IU/mg of protein (equivalent to 3 × 10⁶ Cetus units or 6.9 × 10⁸ Biological Response Modifier Program units). International units (IU) will be used in this paper unless otherwise stated.

**Blood collection.** Blood samples were obtained through an indwelling intravenous device (Venflon® infusion needle 16G), without congestion and collected in 5 ml siliconized glass tubes that contained 10 mm EDTA and 0.05% Polybrene (final concentrations) to prevent activation of the complement and contact coagulation system. The first 4–5 ml of the withdrawn blood were not used for the present study. The blood samples were taken before and at 1, 2, 4, 6, 8, 10, 12 and 24 h after the start of IL-2. Plasma was obtained by centrifugation of blood for 10 min at 1300 g.

All plasma samples were stored at −70°C until tested.

In two healthy volunteers blood samples were drawn using the same protocol. These samples were used to establish that the indwelling catheter and sampling technique did not cause activation of coagulation and fibrinolysis.

**Assays.** Thrombin–antithrombin III (TAT) complexes were measured with an enzyme linked sorbent assay obtained from Behringwerke AG (Marburg, Germany). Results were expressed as μg per litre. Normal values were less than 4 μg/l.

Plasmin–α2-antiplasmin (PAP) complexes were determined with a novel assay that has been described elsewhere (Levi et al. 1992). Briefly, samples were incubated with a monoclonal antibody specific for complexed α2-antiplasmin that was coupled to sepharose. After incubation, non-bound proteins were removed by a washing procedure. Bound PAP complexes were then quantified by an incubation with labelled monoclonal antibody to plasmin. Results were referred to a standard curve of purified complexes and expressed as nmol/l. Normal values were less than 8 nmol/l of PAP complexes per litre.

Antigenic levels of tissue type plasminogen (tPA) and its inhibitor plasminogen-activator inhibitor-1 (PAI-1) were determined with sandwich type radio-immunoassays using appropriate monoclonal antibodies that have been described previously (Lambers et al. 1988; de Boer et al. 1991). For both assays, results were related to standard curves of recombinant t-PA and PAI-1 purified from Hep-G2 cells (de Boer et al. 1991), respectively and expressed as μg/l. Normal values were less than 11 μg/l for tPA. Levels of PAI-1 in normal individuals were 42 ± 19 μg/l (mean ± standard deviation) for PAI-1. tPA–PAI complexes were measured with a differential antibody sandwich assay using monoclonal antibodies against tPA (Zonneveld et al. 1987) on the solid phase (sepharose) and radiolabelled polyclonal rabbit antibodies against human PAI-1 (de Boer et al. 1991) as the second
antibody. Results were related to standard curves of purified complexes and expressed as nmol/l. In short, purified recombinant tPA was incubated with an excess of purified PAI-1 until chromogenic activity was zero (de Boer et al. 1991). The amount of complexes in this mixture was said to equal the amount of tPA added. 0-1 nmol/l tPA–PAI-1 complexes is equivalent to 7.2 ng/l tPA. Normal values of tPA–PAI-1 complexes were less than 0-12 nmol/l.

Antigenic levels of α2-antiplasmin (α2AP), antithrombin III, fibrinogen and plasminogen were measured with a nephelometer (Behringwerke AG) using appropriate antisera and according to manufacturer’s instructions. Results were expressed as mg per litre.

**Statistical methods.** The data in figure and the table are represented as observed means and standard deviations.

The observations were analysed by way of analysis of variance for repeated measures. For some patients, the observations were not entirely complete mainly due to an inability to derive a blood-sample without congestion or the patient’s request to limit the sampling to certain time points (day 1: 0 h n = 14; 1 h n = 6; 2 h n = 11; 4 h n = 13; 6 h n = 12; 8 h n = 12; 10 h n = 4; 12 h n = 8). We assumed that the observations were missing at random: in other words, that the missing observations could be considered as a random subsample. Under these conditions, valid estimates of the average levels can be obtained by way of a method called maximum likelihood estimation (Little & Rubin. 1990).

The analysis was done using program PSV of the statistical software program BMDP (Dixon. 1990). The null hypothesis tested was that on average these levels did not change over the time. For each response variable, separately estimated average levels were compared between time points.

Prior to these analyses, for each variable and each time-point separately, it was tested whether or not the observations were normally distributed. If the data were not normally distributed, they were ignored.

The means of the values of objective toxicities occurring on the fifth day of treatment were compared to their respective baseline values by the use of the Student’s t test. P values of less than 0.05 were considered to represent significant differences for both statistical methods.

**RESULTS**

**Patients**

All patients were evaluated during their first cycle of therapy. None of them required admission to the intensive care unit or vasopressor support for hypotension. Details of the changes occurring in these patients during IL-2 therapy are listed in Table I. The acute side-effects consisted of pyrexia, rigors, tachycardia and mild hypotension. Chronic toxicity was manifested by a small increase in weight, decrease in albumin (Alb) concentration, slight renal and hepatic function disturbances. All toxicity was rapidly reversible upon cessation of the IL-2. None of these patients experienced clinically observable thrombo-embolic events during therapy.

**The activation of coagulation**

As a parameter for coagulation we measured circulating TAT complexes. Eight of 14 patients had mildly elevated TAT complexes before starting therapy (range 10–25 μg/l). Upon IL-2 administration, levels started to increase at 2 h after IL-2 administration, reaching peak levels at 4 h (Fig 1). Thereafter, TAT complexes slowly declined, returning to baseline levels at 12 h after the infusion (Fig 1). In all patients TAT complexes increased upon infusion of IL-2, but the extent of this response varied considerably between the patients both with respect to the peak value reached (from 1.2 to 16.6

Table I. Changes in 14 patients receiving a bolus infusion of IL-2 for 5 consecutive days.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline*</th>
<th>Zenith or nadir*</th>
<th>Time after IL-2</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic BP (mmHg)</td>
<td>121±12</td>
<td>112±7</td>
<td>8 h†</td>
<td>&lt;0.01‡</td>
</tr>
<tr>
<td>Heart rate (beats/min)</td>
<td>81±12</td>
<td>102±9</td>
<td>4 h†</td>
<td>&lt;0.01‡</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>36.1±0.7</td>
<td>37.0±1</td>
<td>6 h†</td>
<td>&lt;0.01‡</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>68.6±7.6</td>
<td>70.0±8.1</td>
<td>Day 5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Albumin (g/l)</td>
<td>30±6</td>
<td>24±4</td>
<td>Day 5</td>
<td>&gt;0.02</td>
</tr>
<tr>
<td>Creatinine (μmol/l)</td>
<td>107±32</td>
<td>122±62</td>
<td>Day 5</td>
<td>&gt;0.02</td>
</tr>
<tr>
<td>γGT (U/l)</td>
<td>127±138</td>
<td>248±142</td>
<td>Day 5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Bili (μmol/l)</td>
<td>7±5</td>
<td>22±18</td>
<td>Day 5</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>AP (U/l)</td>
<td>196±177</td>
<td>318±262</td>
<td>Day 5</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>ASAT (U/l)</td>
<td>29±15</td>
<td>53±32</td>
<td>Day 5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>ALAT (U/l)</td>
<td>29±18</td>
<td>68±46</td>
<td>Day 5</td>
<td>&lt;0.02</td>
</tr>
</tbody>
</table>

* α2AP: gamma-glutamyl transferase; Bili: bilirubin; AP: alkaline phosphatase; ASAT: amino-aspartate transferase; ALAT: amino-alanine transferase.

† Values are given as the mean ± standard deviation.

‡ Day 1 of the treatment.

‡ Testing the hypothesis that on average the levels did not change during day 1.
times the baseline value) as well as to the time on which this maximum occurred (one patient at 2 h, eight patients at 4 h, two patients at 6 h, three patients at 8 h).

The values of TAT changed significantly during the first day (Fig 1).

The course of plasminogen activation
As a parameter for plasminogen activation in vivo, we measured PAP complexes. Thirteen out of 14 patients had slightly elevated PAP complexes before starting the therapy compared to normal controls. The concentration of these complexes rose significantly at 2 h after the IL-2 infusion, reaching maximum values at 4 h after IL-2 administration (Fig 1). PAP complexes returned to baseline levels within 24 h. During the first day, increases in PAP levels were observed in 12 of the 14 patients (range 1·3–15·76 times the baseline value). In four of these 12 patients peak levels of TAT complexes occurred after those of PAP complexes, whereas in six patients both complexes peaked at the same time. In two patients the peak of the TAT complexes occurred before that of the PAP complexes.

The values of PAP changed significantly during the first day (Fig 1).
1. All patients had elevated values during the first day, ranging from 1-3 to 9-8 times the baseline values, occurring at 4 h in six patients, at 6 h in seven patients and at 8 h in one patient. The levels of PAI and tPA—PAI once again showed a significant change during the first day (Fig 1).

The course of other coagulation and fibrinolytic parameters

In addition to the parameters mentioned above, we also measured levels of fibrinogen (FBG), plasminogen (PLG), AT III and α2 antiplasmin (α2AP). To correct for the vascular leakage, levels of these proteins were related to those of albumin.

When we looked at the trend of the mean values during the first day, the AT III/alb and α2AP/alb ratios remained unchanged (data not shown). The mean values of FBG/alb and PLG/alb ratios did, however, show a significant change during the first day. (P<0.01). The P value is based on all observations during day 1: F test instead of t test. The hypothesis tested was that on average the levels did not change during day 1. The baseline value of the FBG/alb ratio was 0.2±0.12, nadir was 0.18±0.1 at 4 h after IL-2. The baseline value of the PLG/alb ratio was 4.55±1.24, nadir 4.31±1.14 at 8 h after IL-2.

The course of measured parameters in healthy volunteers

All values determined in the healthy volunteers were within normal ranges and showed no significant changes during the day.

DISCUSSION

Patients with metastatic malignancies frequently have disturbances in their coagulation and fibrinolytic systems (Patterson, 1990). In agreement herewith, many of the patients studied had mild elevations in their baseline values of TAT, PAP, PAI-1 and tPA—PAI complexes, although their platelet counts, cephalin and prothrombin time were normal before the start of treatment. The first bolus infusion of IL-2 induced significant increases in the levels of TAT, tPA and PAP complexes, followed by rising concentrations of PAI-1 and tPA—PAI-1 complexes. These results indicate that IL-2 caused an activation of coagulation and fibrinolysis, even at doses which do not cause serious side-effects. The levels of the determined parameters returned to values not significantly different from the baseline within 24 h.

Suffredini et al (1989) studied 19 healthy volunteers, receiving low doses of endotoxin and observed activation of the fibrinolytic system, which started with the release of tPA into the circulation within 1 h after the challenge. The levels of tPA antigen reached a maximum at 3 h. At this time, PAI-1 had risen 4-8-fold, whereas tPA antigen and PAP complexes started to decline at the same time, returning to baseline levels by 24 h. Van Deventer et al (1990) confirmed these observations with respect to activation of the fibrinolytic system in experimental human endotoxaemia. In addition, these authors showed that activation of the coagulation system was continuing at the time that the activation of the fibrinolytic system was decreasing due to rising levels of PAI-1. Thus, at 3-4 h after endotoxin administration, a procoagulant state existed, characterized by an increase in PAI activity and diminished tPA-activity (Suffredini et al, 1989; van Deventer et al, 1990). A similar pattern was observed in our patients after IL-2 administration except that the changes in coagulation and fibrinolysis occurred 1 h later compared to the endotoxin induced patterns.

The striking resemblance between the changes in coagulation and fibrinolysis observed after a bolus infusion of IL-2 or endotoxin suggests that it is likely that the same pathogenetic mechanisms are responsible for these perturbations. Furthermore, it virtually excludes the possibility that the observed changes were due to catheter induced activation, which was also substantiated by the fact that these abnormalities did not occur in two healthy volunteers who had the same catheters inserted.


tPA and PAI, both synthesized by endothelial cells, are critical factors in the regulation of fibrinolysis. IL-1 and TNF stimulate production of PAI (Gramse et al, 1986; Emeis & Kooistra, 1986; van Hinsbergh et al, 1988; Schleef et al, 1988) but have no stimulatory effects on production of tPA in vitro (Gramse et al, 1986; Emeis & Kooistra, 1986; van Hinsbergh et al, 1988; Schleef et al, 1988). It has been shown, however, that TNF administration to healthy volunteers can induce tPA secretion in vivo (v.d. Poll et al, 1991). As already mentioned, TNF injection also induces in vivo a rapid and sustained activation of the common pathway of coagulation, probably due to activation of the extrinsic system (v.d. Poll et al, 1990). Furthermore, in 17 patients with metastatic cancer who received TNF, degradation products of both fibrin and fibrinogen were generated (Hinsbergh et al, 1990). It was hypothesized that this was caused by the fact that immediately after the formation of fibrin molecules, plasmin is formed by tPA (Hinsbergh et al, 1990). These data indicate that TNF might be one of the key factors by which IL-2 causes the observed disturbances in the coagulation and fibrinolysis.

Although the intrinsic pathway of the coagulation system might become activated during high dose IL-2 therapy (Hack et al, 1991; Fleischmann et al, 1991), it is unclear how this contributes to the coagulation abnormalities that we observed in our patients. These phenomena might have some implications for the therapy with cytokines including IL-2.
Induction of a procoagulant state could potentially cause fibrin deposits and formation of microthrombi in small vessels. Double-blind controlled studies on the effect of anticoagulants on the side-effects of cytokine therapy may possibly reveal the extent to which these microvascular thrombotic events contribute to the organ toxicity of cytokines. It is, however, not known whether similar changes occur in the blood vessels of tumour tissue and how this relates to the anti-tumour efficacy of cytokines. Experimental studies suggest that abnormal clotting and fibrinolysis may protect cancer cells from destruction by host inflammatory cells (Gunju & Gorelik, 1988; Dvorak, 1987). Thus, whether the altered coagulant state of the endothelium influences the anti-tumour efficacy of IL-2 therapy remains to be established.

Apart from the induction of changes in the coagulation state at the endothelial cell level, high-dose IL-2 treatment also interferes with the hepatic synthesis of clotting factors and their inhibitors (Richard et al., 1991). It is unlikely that this occurred in our patients, in spite of the observed elevations in liver enzymes, because we did not detect major reductions in fibrinogen. AT III or plasminogen levels, all of which are synthesized by the liver.

In summary, we have shown that treatment with IL-2 leads to activation of the coagulation and fibrinolytic systems, in a similar fashion to that observed after endotoxin administration. We therefore advise that parameters of the coagulation system are evaluated prior to and during cytokine therapy. Moreover, further investigations are needed to explore the impact of these changes on the side-effects and anti-tumour efficacy of IL-2 and other cytokine treatments.

REFERENCES


