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REGULATION OF THE PRODUCTION OF PRO-INFLAMMATORY CYTOKINES AND ANTAGONISTS DURING CHEMOTHERAPY-INDUCED NEUTROPENIA IN PATIENTS WITH HAEMATOLOGICAL MALIGNANCIES*



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Cytokine profiles were studied during 19 episodes of chemotherapy-induced neutropenia in 17 patients with haematological malignancies. Circulating concentrations of interleukin 1 α (IL-1 α), tumour necrosis factor α (TNF- α) and IL-1 receptor antagonist (IL-1ra) were measured before chemotherapy and thereafter three times weekly. During and after chemotherapy no significant changes were found in circulating cytokines. After start of chemotherapy, the ex-vivo LPS-stimulated production of cytokines in whole blood decreased and subsequently disappeared completely in all patients, and recovered after the end of treatment. The decrease of cytokine production could not be attributed to the decreased number of cells only, as the net production per circulating neutrophil or monocyte also decreased significantly, and was restored after completion of chemotherapy. These results show that the production of IL-1 β , TNF- α and IL-1ra in blood disappears during chemotherapy-induced neutropenia, not only due to the decreased number of producing cells, but also as a result of a decreased production per cell, suggesting a mechanism of downregulation.

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Interleukin 1 β (IL-1 β) and tumour necrosis factor- α (TNF- α) are pro-inflammatory cytokines which play an important role in inflammation and fever-related processes.^{1,2} These cytokines are central mediators in the pathogenesis of sepsis and septic shock.³ Interleukin 1 receptor antagonist (IL-1ra) is also produced early in the course of infection,² and as an inhibitor of the effects of IL-1 it plays an important role in the normal and pathological activities of IL-1.⁴

Neutropenic patients are severely immunocompromised and are particularly prone to infections, these being the major cause of death in those patients

as established during autopsy.⁵ In granulocytopenic patients with fever, an infection can be demonstrated in about 50%.⁶ Serum concentrations of TNF, IL-1 and IL-6 have been found elevated in neutropenic children with fever, but no correlation could be found with a documented bacterial aetiology.⁷ In another study, serum concentrations of TNF decreased during chemotherapy-induced neutropenia and increased when patients developed a bacterial infection.⁸ In order to enable proper interpretation of cytokine patterns during infections, data are needed about the cytokine response to anticancer chemotherapy and neutropenia itself. It may be hypothesized that leukopenia may lead to reduced production of cytokines due to the reduced number of cytokine-producing cells. Alternatively, regulatory mechanisms may lead to enhanced production of pro-inflammatory cytokines during leukopenia, in view of their role in haematopoiesis. Studies in mice have shown that concentrations of circulating TNF and IL-6 are considerably higher in granulocytopenic mice infected with *Candida albicans* than in normal mice,^{9,10} but the cellular source of these cytokines is unknown. Little information exists about cytokine profiles of pro-inflammatory cytokines during neutropenia in humans.

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TABLE 1. Patient characteristics.

Patient No.	Age (year)/sex	Diagnosis	Clinical status	Chemotherapy and duration (days)	Co-medication	Infection	Duration of neutropenia (d)	Duration of follow up (d)
1	71/F	AML	1st relapse	ARA-C 1-7 DAU 1	no	no	14	17
2	51/F	AML	newly diagnosed	ARA-C 1-6 DAU 4-6	Prednisone day 5-9	no	9	18
3	21/M	AML	1st relapse	ARA-C 1-5 & 6-15 IDA 2 + 4 + 6	no	1) <i>Staphylococcus epidermidis</i> septicaemia 2) <i>Aspergillus</i> pneumonia	42	37
4	50/F	AML	1st complete remission	ARA-C 1-4 IDA 1-4	no	no	19	30
5	38/M	AML	newly diagnosed	ETO 1-5 CYT 1 & 1-10 IDA 1 + 3 + 5	no	1) <i>Staphylococcus aureus</i> lymphadenitis 2) <i>Pseudomonas aeruginosa</i> malignant otitis externa	*	36
6	19/F	AML	1st relapse	ARA-C 1-5 & 6-15 IDA 1 + 3 + 5	Prednisone day 7-15	1) <i>Pseudomonas aeruginosa</i> mucositis 2) <i>Staphylococcus epidermidis</i> septicaemia	60	36
7	50/M	AML	newly diagnosed	ETO 1-5 CYT 1 & 1-10 IDA 1 + 3 + 5	no	no	27	30
8	26/M	NHL	1st complete remission	CFM 1 MTX 1 ARA-C 2-7	no	no	19	11
9	46/M	NHL	1st relapse	ARA-C 1-6 MIT 5-7	G-CSF day 8	no	17	10
			2nd complete remission	CFM 1 MTX 1 ARA-C 2-7	G-CSF day 10	<i>Streptococcus oralis</i> septicaemia	14	31
10	27/F	NHL	2nd complete remission	CAR 1 ETO 2-5 ARA-C 2-5 MEL 6	no	no	12	50

continued

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11	26/F	NHL	refractory	CFM ARA-C ASP	1-4 1-4 5-8	Prednisone day -4/-1	<i>Enterococcus</i> urinary tract infection	6	29
			refractory	ARA-C MIT	1-5 4-6	Solumedrol day 1-3	no	10	18
12	29/M	NHL	refractory	CFM DAU VIN MTX	1 + 8 1-3 1 + 8 + 15 + 22 1 + 8 + 15 + 22	Prednisone day 1-22	no	9	25
13	21/F	ALL	newly diagnosed	CFM DAU VIN MTX	1 + 8 1-3 1 + 8 + 15 + 22 5 + 12 + 19 + 26	Prednisone day 1-23	no	*	12
14	27/M	AUL	1st complete remission	IDA CFM TBI	1-2 11-12 7 + 8	no	<i>Streptococcus mitis</i> septicaemia	50	36
15	39/M	RAEB-t	newly diagnosed	IDA ARA-C ETO	1 + 3 + 5 1-10 1-5	no	<i>Clostridium septicum</i> septicaemia	15	47
16	38/M	HCL	1st relapse	LEU	1-7	no	no	†	9
17	22/M	HL	2nd complete remission	CAR ETO ARA-C MEL	1 2-5 2-5 6	no	<i>Staphylococcus epidermidis</i> septicaemia	16	37

*Neutropenia was present at the time of diagnosis.

†PMN not below $0.5 \times 10^9/l$ during the follow-up period.

M, male; F, female; NHL, non-Hodgkin's lymphoma; AML, acute myelogenous leukaemia; ALL, acute lymphocytic leukaemia; HCL, hairy cell leukaemia; AUL, acute undifferentiated leukaemia; RAEB-t, refractory anaemia with excess of blasts in transmission; HL, Hodgkin's lymphoma; CFM, cyclophosphamide; MTX, methotrexate; ARA-C, cytosine arabinoside; MIT, mitoxantron; DAU, daunorubicin; VIN, vincristine; CAR, carmustin; ETO, etoposide; MEL, melfalan; ASP, asparaginase; IDA, idarubicin; LEU, leustatin; CYT, cytarabine; TBI, total body irradiation.

Human cytokine production is usually studied in vitro using peripheral blood mononuclear cells (PBMC). However, not only PBMC are known to be cytokine producing cells, but also neutrophils produce IL-1ra¹¹ and, although to a far less extent than monocytes, IL-1.¹² The latter is also synthesized by many other cell lineages than leukocytes.¹³ Therefore, we investigated the production capacity of IL-1 β , TNF- α and IL-1ra in whole blood in patients with haematological malignancies at various stages of chemotherapy and neutropenia in the absence of infection.

RESULTS

Patient characteristics

The clinical characteristics of the patients are presented in Table 1. A variety of chemotherapy regimens was given for haematological disorders. The median duration of chemotherapy treatment was 7 days (range 4 to 26 days) with a median patient follow-up period of 30 days (range 9 to 50 days) and a median follow-up period after the end of the chemotherapy of 21 days (range 0 to 44 days). The median duration of neutropenia was 15.5 days (range 6 to 60 days). Two patients (patients 5 and 13) were already neutropenic before start of the chemotherapy and the neutrophil count of patient 16 remained above $0.5 \times 10^9/l$ during the follow-up period. Five patients received systemic corticosteroids as part of their chemotherapy regimen and one patient (patient 11) finished corticosteroid treatment just before start of the chemotherapy. Granulocyte colony-stimulating factor (G-CSF) was given in two episodes immediately after the end of the chemotherapy. Patients 10, 14 and 17 received high-dose preparative treatment for subsequent autologous stem cell transplantation. Eight patients developed a microbiologically proven infection during their hospital stay. In six of these patients, the diagnosis of septicaemia was established by positive blood cultures.

Circulating cytokines

Before chemotherapy, circulating IL-1 β concentrations in non-neutropenic patients were higher than in healthy controls ($P < 0.002$). TNF- α and IL-1ra in the circulation did not differ from controls (Table 2).

During and after chemotherapy, no significant changes in the circulating concentrations of cytokines were found for IL-1 β , TNF- α or IL-1ra (data not shown). Neither treatment with corticosteroids or G-CSF nor the presence of infection had an effect on concentrations of circulating cytokines (stepwise regression analysis; data not shown).

TABLE 2. Cytokines before chemotherapy.

	Patients (n = 17)	Controls (n = 20)
Circulating cytokines (pg/ml)		
IL-1 β	74 \pm 33*	44 \pm 10
TNF- α	94 \pm 20	92 \pm 17
IL-1ra	298 \pm 138	237 \pm 92
LPS-stimulated cytokine production ex vivo (pg/ml)		
IL-1 β	10 982 \pm 7374	7635 \pm 3509
TNF- α	5008 \pm 2261	6804 \pm 2249
IL-1ra	8290 \pm 2465†	12 342 \pm 3374

Results are expressed as mean \pm standard deviation.

*Significantly higher in patients than in controls ($P < 0.002$).

†Significantly lower in patients than in controls ($P < 0.001$).

Ex-vivo cytokine production

Before chemotherapy, the non-neutropenic patients showed a significantly lower ex-vivo IL-1ra production compared to healthy controls ($P < 0.001$). No difference was detected for IL-1 β or TNF- α (Table 2). In the two patients who were already neutropenic before chemotherapy, the ex-vivo production of all cytokines tested was extremely low (< 500 pg/ml). In all patients the net ex-vivo production of the three cytokines decreased below 500 pg/ml and successively disappeared completely after chemotherapy was started. Production of cytokines increased again after end of treatment, upon neutrophil recovery (Fig. 1). This pattern was not influenced by administration of G-CSF or corticosteroids, and there was no significant difference between patients with or without infections (stepwise regression analysis; data not shown). Cytokine production decreased below 500 pg/ml after a median of 4.5 days (range 1–12 days) for IL-1 β , 3.5 days (1–11 days) for TNF- α and 10 days (4–15 days) for IL-1ra. For the cytokines studied, a net production of more than 500 pg/ml was regained after a median duration of 15 days after completion of chemotherapy, with a range of 0–24 days for IL-1 β and TNF- α , and 0–21 days for IL-1ra. In only one patient the cytokine production was restored before the whole chemotherapy cycle was finished.

Ex-vivo cytokine production expressed per cell

In order to assess whether the observed reduction of ex-vivo cytokine production is solely due to a decrease of the number of cytokine-producing cells, the net production capacity measured in whole blood was expressed per neutrophil and per monocyte, respectively. To normalize for the wide variation in neutrophil number among our patients, Figures 2 and 3 depict the time course of the net cytokine production capacity, with numbers of PMN normalized by expressing the PMN count in terms of percentage of the initial count before chemotherapy.

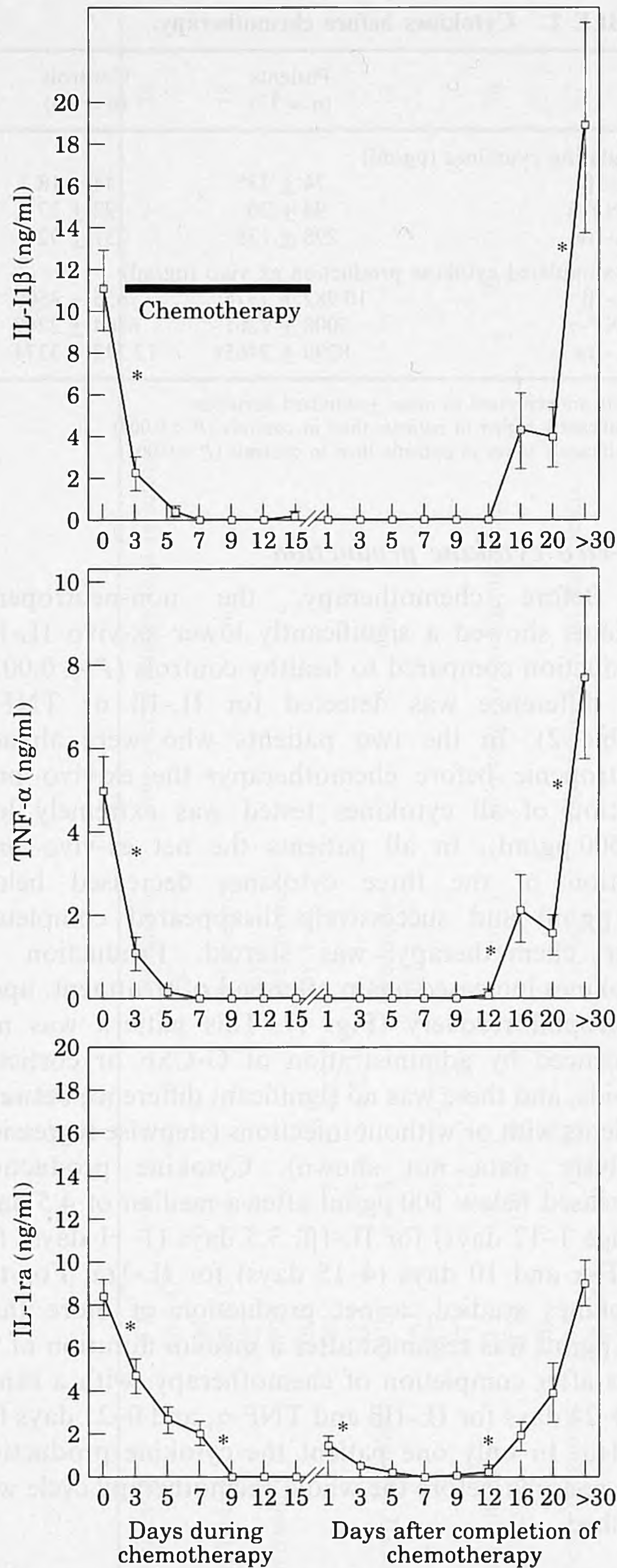


Figure 1. LPS-stimulated net ex-vivo production of IL-1 β , TNF- α and IL-1ra in whole blood before, during and after chemotherapy in 17 patients treated with anticancer chemotherapy (mean \pm SEM).

Significant differences between consecutive time points are indicated (* $P < 0.05$).

Interestingly, the production of all three cytokines measured decreased rapidly before the number of circulating cells began to decrease. Most notably, this decreased production was even observed in those patients who showed an initial increase of the number

of neutrophils after start of chemotherapy. Therefore, the cytokine production as expressed per cell was significantly decreased after the start of chemotherapy, both when expressed per neutrophil (Fig. 2) and per

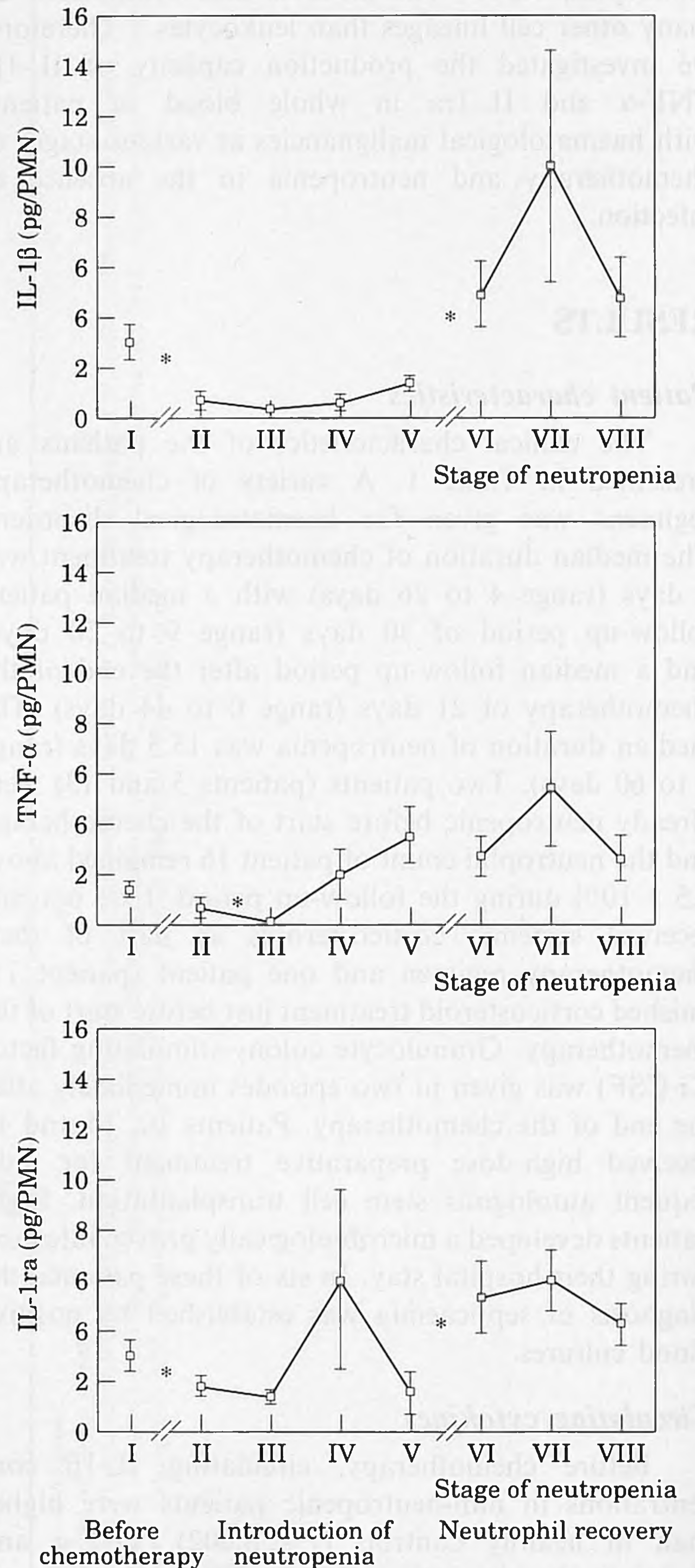


Figure 2. LPS-stimulated net ex-vivo production of IL-1 β , TNF- α and IL-1ra in whole blood expressed per neutrophil during consecutive stages of anticancer chemotherapy treatment in 17 patients (mean \pm SEM).

I, before chemotherapy; II, PMN remaining $\geq 100\%$ of initial value; III, PMN decreasing from 100% to 20% of initial value; IV, PMN 20-2%; V, PMN 1-0%; VI, PMN increasing from 0% to 10% of initial value; VII, PMN 10-50%; VIII, PMN $> 50\%$ of initial value. Significant differences between consecutive time points are indicated (* $P < 0.05$).

monocyte (Fig. 3). In contrast, the production of IL-1ra as expressed per circulating monocyte appeared to increase (Fig. 3), although its total production decreases significantly upon induction of neutropenia (Fig. 1).

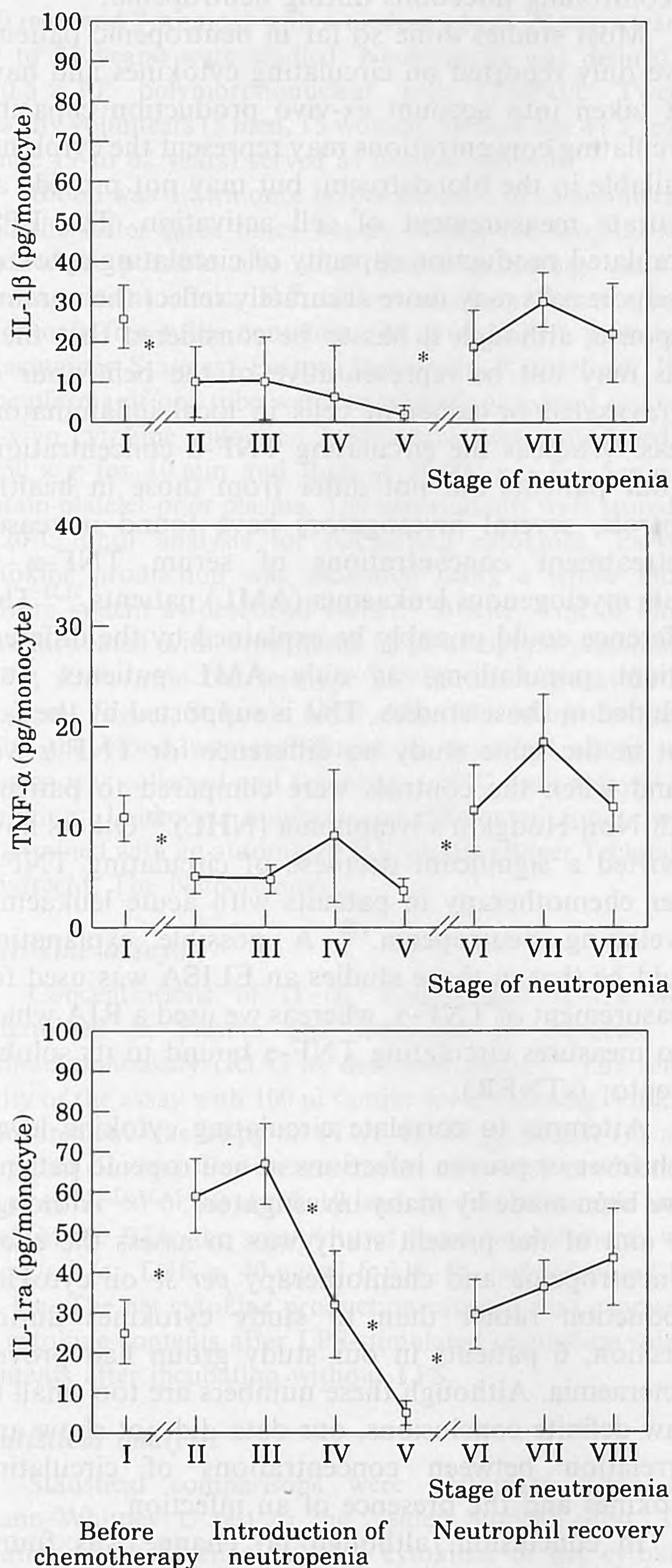


Figure 3. LPS-stimulated net ex-vivo production of IL-1 β , TNF- α and IL-1ra in whole blood expressed per monocyte during consecutive stages of anticancer chemotherapy treatment in 17 patients (mean \pm SEM).

I, before chemotherapy; II, PMN remaining \geq 100% of initial value; III, PMN decreasing from 100% to 20% of initial value; IV, PMN 20–2%; V, PMN 1–0%; VI, PMN increasing from 0% to 10% of initial value; VII, PMN 10–50%; VIII, PMN > 50% of initial value. Significant differences between consecutive time points are indicated (* P < 0.05).

During recovery from neutropenia after completion of the chemotherapy cycle, cytokine production was restored, even before the number of cells began to rise (Fig. 2). When the ex-vivo cytokine production capacity at various PMN counts during induction of neutropenia was compared to the production at corresponding PMN counts during recovery, production of IL-1 β , TNF- α and IL-1ra during the recovery phase was significantly higher than during the induction of neutropenia, even though the total amount of circulating cells was the same in both groups (data not shown).

DISCUSSION

In the present study we investigated cytokine profiles in patients with haematological malignancies before, during and after chemotherapy in a whole blood culture system. Circulating concentrations of IL-1 β were significantly elevated in these patients before treatment, but chemotherapy and induction of neutropenia did not influence IL-1 β , TNF- α or IL-1ra concentrations in plasma. The net production of IL-1 β , TNF- α and IL-1ra in whole blood disappeared rapidly during chemotherapy, already before induction of neutropenia. When expressed per circulating neutrophil or monocyte, the cytokine production capacity decreased immediately after start of the chemotherapy, independently of the number of cells, suggesting a mechanism of downregulation of cytokine production after chemotherapy. After completion of chemotherapy, the net production of the three cytokines increased again before the number of cells did, probably due to an enhanced production per cell.

The downregulation of cytokine production after the start of chemotherapy may be caused by a direct cytotoxic effect of the therapy. The finding that the decrease of cytokine production capacity occurred regardless of the chemotherapy regimen used, suggests that the mechanism for this downregulation is not specific. The fact that this occurred for all three cytokines studied implies a general toxic effect on the cell, interfering with various cellular mechanisms. Ridgway and Borzy¹⁴ have demonstrated that antineoplastic treatment for children with acute lymphocytic leukaemia (ALL) can damage the mechanisms which lead to cytokine production in PBMC. In the present study we have shown that the cytokine production during the recovery of the neutrophils is significantly higher than during the induction of neutropenia, even when the number of circulating PMN is similar in both phases, which supports the hypothesis that a cytotoxic effect plays a role. Alternatively, the rise in cytokine production per cell during the recovery phase may be accounted for by exposure of circulating cells to other

cytokines and growth factors that are produced during this period of increased haematopoietic activity, such as G-CSF.

Our study included two patients with newly diagnosed leukaemia who were already neutropenic before administration of chemotherapy. The ex-vivo production of the three cytokines measured was downregulated in these patients at the time of inclusion. This suggests that other mechanisms than cytotoxic drugs may be responsible for this downregulation as well, which in this case could be the disease or the neutropenia itself. In our study however, there was no difference in cytokine production between non-neutropenic cancer patients and healthy controls, with the exception of a significant lower ex-vivo production of IL-1ra in patients. Although the group of patients studied is relatively small and quite diverse in terms of primary haematological disease, the pattern of reduced ex-vivo cytokine production during induction of neutropenia was found in all patients studied. These observations suggest that the malignant disease itself does not severely affect cytokine production. It cannot be excluded that neutropenia itself, directly or indirectly, may contribute to reduced cytokine production. Therefore, it is obvious that further studies need to be carried out in patients who are neutropenic due to other causes than chemotherapy.

To our knowledge, this study is the first to investigate cytokine production during granulocytopenia using a whole blood culture system. Whole blood can be used as a reliable alternative to PBMC to measure cytokine production upon stimulation with LPS, providing a simple method that requires minimal laboratory facilities and which maintains a physiological environment.^{15,16} PBMC from children treated with continuous maintenance therapy for ALL have a decreased ability to produce IL-1 and GM-CSF,¹⁴ which is consistent with our observations in whole blood. Takamatsu *et al.* showed that a significant increase of IL-1 β , IL-6 and G-CSF production by monocytes occurs in adults with various haematological malignancies during haemopoietic recovery after chemotherapy treatment, when compared with normal healthy subjects.¹⁷

It may be questioned whether the observed down-regulation of cytokine production during chemotherapy-induced neutropenia is beneficial. It could be hypothesized that due to an extremely low production of pro-inflammatory cytokines, these patients are less prone to develop severe sepsis syndrome. However, IL-1ra, which is thought to protect against lethal sepsis, has also been downregulated. If chemotherapy directly affects the ability to produce TNF- α and IL-1 then this could, at least partly, explain the bone marrow toxicity and the greater risk of infections in these patients. Indeed, IL-1 has been

shown to accelerate bone marrow recovery after myelosuppression¹⁸ and TNF- α has protective effects on haematopoiesis in animals given cycle-specific chemotherapy.¹⁹ TNF- α also possesses anti-viral activity²⁰ and enhances neutrophil function²¹ and so may be beneficial in controlling infections during neutropenia.

Most studies done so far in neutropenic patients have only reported on circulating cytokines and have not taken into account ex-vivo production capacity. Circulating concentrations may represent the cytokines available in the bloodstream, but may not provide an accurate measurement of cell activation. The LPS-stimulated production capacity of circulating cytokine producer cells may more accurately reflect the immune response, although it has to be considered that these cells may not be representative of the behaviour of extravasated or adherent cells in local inflammatory areas. Whereas the circulating TNF- α concentrations in our patients did not differ from those in healthy controls, several investigators have found increased pretreatment concentrations of serum TNF- α in acute myelogenous leukaemia (AML) patients.^{22,23} This difference could possibly be explained by the different patient populations, as only AML patients were included in those studies. This is supported by the fact that in the same study no difference for TNF- α was found when the controls were compared to patients with Non-Hodgkin's lymphoma (NHL).²² Others have reported a significant decrease of circulating TNF- α after chemotherapy in patients with acute leukaemia developing neutropenia.^{8,24} A possible explanation could be that in those studies an ELISA was used for measurement of TNF- α , whereas we used a RIA which also measures circulating TNF- α bound to its soluble receptor (sTNFR).

Attempts to correlate circulating cytokine levels with fever or proven infections in neutropenic patients have been made by many investigators.^{7,8,25-27} Although the aim of the present study was to assess the effect of neutropenia and chemotherapy *per se* on cytokine production rather than to study cytokines during infection, 6 patients in our study group had proven bacteraemia. Although these numbers are too small to draw definite conclusions, our data did not show any correlation between concentrations of circulating cytokines and the presence of an infection.

In conclusion, although no change was found in circulating cytokine concentrations, ex-vivo production of IL-1 β , TNF- α and IL-1ra in whole blood disappeared rapidly after start of chemotherapy. This effect is not only due to the decreased number of producing cells, but is also a result of a decreased production per cell. This suggests a mechanism of downregulation of cytokine production during chemotherapy-induced neutropenia, most likely due to a cytotoxic effect of chemotherapy.

MATERIALS AND METHODS

Study protocol

Consecutive inpatients with leukaemia or malignant lymphoma who were eligible for chemotherapy were enrolled. Over a 4-month period 19 neutropenic episodes in 17 patients (10 men and 7 women) with a median age of 29 years (range 19 to 71 years) were studied. Neutropenia was defined as $<0.5 \times 10^9$ polymorphonuclear cells (PMN)/l. Twenty healthy volunteers (5 men, 15 women; median age 44.5 years; range 19 to 62 years) served as normal controls.

Blood was drawn once before the start of chemotherapy and thereafter three times weekly during the hospital stay and when possible also after discharge during visits at the outpatient clinic. For collection of cytokines special endotoxin-free tubes containing 48 μ l of EDTA were used (Vacutainer Systems; Becton Dickinson, Rutherford, NJ). The plasma of one tube was immediately processed to avoid ex-vivo cytokine induction.²⁸ This tube was centrifuged at $2250 \times g$ for 10 min and then at $20\,000 \times g$ for 5 min to obtain platelet-poor plasma. The supernatants were stored at -20°C until analysis for circulating cytokines. Ex-vivo cytokine production was measured using a whole blood culture system as described earlier.²⁹ Briefly, 4 ml of blood was incubated with or without 50 μ l of lipopolysaccharide (LPS; *Escherichia coli* serotype 055:B5, final concentration 10 $\mu\text{g}/\text{ml}$; Sigma, St Louis, MO). After 24 h of incubation at 37°C the blood was centrifuged as described above and plasma was collected and stored at -20°C until assayed for cytokines. Leukocyte numbers and differential counts were determined with an automated H3 counter (Bayer Technicon Mijdrecht, The Netherlands).

Cytokine assays

Concentrations of IL-1 β , TNF- α and IL-1ra were determined in plasma and measured in duplicate by radioimmunoassay (RIA) as described earlier.³⁰ The sensitivity of the assay with 100 μ l sample for circulating cytokine measurement was 20 pg/ml (TNF- α), 40 pg/ml (IL-1 β) and 60 pg/ml (IL-1ra). For measurements of ex-vivo production, 25 μ l (TNF- α) or 10 μ l (IL-1 β and IL-1ra) of sample were used in the RIA; the sensitivity of these measurements was 80 pg/ml for TNF- α , 40 pg/ml for IL-1 β and 600 pg/ml for IL-1ra. The net cytokine production capacity was expressed as cytokine contents after LPS-stimulated incubation minus contents after incubation without LPS.

Statistical analysis

Statistical comparisons were performed using the Mann-Whitney U test or the 2-sided Student *t*-test. The relationship between circulating cytokines or net cytokine production and number of neutrophils, presence of infection and medication was assessed by stepwise regression analysis. Statistical significance was defined as $P < 0.05$.

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