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Decrease of Natural Killer Cell Activity and Monokine Production in Peripheral Blood of Patients Treated With Recombinant Tumor Necrosis Factor

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Tumor necrosis factor (TNF), a protein predominantly produced by activated macrophages/monocytes, is presently available in recombinant, purified form for clinical trials. Intensive studies in many laboratories have shown that besides the tumorcytotoxic effects, TNF acts on a large array of different cells and has potent immunomodulatory activities. In a clinical phase I study, some immunologic functional parameters of blood cells from patients who received 24-hour infusions of recombinant human TNF (rhTNF) were analyzed. Natural killer (NK) cell activity, TNF production, interleukin-1 (IL-1) production and mitogen-induced proliferation were measured either in whole blood samples or in cultures of peripheral mononuclear leukocytes of the patients directly before and after rhTNF infusion. NK cell activity, TNF and IL-1 production capacity and proliferative responses to concanavalin A (Con A) were significantly reduced after rhTNF application. We conclude from these observations that rhTNF in vivo acts directly or indirectly on NK cells and monocytes by either inactivating their functional capacity or by absorbing the relevant cells to the endothelial cell layer, thus removing them from circulation.

TUMOR NECROSIS FACTOR (TNF) was first described by Carswell et al[1] as the active mediator responsible for the endotoxin-induced hemorrhagic necrosis of certain mouse tumors. Studies with purified native TNF as well as with recombinant human-derived and mouse-derived TNF have confirmed the tumor necrotic activity in vivo[2] as well as tumor cytotoxic activity of the molecule in vitro.[2] However, TNF not only mediated the beneficial effects of endotoxin, but also the harmful effects of endotoxic shock, such as fever and hypotension.[3-5] In addition, a plethora of immunomodulatory and cell activating functions of TNF have been described, especially on macrophages/monocytes,[6-9] polymorphonuclear leukocytes,[10-12] and endothelial and epithelial cells and fibroblasts.[13-15]

Therefore, monitoring some immunologic functions of peripheral mononuclear leukocytes (PMNLs) of patients who had received a continuous intravenous (IV) infusion of recombinant human TNF (rhTNF) for 24 hours once in a clinical phase I study appeared interesting. Peripheral blood was drawn before infusion and immediately after completion. A series of immunologic tests were performed with the PMNLs obtained. The ability of PMNLs to exhibit natural killer (NK) cell activity, TNF production, interleukin-1 (IL-1) production, and proliferative responses on mitogenic stimulation was determined, and the values were compared to those obtained after TNF application. Considering the activating effects of TNF on most immunologically important cell types and functions under in vitro conditions, it was surprising that a significant reduction occurred in NK activity, the TNF and IL-1 production capacity, and the concanavalin A (Con-A)-induced proliferation of the PMNLs immediately after infusion of TNF.

MATERIALS AND METHODS

Patients. The patients in this study were treated with recombinant human TNF (rhTNF) according to a protocol for phase I clinical trial. Patients were eligible for the study if they had progressive neoplastic diseases refractory to standard chemotherapy regimens and no alternative treatment was available. They had to have a performance status of 2, normal renal and hepatic functions, and no signs of active infections. No cytostatic or immunosuppressive drugs were given for at least 6 weeks before TNF treatment.

Eligible patients were assigned at random to two regimen arms: In Arm A, patients received a continuous IV infusion of rhTNF for 24 hours once a week (Mondays) for 8 weeks; in arm B, patients received the same dosage of rhTNF in a 24-hour infusion twice a week (Mondays and Thursdays) for 8 weeks. The initial dose for each patient was 0.04 mg/m^2/24 h, and subsequent doses were escalated each week according to a Fibonacci scale until maximum therapeutic dose (MTD) for the patient was reached. In this study, all patients were investigated immediately before and after the first administration of rhTNF (0.04 mg/m^2 for 24 hours continuous infusion) with the exception of one patient, who was studied as he received 0.08 mg/m^2 of rhTNF.

The protocol of the clinical trial was approved by the Ethics Commission of the Medical Faculty of the University of Heidelberg, FRG. Informed consent was obtained from each patient before accrual into the clinical trial and the laboratory studies. Altogether, 12 patients were studied for NK activity and monokine production: eight patients with refractory colorectal carcinoma and four with renal cell carcinoma. Their median age was 57 years (range 38 to 64 years). Ten patients were men and two were women.

Reagents. rhTNF was supplied by Knoll/BASF AG, Ludwigshafen, FRG. The specific activity of the material was 9×10^7 U/mg protein as measured in the biologic tumor cell (L929) cytotoxicity assay in the presence of actinomycin D.[16] The pyrogen content was <1.3 ng/mg protein. Recombinant human IL-1α was provided by J.J. Farrar, Hoffmann La Roche, Nutley, NJ. The specific activity was 1.7×10^7 U/mg protein based on half-maximal stimulation in the costimulator assay.[17]

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Preparation of PMNLs. Human PMNLs were prepared from heparinized blood samples by Ficoll-Paque (Pharmacia, Freiburg, FRG) density-gradient centrifugation.[18]

Determination of NK cell activity. Target cells (K562 tumor cells) were incubated for 90 minutes with 10 μL ^{51}Cr [sodium chromate, specific activity 1.85 GBq/mg (18.5 MBq/mL), Behring AG, Marburg, FRG] in 1 mL RPMI 1640 (GIBCO, Karlsruhe, FRG) at 37°C. Labeled and washed cells were adjusted to 10^5 cells/mL. Whole blood was tested at three different dilutions (1:4, 1:8, 1:16) as previously described.[19] PMNLs were tested at three different concentrations (1×10^6, 5×10^5, 2.5×10^5 cells/mL). One hundred microliters diluted blood or 100 μL PMNL suspension were mixed with 100 μL target cell preparation in round-bottom microtiter plates (Nunc, Wiesbaden, FRG) and incubated for four hours at 37°C. For the maximal release values, the target cells were lysed by treatment with Triton X-100 (Sigma, Deisenhofen, FRG); for the spontaneous release, labeled targets were incubated with medium alone. Radioactivity released into the supernatant was measured and the percentage of specific lysis (% specific lysis) was calculated according to the formula: % specific lysis = 100 × (test release − low control release/high control release − low control release).

Determination of Leu11-positive cells. Human PMNLs (1×10^6) were washed in Hank's balanced salt solution (HBSS) and supplemented with 0.1% sodium azide and 1% bovine serum albumin (BSA). 10 μL of fluorescine conjugated (FITC) anti-Leulla (Becton Dickinson, Heidelberg, FRG) was added to the cells resuspended in 50 μL HBSS. The cell suspension was incubated for 30 minutes on ice. The cells were washed three times with HBSS and fixed with 1% paraformaldehyde before the cells were analyzed with FACS at 488 nm gated on lymphocytes.

Determination of TNF and IL-1 production capacity. PMNLs (2×10^6/mL) were cultured in RPMI 1640 (GIBCO), with 10% heat-inactivated fetal calf serum (FCS) (GIBCO) for 20 hours either with or without 10 μg/mL *Staphylococcus aureus* (Pansorbin, Calbiochem, Frankfurt, FRG). Cell-free supernatants were harvested and stored at −20°C until they were tested for TNF and IL-1 activity.

TNF activity was determined by an enzyme-linked immunospecific assay (ELISA). Plates (96-well flat-bottom, Titertek Immuno Assay-Plate, Flow Laboratories, Meckenheim, FRG) were coated with affinity-purified (Protein A-Diasorb, Diagen, Düsseldorf, FRG), polyclonal rabbit anti-rhTNF antibodies 5 μg/mL in $NaHCO_3$ buffer (0.05 mol/L, pH 9.0) for 16 hours at 4°C. Serial dilutions of the test samples in phosphate buffer (0.1 mol/L, pH 7.5, 2% EDTA, 1% BSA) were applied to the plates for two hours at room temperature after being blocked with 1% BSA in phosphate-buffered saline (PBS) for two hours at room temperature. Plates were washed with PBS containing 0.05% Tween and Biotin (Sigma, Deisenhofen, FRG)-conjugated affinity-purified (Protein A-Diasorb) polyspecific rabbit anti-rhTNF antibodies, 2.7 μg/mL was added to the wells for 1.5 hours at room temperature. After extensive washing with PBS containing 0.05% Tween Streptavidin-peroxidase complex (BRL, Karlsruhe, FRG), a dilution of 1:2,000 was applied for 30 minutes at room temperature. The plates were washed again with PBS containing Tween (0.05%) and the substrate solution [3,3', 5,5' tetramethylbenzidine, Miles Scientific, München, FRG, 10 mg in 100 mL sodium acetate-citric acid buffer (0.1 mol/L, pH 4.9) and 14.7 μL 30% H_2O_2] was added to the complex. The reaction was stopped with 2mol/L H_2SO_4, and absorption was measured at 450 nm. The absorption curves obtained with the test samples were compared with a standard curve obtained with rhTNF and the TNF content expressed as nanograms per milliliter.

IL-1 activity was determined by the conventional costimulator assay.[17] In brief, single-cell suspensions of C3H/HeJ thymocytes (5×10^5/culture) were cultured in the presence of phytohemagglutinin (PHA-M, 50 μg/mL, Sigma) and serial dilutions of the test samples for 3 days. The cultures were pulsed with 1 μCi tritiated thymidine [(6-^{3}H)thymidine, specific activity 50 Ci/mmol (185 GBq/mmol), Amersham Buchler, Braunschweig, FRG] for six hours, and incorporated counts were determined. The curves obtained with the samples were compared with a standard curve obtained with rhIL-1 and the IL-1 content expressed as IL-1 units.

Lymphoproliferation assay. Lymphoproliferation was determined in a whole blood assay as previously described.[20] Whole blood was diluted with RPMI 1640 (GIBCO) at a ratio of 1 to 10. Aliquots (0.2 mL) were cultured without or with mitogen (phytohemagglutinin PHA 6.25 μg/mL or Con A 6.25 μg/mL both from Sigma) for 6 days at 37°C. The cultures were pulsed with tritiated thymidine ([6-^{3}H] thymidine, specific activity 25 Ci/mmol [925 GBq/mmol] Amersham Buchler, Braunschweig, FRG) for four hours and incorporated counts were determined.

Because blood cells of individual donors differ considerably in the absolute values of NK activity as well as in cytokine production, regardless of whether they are obtained from normal controls or patients, mean values of pretreatment *v* postreatment values could not be compared. Therefore, statistical analyses of the data pairs from individual patients obtained before and after TNF treatment were performed using the Wilcoxon signed-rank test. *P* values were calculated for assessment of significance.

RESULTS

Determination of NK cell activity. NK cell activity was determined by a whole blood technique in samples from cancer patients before and immediately after 24-hour infusion of rhTNF. A decrease in NK cell activity was observed in most patients after treatment. This was confirmed in a second series of experiments in which PMNLs were separated from peripheral blood and tested in the NK cell assay (Fig 1). Again, in this experiment using PMNLs, the NK cell activity was significantly decreased after rhTNF treatment of the patients.

Determination of Leu11-positive cells. To determine whether the percentage of cells bearing the surface marker

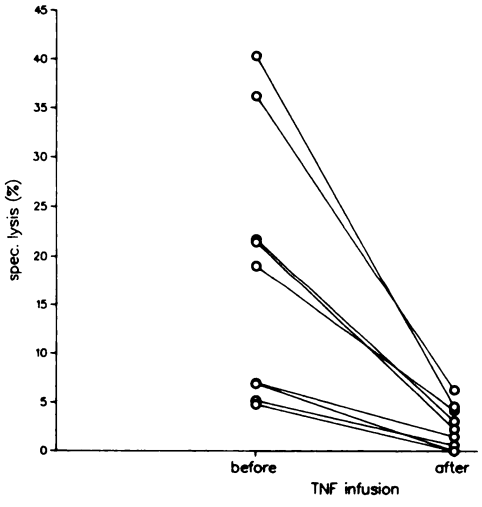

Fig 1. NK cell activity in PMNL preparations obtained from nine patients before and after rhTNF treatment. Reduction in NK cell activity after treatment was highly significant (*P* = .005).

CD16 (Leu11$^+$) that is preferentially responsible for NK activity[21] was changed after treatment, the PMNLs of five patients were stained with FITC-labeled antibodies to Leu-11a, and positive cells were counted (Table 1). rhTFN treatment resulted in a marked decrease in the percentage of CD16-positive cells as compared with the respective pretreatment values. Absolute numbers of CD16-positive cells were also decreased posttreatment (data not shown).

Determination of TNF production. The capacity to produce monokines was used as a measure of monocyte functions in PMNL preparations from cancer patients (Fig 2). Low spontaneous TNF production occurred only in three of nine PMNL preparations, but all nine cultures were inducible for TNF production with *Staph aureus*. TNF production of PMNLs from patients who had received rhTNF infusion was significantly reduced in the three cases with low spontaneous release, as well as in all stimulated cultures. The values obtained with the ELISA test were confirmed by a conventional biologic TNF assay [cytotoxicity on L929 fibrosarcoma cells in the presence of actinomycin D[16] (data not shown)]. The existence of inhibitory substances in the posttreatment samples was excluded by experiments in which these samples were added to defined concentrations of TNF (data not shown).

Determination of IL-1 production. The pattern of IL-1 activity followed closely the TNF activity when the same PMNL supernatants were tested in the IL-1 costimulator assay (Fig 3). Before rhTNF treatment, low spontaneous IL-1 activity was measured in the supernatants of almost all unstimulated cell cultures. High IL-1 titers were detected, however, in all nine cultures when stimulated with *Staph aureus*. Again after treatment with rhTNF, PMNLs significantly lost their capacity to produce IL-1. This phenomenon was even more pronounced in supernatants from *Staph aureus*-stimulated PMNL cultures of the same patients.

Determination of lymphoproliferative responses. PHA-stimulated and Con A-stimulated lymphoproliferation was significantly reduced after rhTNF treatment when compared with the values of PMNLs from the same patients before rhTNF infusion (Table 2). The proliferation stimulated by Con A was more affected by TNF treatment than that stimulated by PHA. The more pronounced reduction in lymphoproliferation after TNF treatment in Con A-stimulated (93%) *v* PHA-stimulated cultures (57%) was significant.

Table 1. Percentage of Leu11-Positive Cells in PMNLs of Patients Before and After rhTNF Treatment

Patient	rhTNF Treatment	
	Before	After
	(% Leu11$^+$ Cells)	
1	8.27	0.33
2	15.67	2.67
3	13.62	1.02
4	19.32	3.19
5	10.12	1.12

The percentages of Leu11$^+$ cells in the PMNL preparations obtained from patients before and after rhTNF treatment were determined by direct immunofluorescence using FITC-labeled Leu11a antibodies.

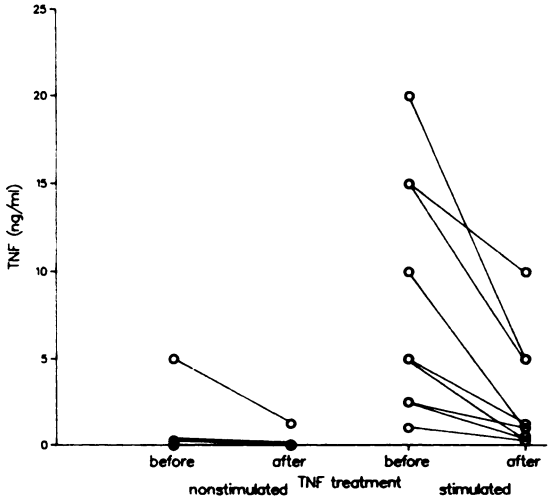

Fig 2. TNF production of PMNL preparations obtained from nine patients before and after rhTNF treatment. Reduction of spontaneous TNF production after treatment was statistically significant (P = .054). For the stimulated cultures, reduction was highly significant (P = .004).

Differential counts of blood cells from the patients did not differ significantly from normal controls. After TNF treatment, some patients had a mild leukopenia (data not shown).

DISCUSSION

NK activity and monokine production capacity were markedly impaired in the PMNLs from individual patients who had received rhTNF infusion as compared with the same patients' pretreatment patients' values. This finding was surprising in light of recent reports that TNF enhanced

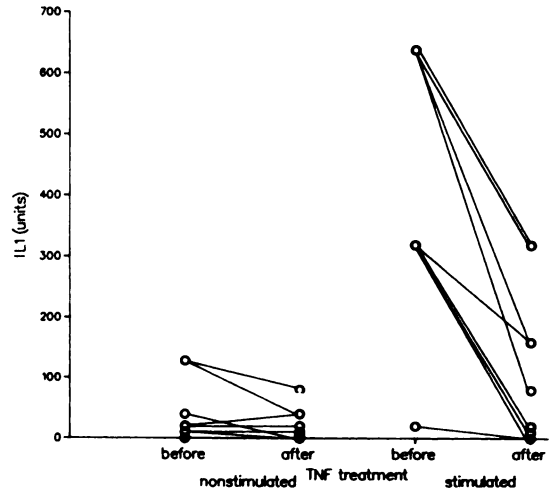

Fig 3. IL-1 production of PMNL preparation as described in legend to Fig 2. Reduction of spontaneous IL-1 production after treatment was statistically significant (P = .046). For the stimulated cultures, reduction was highly significant (P = .004).

Table 2. Lymphoproliferative Responses of Blood Cells From Patients Before and After rhTNF Treatment

	Stimulation*					
			PHA		ConA	
			Before	After		
Patient	Before	After	(cpm × 10^{-3})		Before	After
1	7.5	0.5	4.9	1.8	21.2	6.8
2	0.5	0.8	144.3	49.6	116.0	1.5
3	0.9	1.1	15.4	12.0	6.2	2.1
4	2.0	2.1	41.7	20.6	56.9	2.1
5	1.4	0.4	47.9	3.1	42.2	0.2
6	1.5	0.6	10.5	58.2	46.0	0.4
7	1.8	0.6	46.2	18.2	17.5	0.7

*Whole blood samples obtained from patients before and after rhTNF treatment were cultured either without stimulus or with PHA or ConA. ^{3}H-TdR incorporation was determined as a measure for lymphoproliferation. Reduction of lymphoproliferation after treatment was statistically significant (for the unstimulated cultures $P = .088$ and for PHA-stimulated and ConA-stimulated cultures $P = .009$).

NK activity,[22] and activated monocytes in vitro.[6-9] Our own in vitro data showed that even enhanced TNF production could be expected,[8] and our unpublished in vitro results with human monocytes. However, both NK activity and monokine production were significantly reduced after in vivo rhTNF application. On the assumption that CD16 on large granular lymphocytes (LGLs) represents a specific marker for NK cells,[21] the disappearance of Leu11 positive cells might explain the loss of NK activity on a cellular level. Functional inactivation would be one of the possible mechanisms. However, how rhTNF treatment caused the disappearance of these cells from the circulation is still open to speculation. Reduction of NK activity can also be partly associated with the reduction of TNF production capacity after rhTNF treatment. Recent reports describe partial inhibition of NK activity by antibodies to TNF, implying that part of the NK activity could indeed be caused by TNF.[23,24] However, TNF has never been implicated in the NK-mediated cytotoxicity measured on K562 target cells.

Monocytes and macrophages are the classical producers for TNF and IL-1. However other cell types, like LGLs can also produce these mediators.[24,25] Thus, the disappearance of the CD16-positive cells could partly explain the drop in monokine production as well. Furthermore, it is possible that in addition to the reduced number of CD16 positive cells, monocytes may also have been depleted from the circulation, even though no significant leukopenia or monocytopenia was detectable in routinely performed blood counts. This hypothesis is supported by in vivo experiments in a mouse model in which drastic monocytopenia was found after TNF application.[26] TNF has been described to reorganize human vascular endothelial cell monolayers and to increase adherence of endothelial cells for leukocytes.[27] This could be the molecular mechanism for the drastic reduction of these cells in circulating blood. Another explanation may be a direct toxic effect of rhTNF for the cells responsible for NK and TNF activity. To our knowledge, however, no indication for such toxic action of TNF for the cells relevant for NK activity or monokine production has been described in the literature.

The findings that preferentially Con A-induced and, to a much less extent PHA-induced, lymphoproliferation were reduced after rhTNF treatment also indicated that T cells are not directly affected, but rather that monocyte functions appear to be impaired by the rhTNF treatment. This conclusion is based on the established finding that PHA-induced lymphocyte activation is much less dependent on monocytes than is Con A-induced proliferation.

More detailed studies are in progress concerning the fate of LGLs and monocytes and their functional status in rhTNF-treated patients. In addition, studies on patients who have received several infusions of rhTNF during their treatment will be performed to assemble a comprehensive picture of the effects of rhTNF application during therapy. The decrease in immune function we described was an early transient effect, since the same parameters in six patients were back to pretreatment levels when tested again before the next cycle of TNF application. Thus, the period of depression in function of NK cells and monocytes appears to be a transient phenomenon followed by complete recovery. These findings might provide insight into the complex mechanism of in vivo action of rhTNF. The determination of the clinical implications of the described findings must await completion of the ongoing trials and long-term studies.

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