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Original Paper

Phase II Trial of Intravenous Endotoxin in Patients with Colorectal and Non-small Cell Lung Cancer

F. Otto,[1,*] P. Schmid,[1] A. Mackensen,[1] U. Wehr,[1] A. Seiz,[1] M. Braun,[1] C. Galanos,[2] R. Mertelsmann[1] and R. Engelhardt[1]

[1]Medizinische Klinik I der Albert-Ludwig-Universität, Hugstetterstr. 55; and [2]Max Planck Institut für Immunbiologie, D-79106 Freiburg, Germany

We report the immunological and clinical results of a phase II trial with intravenously administered highly purified endotoxin (*Salmonella abortus equi*) in patients with advanced cancer. 15 patients with non-small cell lung cancer and 27 with colorectal cancer were entered into the study. 37 evaluable patients received at least four injections of endotoxin (4 ng/kg body weight) and 1600 mg ibuprofen orally in 2-week intervals. Transient renal (WHO grade 0–1) and hepatic (WHO grade 0–4) toxicities occurred in several patients. Constitutional side-effects such as fever, chills and hypotension could not be prevented completely by pretreatment with ibuprofen. 3 patients in the colorectal cancer group demonstrated objective responses (1 complete remission (CR), 2 partial remission (PR)). The complete remission has been maintained for more than 3 years, while the partial remissions were stable for 7 and 8 months, respectively. Only marginal antitumour effects were seen in the lung cancer group. Tolerance of the macrophage system to the stimulatory effect of endotoxin, as measured by human necrosis factor alpha (TNF-α) release into serum, built up after the first administration and remained at a steady-state level after each subsequent injection. In contrast, rising CD4:CD8 ratio and release of tumour necrosis factor beta (TNF-β) indicated the continuing activation of the lymphocyte system by repetitive injections of endotoxin.

Key words: endotoxin phase II, non-small cell lung cancer, colorectal cancer, tolerance, T cell
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INTRODUCTION

ENDOTOXIN (lipopolysaccharide, LPS) is a constituent of the outer membrane of gram-negative bacteria. Since the earliest observations by Coley in 1891, that cancer patients had marked regression of their solid tumours during severe bacterial infections, much has been speculated about the antitumoral effect of bacterial compounds [1–3]. Initially, Coley used repeated inoculations of *Erysipelas* to treat inoperable malignant tumours. To circumvent the life-threatening side-effects of infecting patients with life bacteriae, he later used 'Coley's Mixed Toxins', a crude filtrate of *Erysipelas* and *Serratia marcescens*, to treat his patients. In 1943, Shear and colleagues isolated endotoxin from *Serratia marcescens* culture filtrates as a fraction being able to induce haemorrhagic necrosis in tumours [4]. Their study indicated that endotoxin might be the major compound in 'Coley's Mixed Toxins' to evoke the antitumour effect described by Coley. The substance and its derivatives have been used sporadically in the treatment of cancer patients [5–7] as well as in different animal tumour models [8, 9]. To the best of our knowledge, no phase II trial has been reported to evaluate the antitumoral activity of LPS.

A phase I study conducted earlier by our group defined the maximum tolerated dose (MTD-1) as being 1 ng/kg body weight of *Salmonella abortus equi* LPS [10, 11]. Severe constitutional side-effects, such as fever (WHO grade III), chills and hypotension, were the dose limiting toxicities. Pretreatment with the cycloxygenase inhibitor ibuprofen abrogated these toxicities, allowing a further dose escalation to 5 ng/kg body weight. At dose levels >5 ng/kg body weight, fever (WHO grade III) and hepatic toxicity, as defined by an increase in serum transaminase levels (WHO grade III), were the dose-limiting toxicities (MTD-2).

Correspondence to R. Engelhardt.
*Present address: Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London WC2A 3PX, U.K.
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In order to further define the toxicity of LPS and to evaluate its antitumour activity, we performed a phase II clinical trial involving patients with colorectal (CRC) and non-small cell lung cancer (NSCLC). Since our phase I study had shown that the immunological reactions to endotoxin were not changed by pretreatment with ibuprofen, this medication was incorporated into the study protocol. This pretreatment allowed the use of an LPS dose of 4 ng/kg body weight.

Intravenous application of LPS induces the release of high amounts of endogenous macrophage-derived cytokines, such as TNF-α, interleukin-6 (IL-6), IL-8, macrophage-colony stimulating factor (M-CSF) and granulocyte-colony stimulating factor (G-CSF) and leads to acute reversible changes in peripheral blood cell count [10–12]. Repeated applications of LPS induce a state of hyporesponsiveness to its own action with respect to the induction of cytokine release. This effect is more pronounced at daily or weekly intervals as compared to a 2-week interval [10, 13]. For this reason we chose a 2-week application schedule for this trial.

TNF-α serum levels were determined during every exposure to evaluate the interindividual variability in the mononuclear phagocyte system's response to the LPS stimulus.

While endotoxin is a potent B-cell mitogen *in vitro* as well as in animal models [14, 15], no effect of LPS on T lymphocytes has been demonstrated *in vitro*. In order to define the role of the lymphocyte system in the response to an LPS stimulus *in vivo*, we also determined levels of T cell relevant cytokines and performed flow cytometric analysis of lymphocyte subpopulations.

PATIENTS AND METHODS

Preparation of endotoxin

Endotoxin was prepared as described earlier [10]. The purified endotoxin from *Salmonella abortus equi* was essentially free of protein (<0.08%) and free of nucleic acid [16–18]. It was used as single-dose vials containing a sterile solution of 1 μg/ml LPS in phosphate-buffered saline.

Patient selection

Patients eligible for the study included adults older than 18 years of age with histopathologically confirmed metastatic or locally inoperable colorectal or non-small cell lung cancer. Patients had a performance status ≥60% (Karnofsky scale, [19]) and an estimated life expectancy of >3 months. Other selection requirements were adequate baseline physiological function including adequate haematological status (haemoglobin ≥100 g/l, WBC count ≥4000/mm^3, platelet count ≥100000/mm^3), adequate hepatic (serum bilirubin <2 mg/dl, aspartate aminotransferase <80 U/l), and renal function (creatinine <1.5 mg/dl). Exclusion criteria included concomitant different malignant tumours, seizure disorders, central nervous system metastasis, requirement of anticoagulants, corticosteroids or non-steroidal anti-inflammatory drugs, and cardiac or pulmonary failure.

All previous anticancer therapy had to be discontinued for a minimum of 4 weeks before the patient entered the study. All patients underwent a complete medical history and physical examination. The following diagnostic tests were performed: leucocyte, differential and platelet counts; coagulation profile; biochemical screening profile; C-reactive protein; tumour markers-carcinoembryonic antigen and CA 19-9; electrocardiogram; chest X-ray; abdominal sonography; and, where necessary, special diagnostic studies.

The study was approved by the Institutional Review Board and signed informed consent was obtained from all patients.

Study design

The appropriate amount of LPS (4 ng per kg body weight) was diluted with 0.9% saline to a total of 10 ml immediately before use and administered to patients by biweekly bolus intravenous (i.v.) injection. All patients received two doses of 800 mg each of ibuprofen (Hoechst AG, Frankfurt, Germany) orally, the first given 90 min before and the second at the time of LPS injection. All patients were hospitalised for close observation for at least 6 h following LPS injection. Vital signs were monitored before injection and at hourly intervals for 6 h after injection of endotoxin.

Blood samples for complete blood cell counts and immunological parameters were obtained with an i.v. sampling catheter before LPS injection and at postinjection times of 90, 180 and 360 min. For cytokine assays, blood samples were centrifuged and serum was stored at −70°C until use.

A serum chemistry profile, including renal and liver function tests, electrolyte, triglyceride and cholesterol levels, and coagulation profile were obtained before and 6 h after endotoxin injection.

Tumour size was measured by appropriate radiological examination at the end of 8 weeks. In the event of tumour regression or stable disease, patients continued to receive biweekly treatment until tumour progression was observed. The criteria for responses have been previously described [10]. Toxicities were assessed according to the WHO grading criteria [20].

Cytokine assays

Serum levels of TNF-α, TNF-β, IL-2 and IL-7 were determined using specific enzyme-linked immunosorbent assays (ELISA) (TNF-α, T Cell Sciences, Cambridge, Massachusetts, U.S.A.; TNF-β, IL-2, IL-7, R&D Systems, Minneapolis, Minnesota, U.S.A.) with minimum detection limits of 10 pg/ml for each cytokine.

Fluorescence flow cytometry

Heparinised blood samples were collected from 10 patients (5 CRC, 5 NSCLC) before LPS injection and 4 and 24 h after injection for fluorescence flow cytometry. Peripheral blood mononuclear cells were isolated from whole blood using Ficoll Separating Solution (Seromed, Biochrom KG, Berlin, Germany) and immunofluorescence staining was performed as described earlier [13]. Monoclonal antibodies used for two-colour analysis were IOT3, CD3; IOT4a, CD4; IOB1, CD37; IOT2a, HLA-DR; IOT14, CD25 (Dianova Immunotech GmbH, Hamburg, Germany); Leu4, CD3; Leu11a, CD16; Leu19, CD56; Leu7, CD57 (Becton Dickinson, Heidelberg, Germany).

Flow cytometry analysis was performed using a FACScan (Becton Dickinson) flow-cytometer equipped with an argon-ion laser tuned at 488 nm. Data acquisition was performed with FACScan, Research Software (Lysis II) [23].

RESULTS

Patient population

42 patients were entered into the study, 27 with colorectal and 15 with non-small cell lung cancer. All patients were followed for at least an 8-week study period. 5 patients were withdrawn from the study prior to 8 weeks because of pro-

gressive disease. There were 15 female and 22 male patients with a median age of 61 years, ranging from 34 to 70 years. The patients' characteristics are detailed in Table 1.

Toxicity

The most frequent clinical signs of toxicity were low-grade fever (WHO grade 0–2), chills, myalgia and hypotension. The hypotension was generally of short duration (up to 0.5 h) and did not require specific treatment in any case. The biochemical parameters gave evidence of hepatic and mild renal toxicities. Detailed data on the LPS-induced toxicity observed during this study are given in Table 2.

Tumour response

Of the 14 patients with non-small cell lung cancer, 8 had stable disease during the study period (duration 3–4 months). No tumour regression was observed in this group. Within the colorectal carcinoma patient group, 8 patients had stable disease (duration 4–11 months), 2 patients had a partial response, and 1 patient underwent a complete remission (duration +36 months) (Table 3). This 56-year-old woman had undergone an anterior resection of the rectum for an undifferentiated adenocarcinoma of the rectum 8 months before entry into the study. Four months after surgery, a recurrent, histologically proven local tumour growth was observed during radiochemotherapy. An abdomino-perineal rectal resection was carried out. When the patient was entered into the study, locally recurrent disease, multiple enlarged retroperitoneal lymph nodes and an elevated level of the tumour marker CA 19-9 (initial value, 11930 kU/l) were measurable parameters of the disease. During the first 5 months of endotoxin treatment, the tumour manifestations seen in CT scan underwent a complete remission, while the levels of CA 19-9 decreased stepwise to undetectable values (<2.5 kU/l). The therapy was discontinued after four additional LPS injections. Since that time, the patient has undergone periodical examinations and remains in complete remission for 36+ months to this date. Additional data on this patient's reaction to endotoxin in comparison to the reactions of the other patients are given in Table 4.

Table 1. Patient characteristics

Total no. of patients	42
Total no. of patients evaluable	37
Males	22
Females	15
Median age (years)	61
Range	34–70
Performance status (%) (Karnofsky)	
100	16
90	9
80	10
70	2
Diagnosis	
NSCLC	
Adenocarcinoma	6
Squamous cell carcinoma	3
Large cell anaplastic carcinoma	5
Colon carcinoma	11
Rectum carcinoma	12
Previous therapy	
None	7
Surgery	27
Chemotherapy	9
Radiotherapy	10

Table 2. Toxicity of LPS

	Number of LPS treatments*			
	1	2	3	4
Fever†				
WHO grade 0	32	32	30	31
WHO grade 1	5	5	7	5
WHO grade 2				1
Chills	3	3	2	3
Fatigue	1	3	4	3
Headache	1		3	1
Nausea	2		1	1
Myalgia	6	4	5	3
Hypotension‡	8	5	10	8
Hypertension		1		1
Dyspnoea			1	
Hepatic toxicity§				
WHO grade 0	15	27	26	28
WHO grade I	12	5	7	8
WHO grade II	6	5	4	1
WHO grade III	4			
Renal toxicity‖				
WHO grade 0	34	35	34	36
WHO grade I	3	2	3	1

* Results shown are number of patients. †WHO grade 0, none; WHO grade 1, <38°C; WHO grade 2, 38–40°C. ‡Hypotension: systolic blood pressure <100 mmHg. §AST/ALT: WHO grade 0, 0–1.25 × N; WHO grade I, 1.26–2.5 × N; WHO grade II, 2.6–5.0 × N; WHO grade III, 5.1–10 × N. ‖Creatinine: WHO grade 0, 0–1.25 × N; WHO grade 1, 1.26–2.5 × N.

Haematological changes

Endotoxin injection led to an acute reduction in the WBC count at 1.5 h with a subsequent rebound leucocytosis (Figure 1a). The rebound could be attributed to the neutrophil fraction, with numbers doubled 6 h after LPS administration compared to pre-injection levels (Figure 1b). The decrease in numbers of circulating lymphocytes exhibited different kinetics with a plateau after 6 h (Figure 1c). As described earlier, the acute decrease in circulating cell numbers was most pronounced in the monocyte fraction (Figure 1d) [10, 11]. Every injection induced monocytopenia, neutropenia and lymphopenia of comparable magnitude.

Serum levels of cytokines

No TNF-α was detectable in any of the patients' sera before LPS injection. The level of this cytokine increased to reach its peak at 90 min. Levels at this time point ranged between 380 and 17000 pg/ml. Median values of all patients during the 6 hours after LPS injection for the first four therapies are shown in Figure 2a. TNF-β (Figure 2b) exhibited a different time course with peak levels occurring at 3 h after injection. The maximum serum levels were two orders of magnitude lower than those of TNF-α. Values returned to non-detectable levels after 6 h in most of the patients. No circulating IL-2 or IL-7 could be detected at any time point.

Flow cytometry

The most prominent change in lymphocyte subpopulations was an increase in the CD4:CD8 T cell ratio in each patient

*Table 3. Tumour response**

	NSCLC		CRC	
	n	Duration (months)	*n*	Duration (months)
CR	0	—	1	+36
PR	0	—	2	7, 8
NC	8	3 (m4), 4(*n*=4)	8	4 (*n* =4), 5,7,9,11
PD	6		12	
Total	14		23	

*Assessment at the end of protocol (3 months) or as indicated. NSCLC, non-small cell lung cancer; CRC, colorectal cancer.

*Table 4. LPS-induced reactions of the CR patient as compared to the reactions of the other patients**

	CR patient	Other patients
Increase in body temperature [K]†	0.95 (0.2–1.7)	0.5 (0.1–1.5)
Decrease in diastolic blood pressure RR [mmHg]	11 (5–20)	25 (5–50)
Increase in heart rate [min^{-1}]	27 (20–36)	21 (−6–56)
WBC [mm^{-3}]‡	2700 (0.3–100)	2700 (−1300–10400)
Neutrophils [mm^{-3}]‡	2100 (900–2600)	1000 (−1600–7900)
Lymphocytes [mm^{-3}]‡	500 (300–1000)	900 (−200–3200)
Monocytes [mm^{-3}]‡	250 (200–300)	450 (100–1200)
TNF-α (pg/ml)§		
First LPS application	11200	6300 (380–1700)
Second LPS application	600	1700 (100–8000)
Third LPS application	2800	1900 (130–10000)
Fourth LPS application	1200	2100 (300–1000)

*Ranges are given in parentheses. †Changes in physiological parameters between pre-adminstration value and highest or lowest value reached, respectively. ‡Maximal decrease in cell number during the observation period. §TNF-α serum concentrations at 90 min post LPS administration.

at 6 and 24 h after every LPS administration (Table 5). Moreover, there was a trend towards an increase of CD37+ B lymphocytes. Changes in the percentage of natural killer cells (CD3−/CD16+, CD3−/CD56+, CD3−/CD57+) during the course were not significant.

DISCUSSION

This phase II trial was performed to further evaluate the toxicity and define the antitumoral efficacy of intravenously administered endotoxin to cancer patients. Furthermore, LPS-induced immunological changes were investigated, focusing on the lymphocyte system.

Repeated injections of endotoxin were well tolerated by most of our patients. As expected from earlier observations [10, 11], mild adverse events such as mild fever, chills, myalgia and fatigue, were the most pronounced constitutive side-effects. Slight disturbances in blood pressure regulation were observed resulting in either hyper- or hypotension, but no pressor agents had to be administered. No signs of allergic reactions were seen, maybe due to the purity of this LPS preparation [17]. Low grade renal toxicity was observed in only a few patients, whereas transient hepatic toxicity as measured by increased serum transaminase levels was a common event.

Since LPS administration induced very high serum levels of TNF-α in most patients, an overlap in toxicities of both substances could be assumed. Indeed, hepatotoxicity and hypotension were the dose-limiting toxicities in a clinical trial investigating intravenously administered TNF-α [21]. Thus, the increase in transaminase levels in our study might be mediated by TNF-α. However, elevations in transaminase levels and TNF-α peak levels did not correlate significantly in our patients. Moreover, the dynamics of hepatic toxicity and TNF-α peak levels during the study period were different. As discussed later, the first LPS administration generally induced a much higher TNF-α release than one of the following applications. The second and all further therapies provoked nearly constant TNF-α peak levels. In contrast, hepatic toxicity was less pronounced, the more applications had preceded a certain administration. These data suggest the existence of a TNF-α independent mechanism of LPS-induced hepatotoxicity.

Endotoxin administration lead to an acute decrease in the number of circulating white blood cells, most likely due to adhesion of leucocytes to endothelial cells. This leucocyte trapping in the vasculature of different organs might decrease the blood flow in liver, kidneys and lung and thereby account for hepatic and renal toxicities as well as for dyspnoea. However, a significant correlation between the decrease in peripheral leucocyte numbers and extent of the above toxicities could not be established.

Antitumoral activity was only marginal in patients with non-small cell lung cancer. In the colorectal cancer group, a number of patients had no progress in their tumour manifestations for up to 11 months and 3 patients experienced remissions (1 complete and two partial remissions) of their

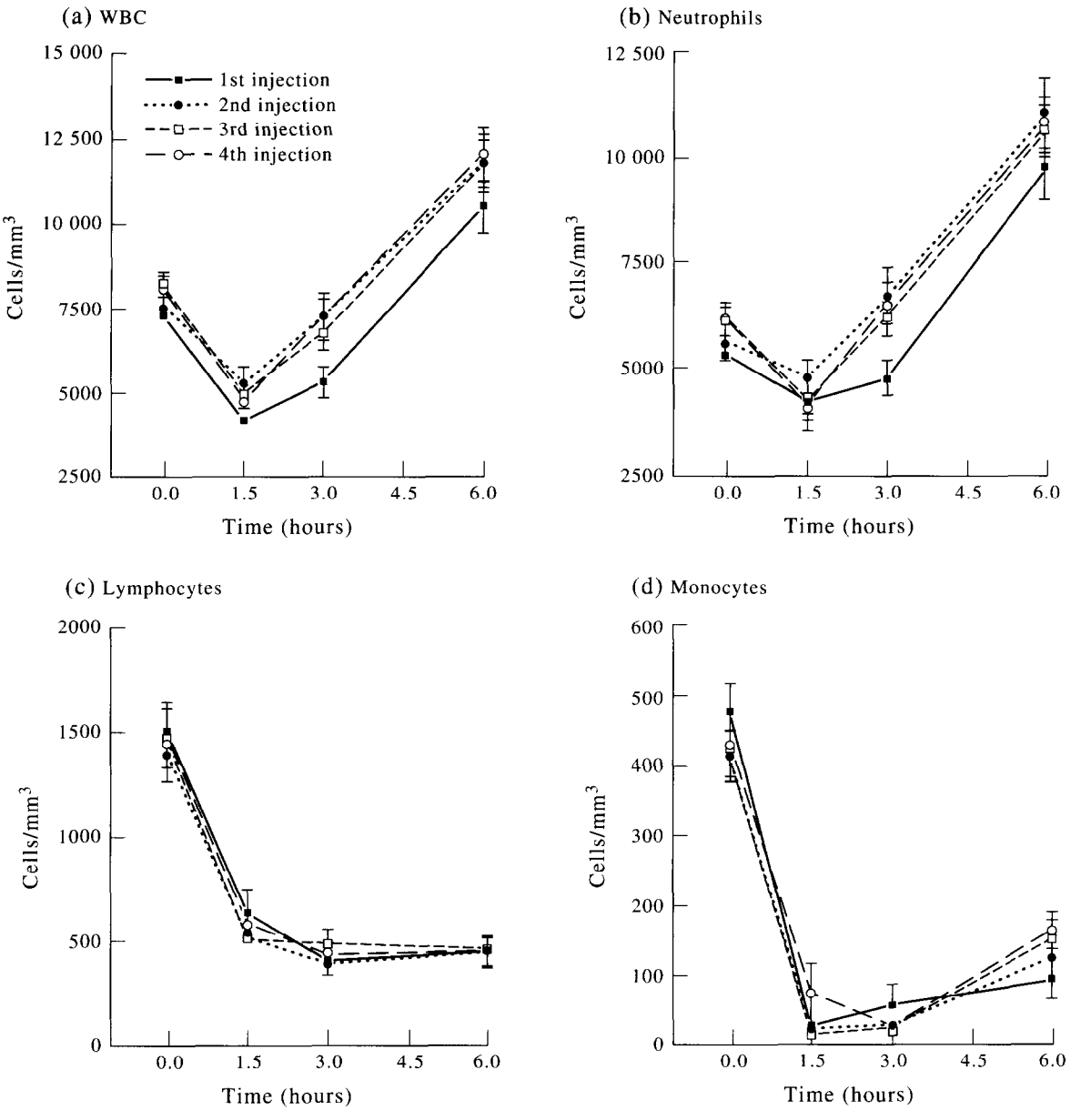

Figure 1. (a) White blood cell (WBC), (b) neutrophil, (c) lymphocyte and (d) monocyte counts following injection of LPS from *Salmonella abortus equi* (4 ng/kg body weight). Median values of 37 patients during the 6 h after LPS injection for the first four injections are shown. S.E.M. are indicated by bars.

tumours. This result is within the range of other non-specific immunotherapeutical approaches (for review see [22]). Several immunological mechanisms activated by LPS might be implicated in the antitumoral activity of endotoxin. Although no direct cytotoxic activity can be attributed to LPS, activation of macrophages, increased expression of endothelial adhesion molecules especially in the tumour vasculature leading to a compartmentalisation of immunologically active cells, release of cytotoxins (e.g. TNF-α), chemoattractants (e.g. interleukin-8) and cytokines recruiting other cells of the immune system (e.g. interleukin-6), may contribute to its antitumoral effects observed in our patients [8, 23–26].

The TNF-α peak levels determined at 90 min after LPS injection varied widely in our series, i.e. by a factor of 50. Since macrophages are the main source of TNF-α, this might reflect the variability in the cancer patients' mononuclear phagocyte system, launching a response to a given stimulus. Factors able to influence the LPS-induced TNF-α release are previous exposure to LPS [13], certain bacteria (e.g. BCG) [9] as well as endogenous or exogenous interferon-gamma [27, 28].

In every patient, the TNF-α peak level after the first LPS administration was higher than all subsequent serum levels. This phenomenon is known as endotoxin tolerance. We showed in this study that 2-weekly administration induces a steady state between the tolerance-inducing LPS stimulus and the regaining of LPS sensitivity in the interval between injections. This is in contrast to the results of a phase I study where tolerance was observed only after the second LPS application, but not after the third and fourth [10]. This discrepancy is probably due to the small respective patient group ($n = 3$) in the phase I study compared to 37 patients

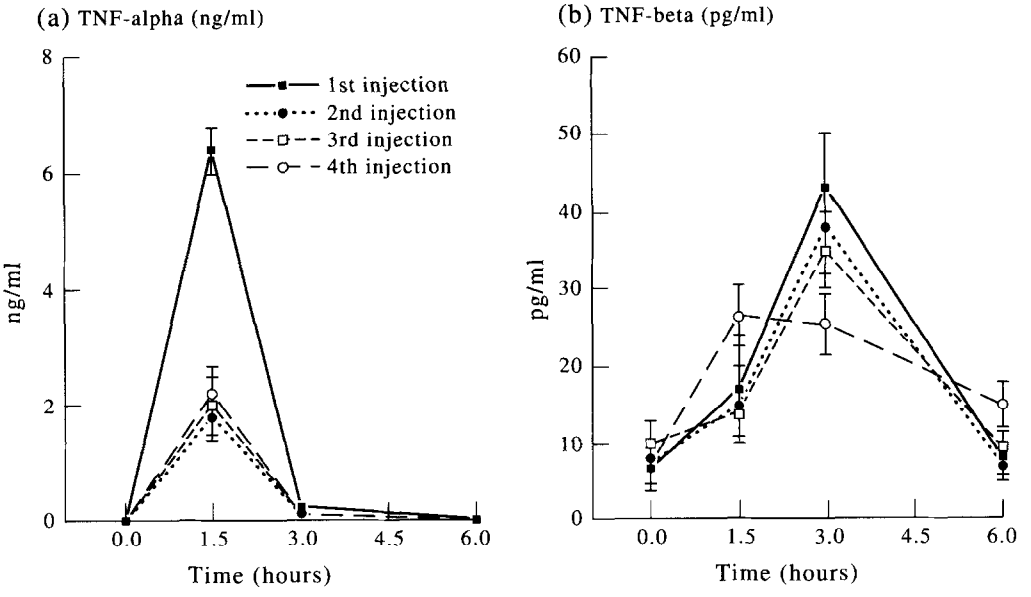

Figure 2. (a)TNF-α and (b) TNF-β serum levels following LPS injection. Median values for 37 patients (TNF-α) and 6 patients (TNF-β), respectively, are shown for the first four injections. S.E.M. values are indicated by bars.

Table 5. Changes in lymphocyte subpopulations

	CD-antigen positive cells within the lymphocyte gate (%)		
	Before injection	4 h post injection	24 h post injection
$CD4^+$	30.6	30.3	37.3
$CD8^+$	27.6	20.5	23.5
CD4:CD8 ratio	1.1	1.5	1.6
$CD37^+$	19.3	29.2	25.9
CD3 $CD16^+$	14.8	10.5	13.4
CD3 $CD56^+$	23.5	18.5	24.6
CD3 $CD57^+$	8.7	6.9	8.4

evaluated here. Therefore, it can be concluded that the application schedule chosen for this clinical trial was optimal with respect to avoiding complete tolerance in spite of administering highly immunologically active doses of LPS. In contrast to the release of TNF, the acute decrease in WBC numbers is not subject to LPS tolerance. This indicates that LPS tolerance is not due to an increased LPS elimination rate.

In addition to an activation of the mononuclear phagocyte system, an influence of LPS on the lymphocyte compartment of the immune system is evident from this study. While the B cell mitogenic activity was not as predominant as might have been predicted from animal and *in vitro* studies, the CD4:CD8 T cell ratio was altered as a response to LPS administration. Tumour patients tend to have a diminished CD4:CD8 ratio, the extent mainly depending on the tumour type (U.Wehr, unpublished observation). In most patients, this ratio returned to normal values during the course of this study. Moreover, a release of TNF-β (lymphotoxin) which is exclusively produced by lymphocytes, was observed after each LPS injection [29]. Neither the IL-2/IL-2 receptor system nor IL-7 [30] seemed to be involved in this response, with circulating IL-2, IL-7 or an increase in the expression of CD25 (IL-2 receptor alpha chain) on the surface of peripheral lymphocytes not found.

In summary, this study has shown that, in spite of an effect of endotoxin on the tumour growth in several patients, response rates comparable to Coley's early studies could not be achieved. This could be due to the use of purified endotoxin as opposed to 'Coley's Mixed Toxins', by administering the substance in 2-week intervals in contrast to Coley's daily or thrice weekly injections, or to the pretreatment of our patients with a cycloxygenase inhibitor. To address the first possibility, we have started a clinical trial of Vaccineurin®, an autolysate of *serratia marcescens* and *streptococcus*. The evidence for an influence of endotoxin on the patients' T cell system, demonstrated in this study, adds to our understanding of the response to an endotoxin stimulus.

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