The effect of antibody against TNFa on cytokine response in Jarisch-Herxheimer reactions of louse-borne relapsing fever

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Summary

Severe Jarisch Herxheimer reaction (J-HR) precipitated by antibiotic treatment of louse-borne relapsing fever (LBRF) is associated with a transient, marked rise in circulating tumour necrosis factor alpha (TNFa), interleukin 6 (IL-6) and interleukin 8 (IL-8). Ovine polyclonal anti-TNFa antibody fragments (Fab) were used in a randomized double blind placebo controlled trial in an attempt to prevent this reaction. Within 4 h after penicillin, in controls ($n = 29$), a several-fold rise in cytokines occurred, concomitant with a fall in spirochaetes and maximal clinical manifestations of the J-HR. An intravenous infusion of anti-TNFa Fab, 30 min before penicillin in 20 patients reduced peak plasma levels of IL-6 and IL-8 (but not IL-1b) compared with controls ($p = 0.01$ and < 0.001, respectively) and the incidence of the J-HR, indicating some neutralization of TNFa. An apparent fall in TNFa reflected interference of anti-TNFa in the immunoassay.

Introduction

The systemic inflammatory response syndrome (SIRS)[1] can be initiated by a number of viral, bacterial, protozoal and fungal toxins, including lipopolysaccharides released from the cell walls of Gram-negative bacteria. Studies in animals,[2,3,4] human volunteers[5] and patients[6] suggest that TNFa is the most important mediator in this syndrome, but that other cytokines, including IL-1b, IL-6 and IL-8, are also involved. Despite treatment with antimicrobials and supportive measures, SIRS continues to have a mortality of up to 60%, and it has been suggested that it might best be treated by neutralizing one or more of the mediating cytokines with, for example, specific antibodies.[7] The effectiveness of this approach remains unproven, because animal models may be inappropriate,[8] while in clinical studies the great diversity of SIRS leads to multiple overlapping subgroups of patients unsuitable for statistical comparison.[9]

We believe that an ethically acceptable and predictable model of SIRS is required to enable the accurate assessment of the potential role of antibodies directed against a single or multiple[10] cytokines. The Jarisch-Herxheimer reaction (J-HR) which usually follows antibiotic treatment of louse-borne relapsing fever (LBRF) meets some of the requirements of such a model. Clinical manifestations of the reaction are predictable in their timing and nature and closely resemble those of a classical endotoxin reaction.[11] Furthermore, Negussie and his colleagues showed that the J-HR which followed penicillin therapy and the resultant clearance of

spirochaetes from the circulation was associated with a several-fold rise in circulating levels of TNFα, IL-6 and IL-8.[12]

The present double-blind, placebo-controlled study was designed to determine whether ovine antibody fragments (Fab) directed against TNFα would suppress circulating cytokine levels and hence the J-HR—since this might be relevant to the treatment of SIRS and other clinical conditions in which high levels of circulating TNFα have been found. This paper relates primarily to plasma cytokine levels, and for reasons of brevity the clinical findings are reported in detail elsewhere.[13]

Methods

Patients

A total of 49 patients, (46 males and 3 females), were recruited from hospitals and health clinics in Addis Ababa. They were between 12 and 60 years old with no clinical evidence of other diseases. All gave informed consent. The diagnosis of LBRF was based on clinical suspicion confirmed by finding *Borrelia recurrentis* spirochaetes in their peripheral blood film stained with Wright's stain. Clinical history and examination were recorded on standard proformas, and patients lay supine in bed throughout the study with an intravenous cannula inserted into both arms for blood sampling and drug administration.

Antibody

Sheep were immunized with recombinant human TNFα in Freund's adjuvant and the antibodies were precipitated with sodium sulphate and cleaved with papain to give Fab and Fc. These were supplied by Therapeutic Antibodies Inc. as lyophilized ovine polyclonal anti-TNFα Fab. Non-specific antibodies from a donor flock were similarly processed and supplied as ovine control Fab. The infusions were prepared by someone not involved in the care or assessment of the patients, who dissolved the contents of 4×1.5 g vials of lyophilized anti-TNFα or control Fab in 100 ml of 0.9% saline.

Protocol and clinical measures

Patients were randomized for treatment with anti-TNFα Fab (20 patients), control Fab (19 patients) or 0.9% saline alone (10 patients). After stabilization of core temperature (i.e. $\pm 0.5\,^\circ$C for 30 min), the test infusion was given over a 30-min period. A curative dose of procaine penicillin (600 000 units) was then administered by deep intramuscular injection into the anterior thigh, and an infusion of 0.9% saline was continued at a rate of 1 L per 8 h for 24 h.

Blood samples (10 ml) were taken into sterile endotoxin-free lithium heparin tubes immediately before starting the first infusion (-0.5 h), at the time of the penicillin injection (0 h) and after a further 1, 1.5, 2, 4, 8 and 24 h. The samples were separated by centrifugation immediately and the plasma was kept at $-20\,^\circ$C until assayed at St Bartholomew's Hospital for TNFα, IL-1β, IL-6 and IL-8. Commercial kits for IFNγ and the two soluble TNFα receptors, sTNF-R1 (p55) and sTNF-R2 (p75) were generously donated, which enabled samples from nine of the patients (six from the control and three from the treated groups) to be assayed. Thin blood smears were prepared at the same time intervals on glass microscope slides, stained using Wright's stain and examined by light microscopy by an independent observer for the presence and number of *B. recurrentis*. Baseline measurements of rectal temperature (using a rectal probe), of blood pressure (by an automatic sphygmomanometer), and of the respiratory and pulse rates were recorded at frequent intervals throughout. The clinical severity of the J-HR was graded as none (no shivering); mild (shivering); moderate (intermittent rigors) or severe (severe sustained rigors) by a clinician who, like the rest of the clinical team, was blinded to the patient's treatment.

Cytokine assays

These were all performed by one person (REC) using commercial immunoassay kits (Medgenix, Belgium) according to the manufacturer's instructions. Each kit is based on several monoclonal antibodies directed against different epitopes on the macromolecule to be measured, as a means of reducing hyperspecificity and incubation times and of increasing sensitivity.

Immunoradiometric Assay (IRMA) for TNFα

Standards or samples (200 ml) were added to plastic tubes coated with several monoclonal antibodies directed against different epitopes on TNFα (the capture antibodies), followed immediately by monoclonal antibodies labelled with [125]I (the signal antibodies). Standards are provided in human serum with levels between 0–5000 ng/l, and one ng of the Medgenix standard is equivalent to 32 U of the International Reference Standard (NIBSC 87/650). After 16 to 24 h at room temperature, the contents of the tubes were aspirated (or decanted) to waste and the tubes were washed carefully and counted using a Hydrogramma 16 (Innotron). The assay measures total TNFα (i.e. free TNFα as well as that bound to natural soluble receptors).[14] The assay has a minimum detectable level (MDL) of 5 ng/l and serum or plasma TNFα levels in normal subjects

range from less than 5 to about 20 ng/l with a mean of 6.3 ng/l.

IRMA for IL-1b

This assay is virtually identical to the TNFa assay. Standards range from 0 to 5000 ng/l and each ng of standard is equivalent to 200 U of an International Reference Preparation (NIBSC 86/552). The MDL is 5 ng/l and values are usually undetectable in plasma or serum from normal subjects but may, on occasion, be as high as 15 ng/l.

IRMA for IL-6

This assay is similar to the above except that it was done in two steps to help avoid a high-dose hook effect. Samples and standards (200 ml) were incubated in the antibody-coated tubes for 16–20 h and then aspirated (or decanted) to waste before addition of 200 ml ^{125}I-labelled monoclonal antibodies. Standards range between 0 and 5000 ng/l and 1 ng is equivalent to 3.5 U of a Reference Preparation (NIBSC 88/514). Levels of IL-6 in 157 normal plasma samples were below the levels of detection (6 ng/l) in 39 and ranged from 6 to 31 ng/l in the remainder.

IRMA for IFNc

This assay is based on the same principle as the TNFa assay. Standards range from 0 to 90 IU/ml and one IU/ml of the Medgenix standard is stated by the manufacturer to be equivalent to 1 IU/ml NIH Gg 23-901-530 standard. The assay has an MDL of 0.2 IU/ml and a mean value of 0.55 ± 0.14 has been reported in 20 normal controls.

Immunoenzymometric Assay (IEMA) for IL-8

The general format differed in that the capture monoclonal antibodies were used to coat the walls of microtitre plates while the signal antibodies were labelled with horseradish peroxidase. The resultant assay was very sensitive, with a MDL of 0.7 ng/l, and used standards ranging between 0 and 750 ng/l, where 1 ng of standard is equivalent to 0.14 U of the International Reference Standard (NIBSC 89/520). Normal values range from < 8 to 47 ng/l.

IEMA for sTNF-R1

This has the same format as the IL-8 assay. Standards were provided in bovine serum with concentrations between 0 and 77 mg/l. sTNF-R2 and TNFa, at concentrations of 1500 mg/l and 400 mg/l, respectively, did not cross-react with sTNF-R1 in the assay. The MDL was 50 ng/l, and levels in 129 normal sera ranged from 0.3 mg/l to 2.9 mg/l with a mean of 1.2 mg/l.

IEMA for sTNF-R2

This assay is virtually the same as that for sTNF-R1 with sTNF-R2 standards (in bovine serum) ranging between 0 and 142 mg/l. There was no interference in the assay due to sTNF-R1 and TNF, at 1500 mg/l and 400 mg/l, respectively. The MDL was 100 ng/l and serum levels in 117 normal subjects ranged from 1.91 to 8.51 mg/l with a mean of 4.17 mg/l.

Statistical analysis

The Mann–Whitney U test was used to analyse the differences in admission and peak cytokine concentrations. For comparison with the data obtained in the group receiving anti-TNFa Fab, the saline control and non-specific Fab groups were combined for analysis, as there was no significant differences between their clinical findings and cytokine levels.

Ethical approval

Ethical approval for the study was given by the Research Ethics Committee, Faculty of Medicine, Addis Ababa University.

Results

There were no significant differences in either the clinical or plasma cytokine findings for patients receiving saline or control Fab. The two have, therefore, been combined to provide what will be referred to as the control group (comprising 29 patients). The remaining 20 patients who received ovine anti-TNFa Fab are referred to as the test group.

Clinical findings

In this paper, for simplicity, the patients are only graded according to the severity of their J-HR as defined earlier. Of the 29 controls, three had no reaction, while 12, 11 and three experienced mild, moderate and severe J-HRs, respectively. In contrast, 10 of the test group had no reaction and the other 10 only mild J-HR. Both the incidence and severity of the J-HR in the two groups differed significantly ($p < 0.01$ and < 0.005, respectively) with the Fab-receiving patients experiencing less severe reactions. Systolic blood pressure, pulse rate and temperature increased less in the test group compared with the control group.[13]

Basal cytokine levels

All 49 patients had cytokine assays performed on at least one basal plasma sample collected before treatment began. There was no significant difference in these basal cytokine levels between the control

Table 1 Basal plasma cytokine levels in patients with LBRF

	TNFa (ng/l)	sTNF-R1 (mg/l)	sTNF-R2 (mg/l)	IL-1b (ng/l)	IL-6 (ng/l)	IL-8 (ng/l)	IFNc (IU/ml)
Number of samples	76	18	16	98	98	98	18
Median	200	17.5	250	ND	2000	37	0.4
(10th–90th percentile)	(100–580)	(8–50)	(120–250)	(0–10)	(900–6400)	(9–350)	(0.2–0.8)
Quoted upper limit of kit normal range	20	2.9	8.5	15	31	47	0–8
Samples with median above upper limit (%)	100%	100%	100%	6%	100%	51%	0%

Basal levels of TNFa and IL-6 were markedly above the quoted upper limit of normal; there was no significant difference between basal cytokine levels in the control and test group; 50% of IL-8 levels were raised and IL-1b levels were mostly unaffected. Nine patients had IFNc and soluble-receptor assays performed on two basal samples; median admission levels for s-TNF-R1 and sTNF-R2 were 6 and 29 times the upper limit of normal, respectively, and IFNc was normal.

and test groups. The results are summarized in Table 1. Every sample tested had TNFa and IL-6 levels markedly above the upper limit of the normal range, with median values more than 10 and 60 times higher, respectively. IL-8 levels were elevated in about 50% of samples while baseline values of IL-1b appeared unaffected. In general, basal admission levels remained stable or fluctuated only slightly between −0.5 and 0 h.

Nine of the patients also had IFNc and soluble-receptor assays performed on two basal samples. The median of admission levels for IFNc was normal and those for sTNF-R1 and sTNF-R2 were markedly raised, with values 6 and more than 29 times the upper limit of normal, respectively (Table 1).

Cytokine profiles during the J-HR

Control patients

Figure 1 shows a typical response in a patient who received a saline infusion before penicillin and developed a severe J-HR. Almost immediately after penicillin treatment, the spirochaete count started to fall and by 8 h spirochaetes were undetectable in the peripheral blood film (in the other patients, spirochaete clearance was unaffected by the test Fab and was the same in both groups[13]). After a delay of about 1 h a rise in TNFa and IL-6 levels was detected, followed by IL-8 (at 1.5 h) and IL-1b between 2 and 3 h. The increase in plasma cytokine levels coincided with or preceded clinical signs of J-HR, but peaked after the rigors had subsided. In this patient, IL-1b and IL-8 levels had returned to baseline by 24 h, and TNFa and IL-6 were lower.

The median plasma levels and percentiles (10th–90th) for TNFa, IL-1b, IL-6 and IL-8 over time for the 29 control patients are listed in Table 2. IL-1b was undetected in 8/29 patients throughout the study period, and levels were much lower and restricted to a narrower range than the other cyto-

kines. Data on the exact time that cytokine levels started to rise are not accurate, because insufficient blood samples were collected at the appropriate times. Plasma levels were raised significantly above baseline for TNFa ($p < 0.0001$) and IL-6 ($p = 0.0001$) by 1.5 h, for IL-8 by 2 h ($p = 0.0003$) and for IL-1b at 4 h ($p = 0.0002$). Median levels for all four cytokines continued to rise until a peak concentration was reached at 4 h, after which levels started to fall and were below admission levels at 24 h. TNFa, IL-6 and IL-8 were detected throughout the study period (−0.5 to 24 h) in every patient, including two who did not have clinical evidence of a J-HR. There was no obvious relationship (although the relative sample size was small) between plasma peak cytokine levels and the severity of the J-HR as shown for each patient in the control group (Figure 2).

There were additional assays in six controls. IFNc plasma concentration levels remained normal throughout the study period. sTNF-R1 and sTNF-R2 levels were markedly elevated throughout, and no obvious rise nor fall in levels, was apparent.

Control group (n = 29) vs. test group (n = 20)

The typical rise to a peak and then fall of circulating cytokine levels seen in the control group (Figure 3a) was not detected in the test group (Figure 3b). Thus, 'measured' plasma levels of TNFa fell dramatically, during the 30-min infusion of anti-TNFa from a median baseline level at −0.5 of 225 ng/l to 3 ng/l at 0 h and remained low throughout the study period. There was no significant change in the median circulating cytokine levels from baseline for IL-8 and IL-1b throughout the study period, but IL-6 levels rose from a median baseline of 2.8 mg/l to 6.5 mg/l ($p = 0.001$) at 2 h. Cytokine levels for this group are listed in Table 2. Median peak levels occurred at the same time (4 h) as in the control group, but were significantly lower for TNFa, IL-6 and IL-8, and are
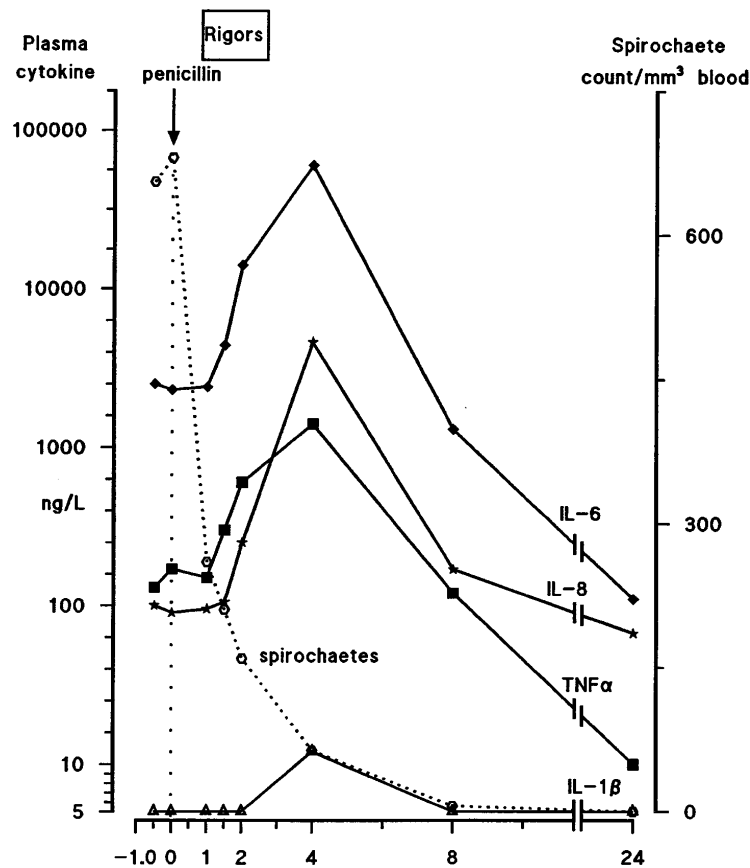
Figure 1. Typical response in a patient receiving a control saline infusion 30 min before penicillin. After penicillin, spirochaetes fell abruptly, and after about 1 h circulating levels of TNFa, IL-6, IL-8, and IL-1b started to rise, and peaked at 4 h. This patient experienced sustained rigors which occurred as cytokine levels were increasing and subsided before peak levels were achieved.

compared in Table 3. The J-HR occurred in 50% of the test group and was mild.

In additional assays, IFNc and sTNF-R1 and sTNF-R2 levels in three patients receiving test Fab were similar to those in the six controls.

In vitro studies of the effect of anti-TNFa Fab on the measurement of TNFa

On endogenous TNFa

Duplicate aliquots of plasma were prepared from the series of samples collected from a control patient who had received only a saline infusion and was known to have had a marked rise in TNFa following his penicillin injection. One series of aliquots was preincubated with control Fab and the other with anti-TNFa Fab in an amount calculated to be similar to that encountered *in vivo* in test subjects. The samples were then assayed in the usual way. As expected, TNFa levels increased markedly from a mean basal level of 780 ng/l to a peak of nearly 5000 ng/l in the samples containing control Fab, but were dramatically lower with a mean basal level of 50 ng/l and a peak of 160 ng/l in samples containing

anti-TNFa Fab. Clearly the latter interfered with the assay.

On TNFa standards

The IRMA for TNFa is based on the use of capture monoclonal antibodies adsorbed to the assay tubes, and signal antibodies labelled with ^{125}I which are added to the tubes immediately after the standards (or sample). The more TNFa present in a standard, the more will bind to the capture antibodies and provide a link so that the amount of radioactivity (cpm) in the tube after washing is directly related to the amount of TNFa in the sample. Each TNFa standard provided in the kit was preincubated with saline, Fab control or specific anti-TNFa Fab and then assayed in the usual way. The maximal amount of TNFa (i.e. with the highest cpm) was detected in the standards preincubated with saline or control Fab, while TNFa was almost undetectable in the standards containing anti-TNFa. Finally, a high TNFa standard was preincubated with doubling dilutions of control or anti-TNFa Fab prior to assay. As shown in Figure 4, there was a gradual increase in the amount of TNFa apparently being measured as the amount of anti-TNFa was reduced.

Table 2 Median plasma cytokine concentrations

Time (h)		Combined control group ($n=29$)				Test group ($n=20$)			
		TNFa (ng/l)	IL-1b (ng/l)	IL-6 (mg/l)	IL-8 (ng/l)	TNFa (ng/l)	IL-1b (ng/l)	IL-6 (mg/l)	IL-8 (ng/l)
−30	Median	170	0	1.7	54	225	0	2.8	29.5
	Percentile*	(70–580)	(0–11)	(0.6–6.8)	(7–420)	(120–600)	(0–9)	(1.3–6.8)	(12.5–405)
0	Median	180	0	2.1	42	3	0	2.5	33.5
	Percentile	(80–540)	(0–18)	(0.75–5)	(6–350)	(0.5–8.5)	(0–4)	(1.4–6.4)	(11–435)
1	Median	230	0	1.9	46	4	0	1.8	25
	Percentile	(120–520)	(0–13)	(1.2–6)	(10–380)	(1.5–20)	(0–9)	(1.1–5.7)	(8.5–483)
1.5	Median	520	0	4.8	61	4	0	3.9	19.5
	Percentile	(270–1400)	(0–15)	(2–16)	(6–390)	(0.5–10.5)	(0–8)	(1.3–15)	(5–483)
2	Median	800	0	13	240	4	0	6.5	22.5
	Percentile	(430–2200)	(0–16)	(4.4–36)	(25–2700)	(0–11.5)	(0–10)	(2.8–24.5)	(5.5–538)
4	Median	1350	8	30	925	4	0	12	33
	Percentile	(520–3500)	(0–40)	(5–135)	(90–4600)	(2–11)	(0–28)	(2–36)	(8–715)
8	Median	518	2	3	160	6	0	3.6	18
	Percentile	(140–2200)	(0–27)	(0.1–13)	(24–2100)	(0–11.5)	(0–7)	(0.06–10.4)	(5.5–433)
24	Median	50	0	3	25	5	0	0.11	23.5
	Percentile	(16–190)	(0–3)	(0.04–0.6)	(8–100)	(1–11)	(0–5)	(0.04–0.06)	(6–325)

In the combined control group ($n=29$), median TNFa and IL-6 levels were raised significantly above baseline by 1.5 h, for IL-8 by 2 h and IL-1b by 4 h. Median peak levels occurred at 4 h for all of these cytokines.
* Range of 10th–90th percentiles.

Discussion

Negussie and his colleagues were the first to report that severe J-HRs, which occurred in 14 of their 17 patients (82%) with LBRF treated with penicillin, are associated with, and probably caused by, a transient, marked rise in plasma levels of TNFa followed by IL-6 and IL-8. The plasma levels of these cytokines increased between four- and ten-fold compared to admission, as determined by bioassay (for TNFa and IL-6) or an immunoassay (for IL-8). The three patients who did not develop a typical J-HR showed no cytokine response. We have, in general, confirmed these findings in a larger control group. Although it is not possible to compare actual cytokine levels directly, because of differences in the assays and standards used, the profile of the cytokine release in the two studies was remarkably similar. Median values for TNFa, IL-6 and IL-8 at each time-point for both studies had increased significantly above baseline by 1.5 h for TNFa and IL-6, and by 2 h, IL-8 showed a four-fold increase. Each cytokine had peaked at 4 h, when TNFa, IL-6 and IL-8 had increased 8-, 18- and 17-fold over baseline, respectively. However, the three control patients in our study who did not develop symptoms of a J-HR showed a typical cytokine response, which is in agreement with Cuevas and colleagues.[15] IFNc levels were normal throughout the study and there was no relationship to the J-HR.

IL-1b assays were also performed in the present study, but admission plasma levels were normal, and only 12 of the 29 control subjects (41%) showed a rise following penicillin injection and then only of a minor degree. These findings accord with those of Cannon and his colleagues[16] who used a radio-immunoassay to determine plasma levels of IL-1b in patients with septic shock and in normal subjects infused with endotoxin.

Great care must be taken in interpreting assay results, since various factors can influence different types of assay to different degrees. For example, the two soluble receptors to TNFa found in the circulation (sTNF-R1 and sTNF-R2) are known to be raised markedly in septic shock, and were present in extremely high concentrations in the nine patients whose samples were assayed in this study. Engelberts[14] and his colleagues have shown that such levels had profound and differing effects on a WEHI 164 bioassay, an enzymoimmunoassay and an IRMA for TNFa. However, the TNFa IRMA used in the present study was chosen because the results obtained are not influenced by the presence of soluble receptors.

One reason for undertaking the present study is that the J-HR that complicates antibiotic treatment of LBRF resembles other forms of SIRS in its clinical features and cytokine changes. Also, the J-HR has the advantage of predictability, which contrasts with previous clinical trials in SIRS, where it is unlikely that patients would be diagnosed and available for
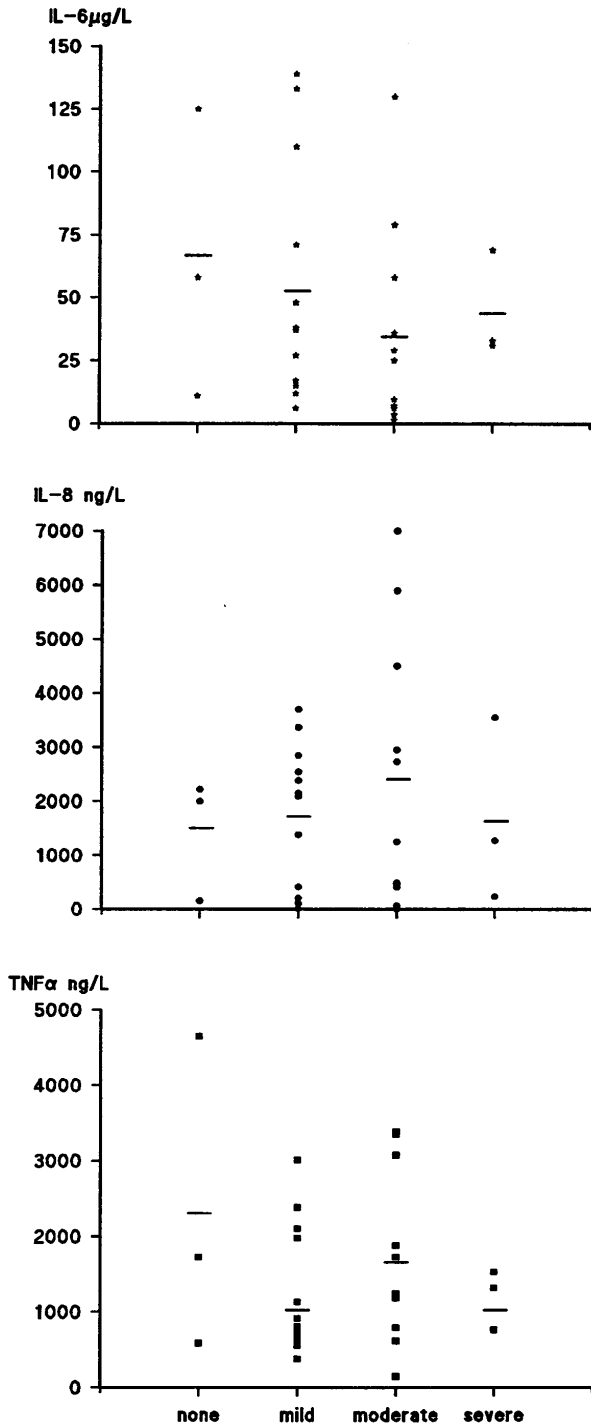
IL−6μg/L

IL−8 ng/L

TNFα ng/L

none   mild   moderate   severe

Figure 2. Cytokine peak levels and clinical severity of the J-HR in the control group. Clinical severity of the J-HR was graded as none (no shivering), mild (shivering), moderate (intermittent rigors) and severe (sustained rigors). There was no obvious relationship between peak plasma cytokine levels and the severity of the reaction, but the relative sample size was small.

Patient A

penicillin

Control Fab

IL−6 ◆
IL−8 ★
TNFα ■
IL−1β ▲

Patient B

penicillin

anti−TNFα Fab

TNFα □
IL−1β ▲

h

Figure 3. Comparison of cytokine profiles in patient A receiving control Fab and on patient B, receiving anti-TNFa Fab. In Patient A cytokine levels rose to a peak and then fell back to below admission levels. During the 30 min infusion of anti-TNFa (patient B), TNFa levels fell suddenly and remained low throughout the study period, peak IL-6 and IL-8 levels were suppressed.

treatment before the onset of their inflammatory response. Recent experience with the use of specific monoclonal antibodies for the treatment of septic shock has been disappointing, especially when such
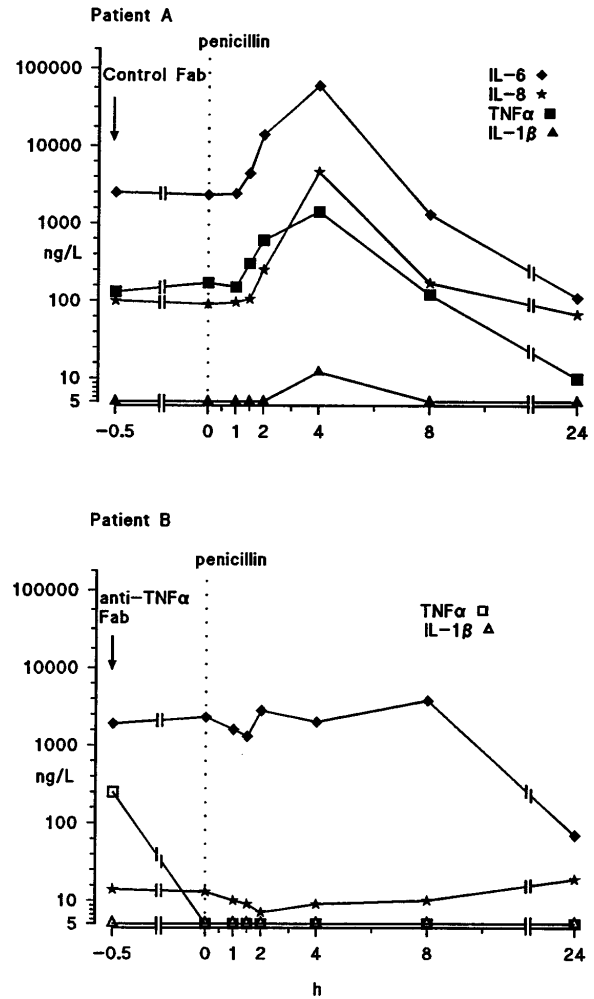
antibodies have been directed against lipopoly-saccharides (LPS).[17] This lack of success may reflect the heterogeneous nature of septic shock. Alternatively, as we have suggested,[18] greater care may need to be taken with the selection of mono-clonal antibodies suitable for use in SIRS, and this would be facilitated greatly by the availability of a suitable model.

We have used Fab prepared from polyclonal antibodies obtained from sheep immunized with rhTNFa for use in the present double-blind placebo-controlled trial. Nature invariably mounts a poly-clonal response to antigens and, in our experience, such antibodies are usually more effective clinically and require less rigorous selection than their mono-clonal counterparts.[18] It was hoped that Fab, with their much more rapid rate and larger volume of

Table 3 Comparison of median plasma cytokine peak levels in control and test

Cytokine	Group (n)	Median	10th–90th percentile	p
TNFa (ng/l)	Control 29	1350	520–3500	
TNFa (ng/l)	Test 20	4*	2–11	<0.0001
IL-1b (ng/l)	Control 29	8	0–40	
IL-1b (ng/l)	Test 20	0	0–28	NS
IL-6 (ng/l)	Control 29	30	5–135	
IL-6 (ng/l)	Test 20	12	2–36	0.01
IL-8 (ng/l)	Control 29	925	90–4600	
IL-8 (ng/l)	Test 20	33	8–715	<0.001

Median peak levels for TNFa, IL-6, IL-8 and IL-1b occurred at the same time (4 h) in both groups but were significantly lower in patients given anti-TNFa Fab. NS, not significant.
*Apparent low levels of TNFa in the test group may be due to interference by the anti-TNFa Fab in the assay.
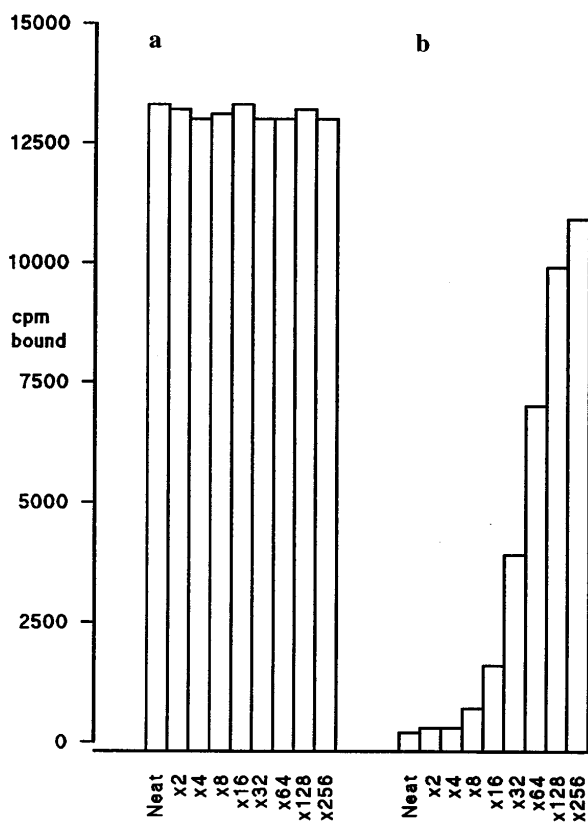

Figure 4. Effect of different concentrations of **a** control Fab and **b** anti-TNFa Fab on measured TNFa. A high TNFa standard was preincubated with doubling dilutions of **a** and **b** and then assayed in the usual way. Maximal TNFa (highest cpm) was detected in the standard preincubated with **a** and was detected in increasing amounts as the amount of anti-TNFa preincubated with the standard was reduced.

distribution than intact immunoglobulins, might offer advantages when used in an attempt to block the biological effects of TNFa. This cytokine probably operates largely in the interstitial rather than the vascular compartment of the extracellular fluid volume. This is, as far as we are aware, the first occasion in which a polyclonal product has been used clinically in a large group of patients with SIRS.

Ovine anti-TNFa Fab produced a rapid and profound reduction in 'measured' plasma levels of TNFa which had approached baseline by the end of the 30-min infusion, and remained low throughout the rest of the study. A series of in vitro studies indicated that the polyclonal Fab had, as would be expected, bound to most, or all, of the surface epitopes in TNFa. As a result, neither the capture nor the signal antibodies provided in the immunoradiometric kit could bind to the endogenous TNFa whose disappearance from the circulation was, therefore, probably apparent rather than real. The rate of clearance of circulating Fab-TNFa complexes has not been studied; however, their rapid clearance may be another reason for the apparently low TNFa levels. There is substantial evidence that the anti-TNFa Fab also suppressed the biological activity of the endogenous TNFa. Thus, both the incidence and severity of the clinical manifestations of the J-HR were less than in the control group, and circulating levels of IL-6 and IL-8 (but not of IL-1b) were reduced significantly. The changes in interleukin levels are probably real, because anti-TNFa Fab does not interfere with their assay. In addition, rats receiving an intraperitoneal dose of monoclonal antibody directed against TNFa prior to receiving a bolus dose of endotoxin had markedly altered plasma cytokine profiles resembling those demonstrated in the J-HR patients receiving Fab.[19] In these animal experiments, antibody against TNFa abolished plasma TNFa bioactivity and considerably reduced the IL-6 detected in plasma. Macromolecules such as TNFa are thought to exert their biological effects by means of an active site(s) which binds to, and activate, specific cell-wall receptors. The most likely mechan-

ism by which anti-TNFα Fab impairs these effects is to bind to an epitope(s) in close proximity to the active site, and thereby hinder its binding to cell receptors—the steric hindrance mechanism. Fisher and his colleagues[20] have infused a monoclonal antibody directed against TNFα into patients with sepsis, and shown that plasma TNFα levels could no longer be detected by means of an L929 cell-line bioassay, but could be detected by an IRMA. These apparently divergent findings are easy to explain by means of the steric hindrance mechanism. Thus, the monoclonal antibody had been chosen with great care to ensure that it impaired the biological effect of TNFα (i.e. it bound to an epitope in the immediate vicinity of the active site). However, TNFα has a multiplicity of different epitopes, so that it is not surprising that the capture and signal monoclonal antibody used in the IRMA could bind to the cytokine and, therefore, enable its measurement.

A further study suggests that the steric hindrance mechanism is not the only one which may be involved. Kwiatkowski and his colleagues[21] recently infused a carefully selected monoclonal antibody into patients who had high circulating TNFα values due to cerebral malaria. Again the endogenous TNFα could no longer be assayed using a WEHI bioassay (as expected according to the steric hindrance theory) but, in contrast to the Fisher study, TNFα levels increased when measured by an IRMA. This suggests that the monoclonal antibody was also binding to (and thereby delaying the movement of) the cytokine, out from the vascular compartment where most TNFα is usually formed. Even if the TNFα had retained all its bioactivity, it still might not have produced its usual biological effects if it had been restrained or sequestered within the circulation rather than reaching the interstitial fluid.

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