

## Enhancement of lipopolysaccharide-induced tumor necrosis factor secretion by hyperimmune serum from chronic infected patients

Gitte Kronborg, Anders Fomsgaard, Niels Høiby

Department of Clinical Microbiology and The Danish CF Center at Rigshospitalet, Juliane Maries vej 28,2 DK-2100 Copenhagen Ø, Denmark

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**Abstract.** Patients with cystic fibrosis (CF) and chronic *Pseudomonas aeruginosa* lung infection have a very high load of endotoxins in their lungs. However, sepsis practically never occurs in this group of patients and the presence of tumor necrosis factor (TNF)- $\alpha$  (one of the mediators of septic shock) in serum from chronically infected CF patients is contentious. The purpose of this study was to investigate the effect of hyperimmune serum from patients with CF on lipopolysaccharide (LPS, endotoxin)-induced TNF secretion from human peripheral blood mononuclear cells (PBMC). PBMC were purified from healthy donors and stimulated with a mixture of purified LPS from *P. aeruginosa* and serum from chronically infected CF patients or healthy controls. TNF in the cell supernatants was detected by an enzyme-linked immunosorbent assay method. CF sera showed a pronounced potentiating effect on TNF secretion from human PBMC induced by LPS from *P. aeruginosa*. In comparison, serum from healthy controls did not have this effect. By contrast, CF serum and serum from healthy controls showed only little potentiating effect when using LPS from *Salmonella abortus equi* at concentrations above 0.01  $\mu\text{g}/\text{ml}$  per  $2 \times 10^6$  PBMC. This indicates a specific interaction between *P. aeruginosa* LPS and CF serum which enhances TNF secretion. The TNF responses varied depending on the sera used in the preincubation with LPS, and correlated positively to the specific IgG, IgA, and IgM anti-*P. aeruginosa* LPS titers of the sera. However, since TNF is hardly detectable in sera from these patients another LPS- and/or TNF-inhibitory activity may be present in these sera.

### Introduction

Immune complexes (ICs) involving antigens from *Pseudomonas aeruginosa* are thought to play a central role in the pathogenesis of the chronic inflammatory lung

disease seen in patients with cystic fibrosis (CF) and chronic *P. aeruginosa* lung infection [16, 17, 19, 28, 36]. We have previously identified *P. aeruginosa* lipopolysaccharide (LPS) as an antigenic component in ICs isolated from sputum of chronically infected CF patients [19, 21].

Tumor necrosis factor-(TNF)- $\alpha$  is one of the most important inflammatory mediators because of its pivotal position in the cytokine network. Both LPS and ICs are known to induce TNF secretion from monocytes and macrophages [8]. TNF has been detected in high concentrations in sputum samples from chronically infected CF patients, and the TNF concentration was significantly higher in those samples which also contained ICs involving *P. aeruginosa* LPS as an antigen [21]. No TNF was detectable in serum samples from these patients [21, 22].

LPS is a biologically active molecule and one purpose of this study was to investigate if IC formation acted to neutralize suggested harmful effects of LPS in the chronic CF infection. Inhibition of LPS effects by serum from healthy donors has been demonstrated by several authors [13, 27, 38, 41], using the *Limulus ameobocyte* lysate assay. Under certain conditions interaction of serum with LPS has also been shown to prevent LPS-induced mediator release [5, 9, 29, 42] and this inhibitory effect has been shown to involve the lipoprotein fraction of the serum [9], the alpha globulins [38] and naturally occurring IgG anti-LPS antibodies [13].

CF patients with chronic *P. aeruginosa* lung infection have a very high load of bacteria and LPS locally in the lungs [2, 19] but symptoms of endotoxemia are practically never seen. However, the findings of TNF in serum from these patients are contradictory [21, 22, 39]. It is possible that the immune response of the CF patients in part explains this observation since high levels of anti-*P. aeruginosa* LPS antibodies have been detected systemically and locally in the lungs of CF patients [11, 20]. However, the effect of these antibodies, in terms of binding of LPS and consequent IC formation, may be an important factor in the continually proceeding destructive inflammatory reactions in the lungs which eventually cause the death of these patients. It is not known whether the formation of LPS- anti-LPS ICs in the lungs and in the circulation reduces or enhances the cytokine-inducing properties of LPS.

The purpose of this study was to investigate the effect of serum from chronically infected CF patients on *P. aeruginosa* LPS induced TNF secretion from human peripheral blood mononuclear cells (PBMCs), and to compare this with the effect of *P. aeruginosa* LPS alone.

## Materials and methods

### Sera

Sera from eight chronically *P. aeruginosa*-infected CF patients and eight age-matched healthy controls were obtained by venipuncture and collection of blood in endotoxin free vacuocontainers. The *P. aeruginosa* infection was defined as chronic when *P. aeruginosa* has been cultured continuously for 6 months in samples of bronchial secretions, which usually coincides with a significantly increased antibody response against *P. aeruginosa* [31]. All samples were handled under sterile conditions to avoid endotoxin contamination. The sera were heat inactivated at 56°C for 30 min and stored at -20°C until use. IgG, IgA, and IgM anti-*P. aeruginosa* LPS antibodies in the sera were measured by ELISA as described previously [20].

## *LPS preparations*

Smooth form LPS (S-LPS) from *P. aeruginosa* and *Salmonella abortus equi* were both extracted by the hot phenol-water method [43], purified by ultracentrifugation and converted to their triethylamine salt form by electro dialysis as described earlier [10]. Both LPS preparations contained less than 3% proteins and no detectable nucleic acids [10]. The *P. aeruginosa* 1118 Habs O group 3 (O:3) was isolated from a patient with CF and chronic lung infection. The O:3 strain was chosen because it is the most common among CF patients in Denmark [30].

## *"IC formation"*

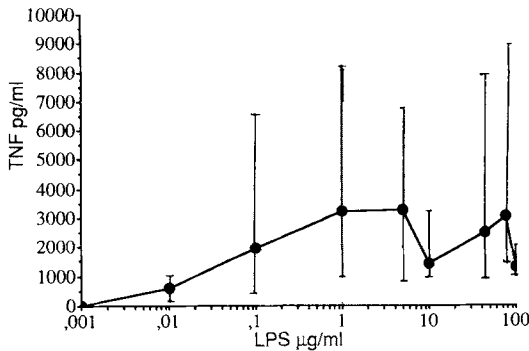
To determine the optimum antigen/antiserum ratio for IC formation, using *P. aeruginosa* LPS and CF serum a quantitative precipitation test was carried out as described previously [18]. LPS and sera were serially diluted in endotoxin-free 0.15 M NaCl in sterile endotoxin free glass tubes, mixed, and incubated at 37°C for 1 h in a shaker. Absorption at 599 nm was measured, and the optimum concentration of LPS determined by this method was 44 µg/ml in sera diluted to 20%. In control experiments serum and LPS were treated separately using the same methods. To confirm the presence of ICs in the mixture, PEG precipitations were carried out as previously described [18], and the identity of the components involved in the PEG-precipitated samples were confirmed using SDS-PAGE and immunoblotting [21] or a more sensitive dot blot assay [21]. The LPS component was identified using a monoclonal anti-*P. aeruginosa* O:3 LPS antibody (produced at the State Serum Institute, Copenhagen, Denmark). The IgG components were identified with monoclonal anti-IgG1, 2, 3, and 4 (Janssen Biochimica, Belgium), and IgA and IgM were demonstrated by monospecific rabbit anti-human IgA and IgM antibodies (Dako, Glostrup, Denmark). The ICs were either used for cell stimulation immediately after formation or stored at -80°C and used within a week after preparation. LPS from *S. abortus equi* and sera from healthy controls (NHS) were used at the same concentrations as *P. aeruginosa* LPS and CF sera.

## *Cell activation*

PBMC were isolated from heparinized blood of healthy individuals by density gradient centrifugation on Lymphoprep (Nyegaard, Oslo, Norway), washed three times in medium (RPMI 1640 with 25 mM Hepes and L-glutamine from Biological Industries, Kibbutz Beth Haemek, Israel, supplemented with penicillin and streptomycin) and resuspended in medium containing 0.2% NHS to a cell concentration of  $4 \times 10^6$ /ml. Cell suspension (100 µl) was incubated with 100 µl of the sample under investigation in the wells of a 96-well flat-bottom cell culture plate (Nunc, Roskilde, Denmark) for 18 h at 37°C. An 18-h incubation was chosen because of convenience, and because a plateau was seen in the TNF secretion. After incubation the cell supernatants were analyzed for TNF. Each experiment was performed in duplicate, and each set of experiments was performed at least two times using different cell donors. Comparisons between the TNF responses produced by each sample were only made in experiments carried out using the same cell donor on the same day.

## *TNF assay*

TNF was detected by a double-antibody sandwich ELISA as described previously [12]. Briefly, 96-well microtest plates (maxisorp, Nunc, Roskilde, Denmark) were coated overnight with rabbit anti-TNF in carbonate buffer, pH 9.8. After washing, samples and a human recombinant (r) TNF standard (Genzyme, corp. Mass. USA) diluted in buffer [PBS, pH 7.4, 0.5% HSA (w/v) containing 10% normal rabbit serum (v/v) (Dako) and 0.05% (w/v) Tween 20], were incubated on a shaker for 1 h at room temperature, followed by biotinylated rabbit anti-TNF, streptavidin-



**Fig. 1.** The mean and range of *Pseudomonas aeruginosa* lipopolysaccharide (LPS)-induced tumor necrosis factor (TNF) secretion from human mononuclear cells isolated from 11 healthy donors

labelled peroxidase and enzyme substrate [12 mg of *o*-phenylenediamine (Dako) in 18 ml of distilled water plus 7.5 µl hydrogen peroxide 30% (v/v)]. Washing between the steps was performed in 0.15 mol/l sodium chloride containing 0.1% Tween 20. The enzyme reaction was stopped with 100 µl sulfuric acid 25% (w/v) per well and the absorbance was measured at 492 nm with a microtest plate photometer (2550 enzyme immunoassay reader; Bio-Rad Laboratories). The sensitivity was 30 pg/ml rTNF.

### Statistics

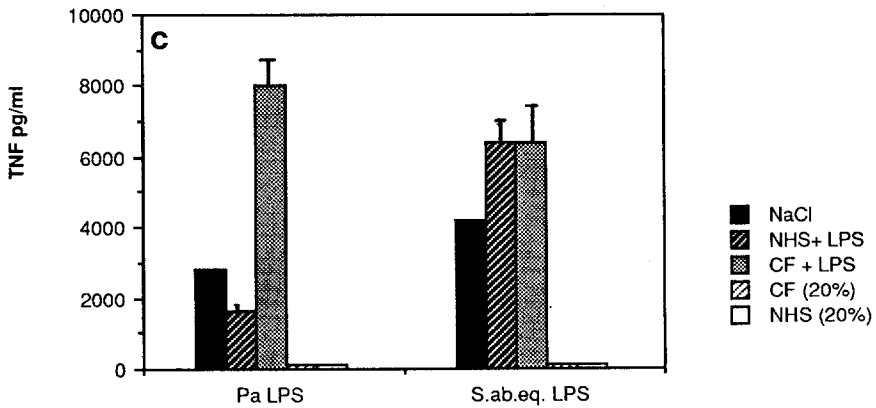
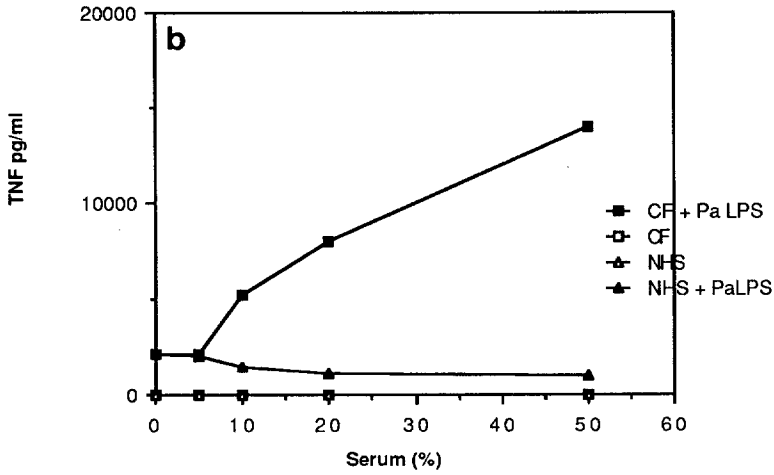
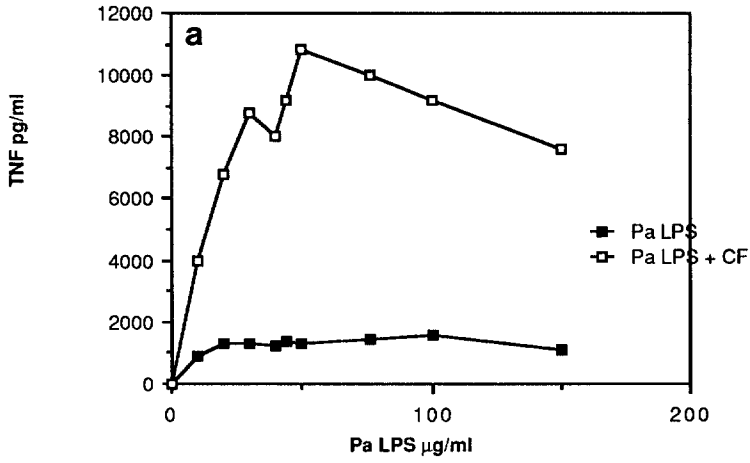
Mann-Whitney U test was used for non-parametric unpaired data, Wilcoxon signed rank test for non-parametric paired data, and Spearman correlation test for correlation analysis of non-parametric data (Macintosh Statview and graphic software).

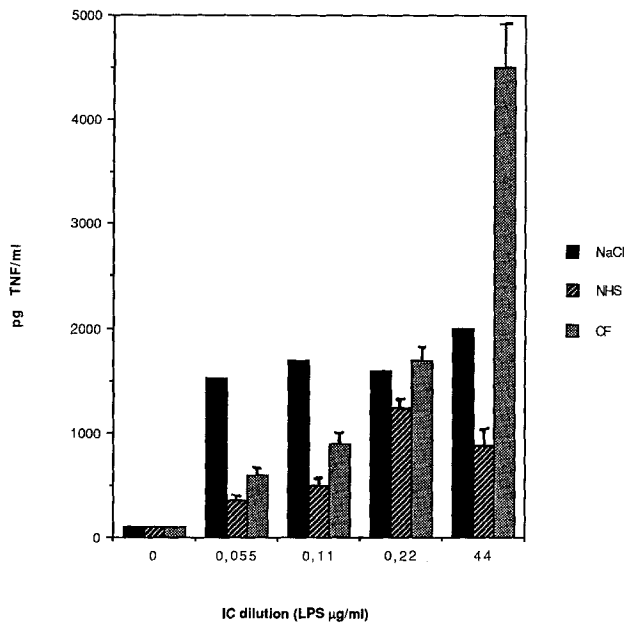
## Results

### *LPS-induced TNF response*

S-LPS from *P. aeruginosa* O:3 stimulated PBMC to release TNF. The TNF response was dose dependent, with increasing amounts of TNF released from  $2 \times 10^6$  PBMC/ml when the LPS concentrations were increased from 0.001 to 1.0 µg/ml. No further increase was seen above this concentration, and in most cell donors a decreasing TNF response were observed when 1.0 µg/ml of *P. aeruginosa* LPS was exceeded (Fig. 1). The amount of TNF released at a given LPS concentration varied within a wide range dependent on the cell donor (Fig. 1).

**Fig. 2. a** Induction of TNF by *P. aeruginosa* LPS and LPS preincubated with 20% serum from one representative cystic fibrosis (CF) patient, chronically infected with *P. aeruginosa*. **b** Induction of TNF by 44 µg/ml *P. aeruginosa* LPS with varying concentrations of serum from one representative CF patient, chronically infected with *P. aeruginosa*, and from one healthy control (NHS), and the varying concentrations of the two serum samples without any addition and preincubation with LPS. **c** TNF response induced by 44 µg/ml LPS from *P. aeruginosa* (*Pa*) and *S. abortus equi* (*S.ab.eq.*), the same amount of LPS preincubated with 20% serum from CF patients or 20% serum from healthy controls, and 20% serum without LPS. The TNF concentrations are median values from one experiment using sera from eight different CF patients and eight different healthy controls for individual incubations with the respective LPSs followed by cell stimulation. The ranges for the four serum + LPS groups were as follows: *Pa* LPS + NHS 940–3100, *Pa* LPS + CF 2400–10000, *S.ab.eq.* LPS + NHS 3200–9000, *S.ab.eq.* LPS + CF 3500–13600. Standard error of deviations are indicated

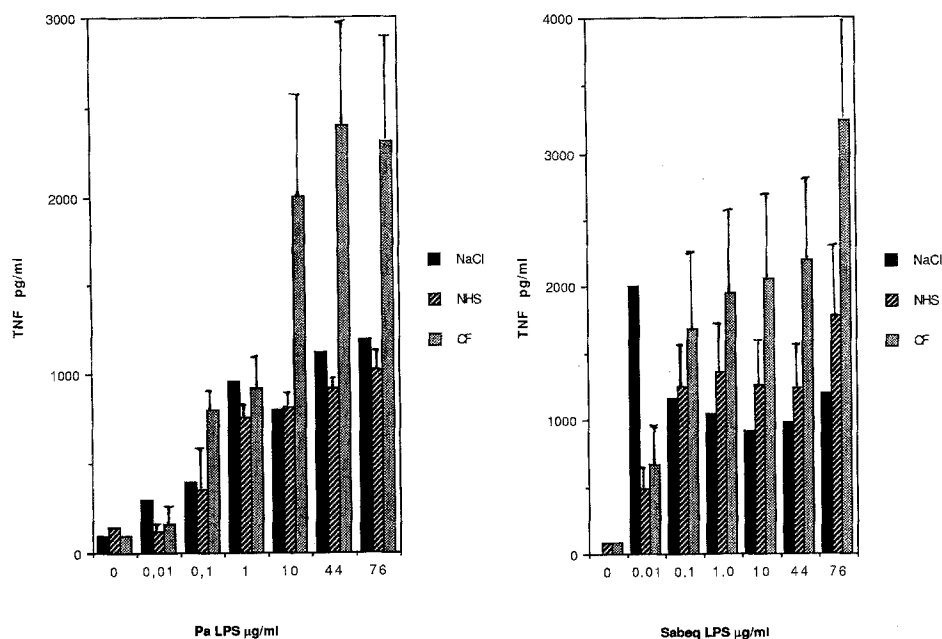




**Fig. 3.** TNF response induced by 44 µg/ml *P. aeruginosa* LPS preincubated with 20% serum from either CF patients or healthy controls. The immune complex (IC) samples were diluted  $\times 200$ ,  $\times 400$ , and  $\times 800$  and are expressed as the equivalent LPS concentration. The serum concentrations were in the dilutions 0.10%, 0.05%, and 0.025%, respectively. The TNF concentrations are median values from one experiment using sera from eight different CF patients and eight different healthy controls for individual incubations with the respective LPSs followed by cell stimulation. The ranges of the median values were as follows: 0.055 NHS 240–440, CF 440–920, 0.11 NHS 320–800, CF 560–1400, 0.22 NHS 440–1520, CF 1120–2000, 44 NHS 580–1180, CF 1400–5200. Standard error of deviations are indicated

#### "IC"-induced TNF response

For optimal IC formation we chose 44 µg/ml LPS and 20% serum since these concentrations were the point of equivalence using sera from CF patients and *P. aeruginosa* LPS in the turbidimetric method. When the LPS preparations were incubated with human serum and the mixture then used for cell stimulation the TNF response changed depending on the source of the LPS (*P. aeruginosa* versus *S. abortus equi*), the LPS concentration, the serum concentration, and whether the sera were from healthy controls or chronically infected patients with CF (Fig. 2). The eight CF sera and eight NHS used in the experiments did not induce any TNF secretion by themselves in the relevant concentrations (Fig. 2b). Using 44 µg/ml of LPS from *P. aeruginosa* together with CF sera potentiated the TNF response as compared to the same concentration of LPS without any preincubation with serum (Fig. 2a, c,  $P = 0.007$ , using Wilcoxon signed rank test). Increasing the concentration of CF serum while keeping the *P. aeruginosa* LPS concentration fixed induced increasing amounts of TNF (Fig. 2b). This was in contrast to the TNF response seen when PBMCs were stimulated with *P. aeruginosa* LPS and NHS together (Fig. 2b, c,  $P < 0.0011$  comparing the two groups of sera, using Mann-Whitney U



**Fig. 4.** TNF response induced by varying amounts of LPS from *P. aeruginosa* (*Pa*) and *S. abortus equi* (*Sabeq*), the same amounts of LPS preincubated with 20% serum from CF patients or healthy controls (NHS), and 20% serum without LPS (= LPS concentration 0). The TNF concentrations are median values from one experiment using sera from eight different CF patients and eight different healthy controls for individual incubations with the respective LPSs followed by cell stimulation. The ranges of the median values were as follows in the order of increasing LPS concentrations: Pa/NHS (0–200), (90–520), (440–2200), (1600–2600), (1680–3800), 1760–3900), (2000–3400). Pa/CF (480–3600), (1000–4800), (900–6000), (2100–10800), (2400–10000), (2200–8800). Sabeq/NHS (0–240), (192–920), (800–2500), (1000–2800), (940–2500), (1000–2900), (1200–4300). Sabeq/CF (90–1680), (200–3400), (480–4000), (820–4400), (800–4600), (900–5800). Standard error of deviations are indicated

test). The TNF response decreased with dilution of the ICs which was not due to a decrease in just *P. aeruginosa* LPS (Fig. 3), since the TNF response was not decreasing when diluting the *P. aeruginosa* LPS from 0.22 µg/ml to 0.11 µg/ml and 0.055 µg/ml in contrast to the decreasing TNF response when the CF serum/LPS mixture was diluted (Fig. 3). Applying LPS from *S. abortus equi* to the cells together with serum having low anti-*S. abortus equi* LPS titers induced a higher TNF response than LPS alone when more than 0.01 µg/ml LPS was used (Fig. 4), indicating a general potentiating effect of serum on LPS-induced TNF secretion, using high amounts of LPS. Increasing amounts of CF sera or NHS together with 44 µg/ml *S. abortus equi* LPS did not increase the TNF response above the level seen using 5% serum (not shown), probably because of lack of IC formation. In some of the experiments a difference in the TNF induction was seen between using either NHS or CF sera together with *S. abortus equi* LPS, but this difference did never reach significant levels (Figs. 2c, 4).

The TNF response induced by *P. aeruginosa* LPS mixed with the different sera showed an individual variation which depended on the serum and correlated

**Table 1.** Anti-LPS ELISA titers

	Anti- <i>P. aeruginosa</i> LPS			Anti- <i>S. abortus equi</i> LPS
	IgG	IgA	IgM	IgG
Healthy controls ( <i>n</i> = 8)				
median	80	32	352	1.7
(range)	(53–127)	(0–69)	(173–647)	(0–4.5)
CF patients ( <i>n</i> = 8)				
median	3200	1018	733	0.76
(range)	(1067–13332)	(333–1666)	(186–1173)	(0.22–5.4)

The differences in anti-*Pseudomonas aeruginosa* lipopolysaccharide (LPS) titers between the two groups were significant for all three classes of immunoglobulins, ( $P=0.0005$  for IgG and IgA,  $P=0.05$  for IgM, Mann-Whitney U test). There was no significant difference in IgG anti-*Salmonella abortus equi* LPS between the two groups

positively to the specific anti-*P. aeruginosa* LPS ELISA titers of the respective sera. The correlations were shown with IgG, IgA and IgM and were most pronounced with IgA anti-*P. aeruginosa* LPS (coefficients of correlation were: 0.56, 0.80, and 0.54, respectively, and the corresponding  $P$  values: 0.03, 0.002, and 0.04, using Spearman's correlation test). The IgG, IgA, and IgM anti-*P. aeruginosa* LPS titers, IgG anti-*S. abortus equi* LPS are given in Table 1.

In control experiments to investigate if the sera used together with LPS for cell stimulation had any effect on the TNF concentration after secretion from the PBMCs, supernatants from cell stimulation experiments were re-incubated with homologous sera, in the same concentration as in the primary experiment (20%), and re-incubated for another 18 h at 37°C. TNF recovery after re-incubation measured by ELISA varied from 81% to 114% demonstrating a lack of TNF inhibitory components in the sera.

## Discussion

Although the levels of anti-LPS antibodies are increased considerably during chronic *P. aeruginosa* infection the bacteria are not eradicated so that LPS antigen remains in the lung. IC formation by LPS and anti-LPS antibodies could modify the biological effects of LPS alone. LPS is a potent inducer of TNF and in this study we have investigated the effect of LPS together with hyperimmune anti-LPS sera on TNF induction from PBMCs. Our results showed a potentiating effect of hyperimmune serum from chronically infected CF patients on high concentrations (> 100–1000 ng/ml) of purified *P. aeruginosa* LPS induced TNF secretion from PBMCs. The results indicate that this effect could be due to IC formation between *P. aeruginosa* LPS and specific antibodies present in these sera. The IC-induced TNF response was significantly higher than the TNF response elicited by the same amount of *P. aeruginosa* LPS alone. The IC-induced cytokine release from PBMCs seen in these experiments is in agreement with earlier studies showing an induction of either interleukin-1 (IL-1) or TNF when PBMCs were stimulated with ICs [1, 6,



8, 33]. Arend et al. [1] showed that the IL-1 inducing capacity of ICs was dependent on the presence of complement. However, Remvig et al. [33] showed an IC-induced IL-1 production in PBMCs also without any complement in the system. In our experiments we used heat-inactivated sera and, thus, complement was not needed for the TNF induction by ICs. The effect of complement was not studied, but from recently published experiments by Heumann et al. [15] it is likely that addition of complement would further enhance the TNF secretion. Debets et al. [8] have shown that cross-linking of Fc receptors on human PBMCs by anti-human kappa-chain antibodies induced a rapid secretion of TNF, and that a TNF response was also induced by a standard IC preparation consisting of tetanus toxoid and human IgG anti-tetanus toxoid [8].

The considerable variation in LPS-induced TNF response among PBMC donors has been described before [3] and may be related to genetic HLA-DR2 variations [3]. However, the phenomenon of IC-induced TNF secretion was seen with all cell donors used. To minimize the variation due to this donor variation we, therefore, only compared results from experiments made with the same cell donor the same day.

LPS from *S. abortus equi* was chosen as a control since there was no difference in IgG anti-*S. abortus equi* LPS titers between the CF patients and the healthy controls. The inactivation of small amounts of *S. abortus equi* LPS (0.01 µg/ml) by serum from healthy controls described in this study supports previous studies, in which the LPS-induced IL-1 or TNF response was inhibited by normal human serum or purified serum proteins as lipoproteins, IgG or alpha globulins [9, 13, 38]. Interestingly, NHS seemed to have a slightly neutralizing effect on even high concentrations of *P. aeruginosa* LPS compared to that of *S. abortus equi* LPS [0.01–76 µg/ml of *P. aeruginosa* LPS compared with 0.01 µg/ml *S. abortus equi* LPS (Fig. 4)]. This is in agreement with the lower potency of *P. aeruginosa* LPS for TNF induction [13], which could be due to the slightly different structure of this LPS molecule compared to the *S. abortus equi* LPS [24]. *P. aeruginosa* LPS differs from enterobacterial LPS by having a large number of phosphate residues, by the presence of L-alanine in the core, by the occurrence of unusual sugars and amino compounds in the O chain [32, 35], and by having fewer and shorter fatty acid residues in the lipid A part arranged asymmetric on the glucosamine backbone [4, 10, 25].

We have previously determined *P. aeruginosa* LPS, IgG and IgA to be the most characteristic components of ICs isolated from sputum in patients with CF and chronic pulmonary infection [19, 21]. Our results suggest indirectly that ICs formed from LPS and CF anti-LPS antibodies are in fact more potent activators in terms of inducing a TNF response from PBMCs than *P. aeruginosa* LPS alone. The lack of TNF inhibiting effect of the specific anti-LPS antibodies in CF serum was further supported when we did not find any protective effect to lethality using the CF sera with *P. aeruginosa* LPS in a D-galactosamine-sensitized mouse model (unpublished data). Using serum as the antibody source in the IC formation could lead to binding of LPS by serum proteins other than immunoglobulins. Thus, lipoproteins and LPS-binding protein are serum proteins which are known to bind specifically to LPS [40, 44]. LPS bound to LPS-binding protein has been shown to activate PBMCs via the CD14 receptor for TNF production, and LPS-binding protein has been shown to be necessary for LPS-induced TNF production, but only when the LPS concentrations were below 0.01 µg/ml (37, 45). With higher concentrations of LPS the cell stimulation might occur via putative LPS receptors

[7, 14, 34]. Bactericidal/permeability-increasing protein is another specific LPS-binding protein, but it is mainly contained in neutrophil granules [26], and it has a neutralizing effect on the biological activity of LPS rather than potentiating. Our results indicate a potentiating effect of CF sera on TNF secretion from PBMCs induced by *P. aeruginosa* LPS at concentrations above 0.01 µg/ml, this could be due to IC formation between *P. aeruginosa* LPS and specific antibodies in the sera, followed by binding to Fc receptors on the cells and the induction of TNF secretion. Since no significant difference in TNF induction was seen between sera from CF patients and healthy controls together with *S. abortus equi* LPS a specific interaction between *P. aeruginosa* LPS and CF serum is indicated.

We have previously used the same IC preparations made from CF sera and *P. aeruginosa* LPS for stimulation of neutrophil granulocytes and showed an oxidative burst response induced by the ICs compared to no response when the cells were stimulated with *P. aeruginosa* LPS or CF serum alone [23]. It is interesting that the interaction of anti-LPS antibodies apparently augments the potential of these endotoxins to induce TNF in vitro. However, other endotoxic effects may be inhibited in the circulation and the molecules are finally opsonized by antibodies and complement and cleared by the reticuloendothelial system in vivo, depending on antibody specificity, isotype, and host species. This is in agreement with our previous results from sputum and serum of CF patients with chronic *P. aeruginosa* infection, where we showed a high local TNF concentration in the patients with LPS ICs in the lungs, whereas no systemic TNF was detected in these patients [21].

In summary, our results are in agreement with the hypothesis, drawn from these experiments, that ICs stimulates TNF secretion in PBMCs via Fc receptors (which do not respond to LPS alone) independent on complement, and, thereby, may contribute to the lung tissue damage seen in chronic *P. aeruginosa*-infected patients with CF. However, the results do not give any explanation of the non-detectable levels of TNF in the serum samples from our group of chronically infected CF patients, and the extrapolation from our in vitro experiments to the in vivo situation of the CF lung disease can only be related to the possible mechanism of IC-induced inflammation.

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