

## Phospholipase A2 Is a Circulating Mediator in Typhoid Fever

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Circulating proinflammatory mediators have not been found in studies on typhoid fever, although the patients suffer from a systemic disease with characteristic protracted fever. The 14-kDa group II extracellular phospholipase A2 (PLA2) is induced by interleukin-1 and tumor necrosis factor and may mediate some of the effects of these cytokines. Circulating PLA2 concentrations were measured in 12 typhoid fever patients on various days after admission and after recovery. On admission, mean concentrations of PLA2 were elevated ( $1444 \pm 1560$  ng/mL) and decreased gradually and significantly to day 14 ( $55 \pm 48$  ng/mL). Patients with complicated disease had significantly higher PLA2 levels on admission. PLA2 was not produced in a lipopolysaccharide-stimulated whole blood culture, indicating that PLA2 originates from other types of cells. These data indicate that PLA2 may be a mediator of disease in protracted inflammatory diseases such as typhoid fever.

Despite the presence of protracted fever and other generalized signs of illness in typhoid fever, several studies have failed to demonstrate circulating proinflammatory mediators in these patients. In a previous report, we presented evidence for cytokine activation in terms of raised interleukin (IL)-1 receptor antagonist (IL-1RA) and soluble (s) tumor necrosis factor (TNF) receptors (sTNF-R) in patients with typhoid fever but no apparent rise in circulating concentrations of proinflammatory cytokines IL-1 $\beta$ , TNF- $\alpha$ , and IL-6 [1]. This is in agreement with other observations in patients with typhoid fever in Nepal and Indonesia [2, 3], although one study revealed elevated cytokine levels in a minority of patients [4].

Phospholipases are lipolytic enzymes that catalyze the degradation of phospholipids. To date, three varieties of phospholipase A2 (PLA2) have been characterized: group I (pancreatic) and group II (nonpancreatic) 14-kDa PLA2 and a cytosolic (85-kDa) PLA2. The group II PLA2 occurs in and is secreted by a variety of cells and has been implicated in the generalized inflammatory responses in several experimental models and clinical syndromes, such as sepsis and adult respiratory distress syndrome (ARDS) [5]. Its release is induced by IL-1 and TNF- $\alpha$ , and the enzyme mediates the production of arachidonic acid [6, 7]. Some of the metabolic effects of these cytokines during infection may therefore be mediated by PLA2, potentially giving this enzyme a central role in inflammation [8]. The absence

of sizable proinflammatory cytokinemia in typhoid fever made us consider extracellular PLA2 a possible circulating mediator of the systemic response. Thus, we studied the relation of its presence to cytokine responses and severity of disease.

### Patients and Methods

The study was conducted in Dr. Kariadi Hospital, Diponegoro University, from December 1990 onward. Patient selection and treatment has been described elsewhere [1]. In brief, the diagnosis of typhoid fever was confirmed by positive blood or bone marrow cultures in all patients, and treatment was with chloramphenicol. Most patients were discharged 7–10 days after defervescence, which was the definition of convalescence. None of the patients died.

*Measurements of cytokines and other mediators.* On admission, on days 2, 4, and 6, and after recovery (day 14), blood was drawn for cytokine, endotoxin, and PLA2 measurements. Circulating concentrations and ex vivo production of cytokines were determined as described earlier [1, 9]. Endotoxin was measured in platelet-rich plasma by a chromogenic limulus amoebocyte lysate assay (Kabi Vitrum, Stockholm). TNF- $\alpha$  was measured by RIA as described [10] (detection level, 30 pg/mL). This RIA detects both free TNF- $\alpha$  and TNF- $\alpha$  bound to its soluble receptors. IL-1 $\beta$  was measured by RIA according to Lisi et al. [11] but without chloroform extraction (detection level, 30 pg/mL). IL-1RA was determined by RIA as described by Poutsika et al. [12] (detection level, 300 pg/mL). sTNF-Rs were measured by an enzyme-linked immunobinding assay (ELIBA; Hoffmann-La Roche, Basel, Switzerland; detection level, 80 pg/mL for p55 and 300 pg/mL for p75).

In a group of healthy controls, normal values for our laboratory were as follows: TNF- $\alpha$ , circulating concentrations and ex vivo production without lipopolysaccharide (LPS),  $106 \pm 25$  pg/mL, and ex vivo production after 24 h of stimulation with LPS,  $3780 \pm 950$  pg/mL; IL-1 $\beta$ , circulating concentrations and ex vivo production without LPS, below detection limit, and ex vivo production after 24 h of stimulation with LPS,  $6930 \pm 3160$  pg/mL; IL-1RA, circulating concentrations and ex vivo production without LPS,

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below detection limit, and ex vivo production after 24 h of stimulation with LPS,  $5757 \pm 1060$  pg/mL; sTNF-R, circulating concentrations, 1.50 ng/mL (p55) and 2.51 ng/mL (p75).

**PLA2 measurements.** PLA2 activity was assayed with 0.2 mM 1-acyl-2-(1-C14)-linoleoylphosphatidylethanolamine (specific activity, 1000 dpm/nmol) in 0.1 M TRIS-HCl (pH 8.5) containing 10 mM CaCl<sub>2</sub> and 0.05% Triton X-100. After incubation for 30 min at 37°C, the [<sup>14</sup>C]linoleate released was extracted by a modified Dole extraction procedure [13]. The radioactive substrate was prepared biosynthetically as described previously [13].

Immunoreactive group II PLA2 was determined with an ELISA modified from that described by Smith et al. [14]. Two monoclonal antibodies to human group II PLA2 (provided by F. B. Taylor, Jr., Oklahoma Medical Research Foundation, Oklahoma City) were used as coating and catching antibodies. Results were compared with those obtained with culture medium from HEp-G2 cells stimulated with human IL-6. The amount of group II PLA2 in this culture medium was assessed by comparison with purified recombinant human group II PLA2. The lower limit of detection was 1 ng/mL, and mean normal plasma values in 19 healthy volunteers were  $20 \pm 7$  ng/mL (range, 9–30).

**Statistics.** All samples from the same patient were analyzed in the same run in duplicate to minimize analytical errors. Values are expressed as mean  $\pm$  SD unless otherwise indicated. When frequency distribution was parametric, paired and unpaired Student's *t* tests were used. If not, the Wilcoxon signed rank test or Mann-Whitney *U* test was used. Analysis of variance was done when considered suitable.  $P < .05$  was considered significant.

## Results

Of 12 patients studied, 4 had a complicated course of disease (3 pneumonia, 1 delirium). The 9 female and 3 male (3:1) patients had a mean age of 16 years (range, 14–53). There was no difference in white blood cell count, female-to-male ratio, or hemoglobin level between patients with a complicated or uncomplicated disease course. No circulating endotoxin was found in any of the samples.

**Circulating PLA2 concentrations.** On admission, mean concentrations of immunoreactive PLA2 were elevated ( $1444 \pm 1560$  ng/mL) and decreased gradually and significantly to day 14 ( $55 \pm 48$  ng/mL; figure 1). Patients with complicated disease had significantly higher mean PLA2 values on admission ( $2520 \pm 1284$  ng/mL) than did patients with uncomplicated disease ( $235 \pm 206$  ng/mL;  $P < .001$ ). Immunoreactive PLA2 concentrations correlated well with PLA2 found in the bioassay ( $r = .894$ ).

**Ex vivo production of PLA2.** Unstimulated whole blood cultures showed PLA2 concentrations virtually equal to circulating concentrations. After incubation with LPS for 24 h, concentrations of enzyme in the supernatants did not change, indicating that no additional PLA2 had been produced by peripheral blood cells. Cells incubated with LPS after removal of plasma did not produce PLA2 either (data not shown).

**Circulating cytokines and inhibitors.** On admission, the mean concentrations of circulating proinflammatory cytokines

were as follows: IL-1 $\beta$ ,  $95 \pm 24$  pg/mL; TNF- $\alpha$ ,  $183 \pm 56$  pg/mL. IL-1 $\beta$  concentrations remained low; TNF- $\alpha$  concentrations decreased gradually but significantly to  $120 \pm 53$  by day 14 ( $P < .05$ ). There was no difference in circulating concentrations of TNF- $\alpha$  on admission between patients with complicated disease and those with uncomplicated disease.

Relatively high concentrations of circulating inhibitors were found on admission and also decreased gradually but significantly during the hospital stay. The mean concentration of IL-1RA decreased from  $1329 \pm 498$  pg/mL on day 0 to  $318 \pm 129$  pg/mL on day 14 ( $P < .001$ ), sTNF-R (p55) from  $7875 \pm 2733$  pg/mL on day 0 to  $2848 \pm 578$  pg/mL on day 14 ( $P < .001$ ), and sTNF-R (p75) from  $27,395 \pm 9796$  pg/mL on day 0 to  $7938 \pm 2138$  pg/mL on day 14 ( $P < .001$ ). Patients with complicated disease had significantly higher concentrations of inhibitors on admission than did patients with uncomplicated disease course ( $P < .05$ ; table 1).

**Ex vivo production of cytokines and inhibitors.** Similar to what we reported earlier [1], the production capacity of proinflammatory cytokines was significantly suppressed during the acute phase of typhoid fever. In these sequential samples, the production returned to normal after day 4 for TNF- $\alpha$  and after day 6 for IL-1 $\beta$  (data not shown). Throughout the period of illness, the LPS-stimulated production capacity of IL-1RA was preserved (mean, 9483 pg/mL) and did not change.

## Discussion

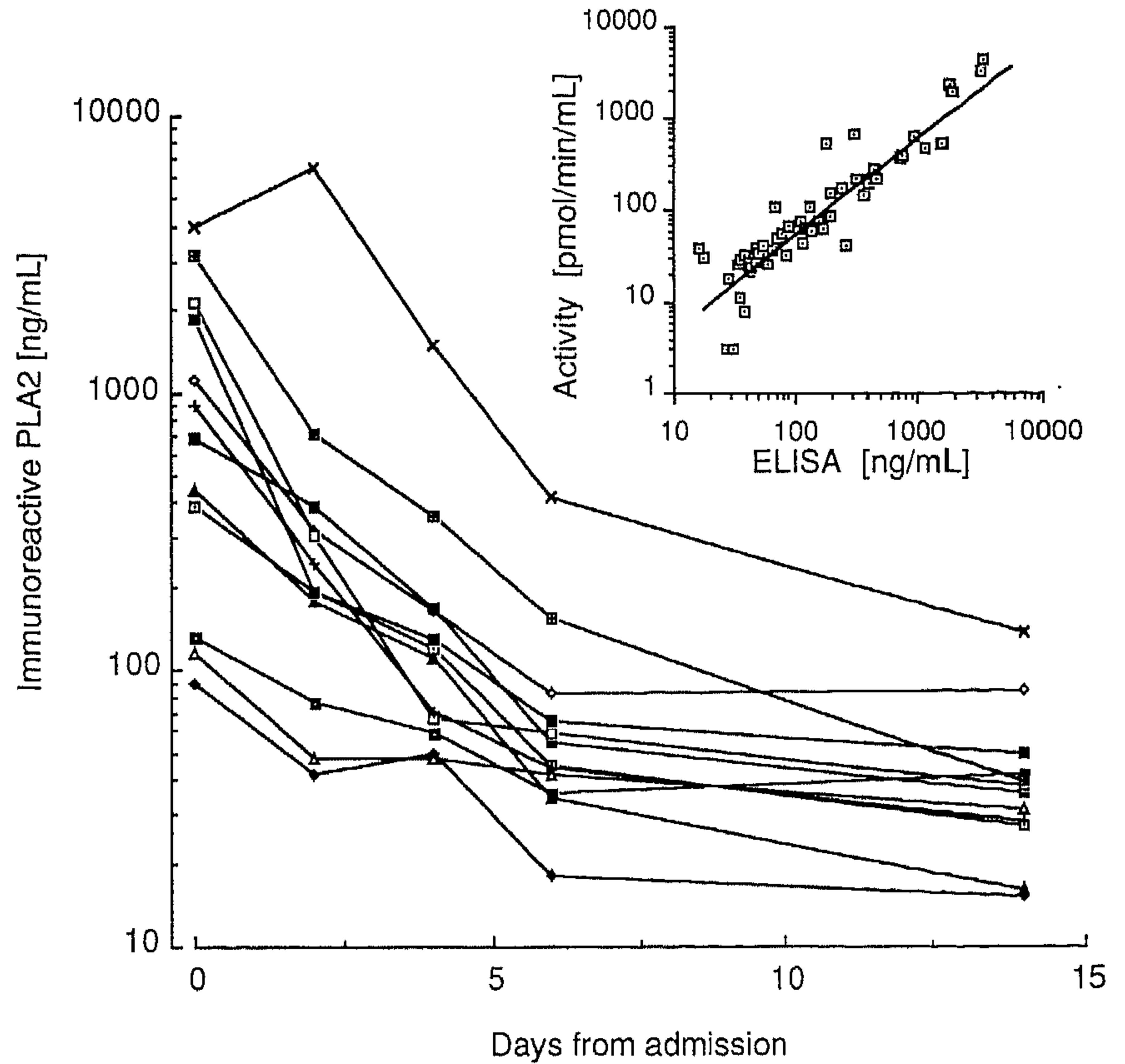
In the 12 patients with proven typhoid fever in the present study, extracellular group II PLA2 circulated in high concentrations during the febrile phase of the infection. PLA2 concentrations were  $\sim$ 10-fold higher in patients with complicated disease. During the course of disease, PLA2 concentrations fell significantly. PLA2 detected by immunoassay was bioactive. The relatively low or undetectable concentrations of proinflammatory cytokines in typhoid fever [1–3] suggest that this bioactive PLA2 is responsible for at least some of the systemic signs in typhoid fever.

Although circulating concentrations of TNF- $\alpha$  in the present series were slightly albeit significantly elevated in the acute phase compared with convalescence, it should be stressed that these concentrations probably reflect mainly the TNF- $\alpha$  bound to soluble receptors, as extremely high concentrations of sTNF-Rs were found on admission.

Support for a role for PLA2 as a mediator of the systemic inflammatory response comes from studies in rabbits, in which PLA2 induced a fall in blood pressure similar to that found with endotoxin infusion and PLA2 inhibitor *p*-bromophenacyl bromide protected against this hypotensive effect [5].

The results of our ex vivo production assay suggest that PLA2 is not being produced by circulating cells but rather outside the bloodstream (e.g., in the liver or endothelial cells). As TNF- $\alpha$  and IL-1 $\beta$  are proximal signals for PLA2 [6, 7], it is conceivable that in typhoid fever, proinflammatory cytokines

**Figure 1.** Circulating concentrations of immunoreactive PLA2 in 12 patients with typhoid fever. Inset: correlation between bioactive and immunoreactive PLA2 in patients with typhoid fever.



are first produced. Production of these cytokines may be confined to the site of infection in the tissues, rather than in the circulation. PLA2 locally induced by these cytokines may reach the circulation and mediate the systemic signs of the disease [6, 15]. This concept of PLA2 as a circulating mediator could explain why circulating cytokines are not found in clinical syndromes that are thought to be cytokine-mediated and that can easily be mimicked by injection of cytokines.

Important issues remain to be investigated. For example, it is not clear how intravascular release of PLA2 is counterregulated, nor has the role of group II PLA2 in the production of inflammatory arachidonate derivatives been satisfactorily defined, especially in view of the lack of specificity of group

II PLA2 for arachidonate. Moreover, the role of other secreted (group I) PLA2 as a mediator of inflammatory processes has to be elucidated.

Our study suggests that PLA2 is a crucial mediator in a protracted inflammatory disease such as typhoid fever. Inhibition of PLA2 may provide a new therapeutic intervention for febrile diseases mediated by this enzyme.

**Table 1.** Circulating concentrations of antiinflammatory mediators in the acute phase of typhoid fever in patients with complicated or uncomplicated disease.

Inhibitor	Complicated (n = 4)	Uncomplicated (n = 8)	P
IL-1RA	1825 ± 484	1081 ± 280	<.01
sTNF-R55	10,380 ± 2345	6621 ± 1712	<.01
sTNF-R75	34,813 ± 10,108	23,656 ± 6775	<.05

NOTE. Data are pg/mL, mean ± SD. IL-1RA, interleukin-1 receptor agonist; sTNF-R, soluble tumor necrosis factor receptor.

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## Coagulation of Whole Blood Stimulates Interleukin-1 $\beta$ Gene Expression

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To study interleukin (IL)-1 $\beta$  gene expression, reverse transcription-polymerase chain reaction was used on 25- $\mu$ L whole blood samples from 11 healthy subjects. Coagulated and unclotted whole blood was compared. There was no evidence of IL-1 $\beta$  gene expression in any time zero samples (i.e., whole blood from which mRNA was immediately extracted) from 11 subjects, whereas a 388-bp band representing IL-1 $\beta$  mRNA was detected in all coagulated samples. No mRNA for IL-1 $\beta$  was detected in EDTA-anticoagulated whole blood, although in these samples the addition of lipopolysaccharide as a positive control induced the expression of IL-1 $\beta$ . In time course studies on samples allowed to clot, mRNA for IL-1 $\beta$  was detectable after 30 min. These findings demonstrate that IL-1 $\beta$  gene expression is not present in circulating cells of healthy subjects and that coagulation is a stimulus for IL-1 $\beta$  gene expression. This may be a mechanism by which thrombosis produces inflammation and fever.

Interleukin-1 (IL-1) has multiple biologic activities that suggest a role in a variety of infectious and inflammatory diseases. Recent studies provide convincing evidence that IL-1 mediates some diseases [1]. The IL-1 receptor antagonist, a naturally occurring specific inhibitor of IL-1, reduces the severity of disease in various animal models as well as in humans with rheumatoid arthritis, sepsis, and graft-versus-host disease [1, 2]. Therefore, studies on the expression of IL-1 genes in human disease should provide valuable information on the pathophysi-

ology or progress of a variety of diseases involving inflammation. However, current methods of studying IL-1 $\beta$  gene expression use isolated peripheral blood mononuclear cells (PBMC), which is problematic in that preparation of these cells or their contact with plastic or glass results in stimulation of IL-1 $\beta$  genes in cells from healthy subjects in the absence of a microbial stimulus [3]. Furthermore, there are conflicting data as to whether IL-1 $\beta$  is expressed in circulating cells in healthy persons. Recent studies suggest that IL-1 $\beta$  is not constitutively produced in healthy humans. Therefore, to resolve the issue of constitutive production of IL-1 $\beta$  and to assess the possibility of artifacts related to assessing IL-1 $\beta$  gene expression in disease, we developed and validated a rapid method for assessing constitutive and inducible IL-1 $\beta$  gene expression using 25- $\mu$ L samples of whole blood not exposed to typical preparative procedures.

### Materials and Methods

*Subjects.* Healthy men and women aged 23–33 years donated blood. No dietary restrictions or requirements for fasting were

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