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Interleukin-1 β (IL-1 β), IL-1 receptor antagonist, and TNF α production in whole blood

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Abstract: The ability of an individual to mount defense responses to infection depend in part on the capacity to produce cytokines such as interleukin 1 (IL-1) and tumor necrosis factor (TNF). The specialized equipment, labor intensity, and sterile practice required for the standard in vitro evaluation of cytokine production can make such evaluation impractical in some clinical situations. We report a method for stimulating whole blood to produce cytokines that can be implemented in laboratories without tissue culture facilities and requires minimal sample preparation. IL-1 β and TNF α production in whole blood samples was stimulated with endotoxin and/or phytohemagglutinin in standard EDTA-containing vacuum collection tubes. After incubation, plasma was removed and frozen for later assay. Comparison of this whole blood method with isolated mononuclear cell cultures indicated a significant correlation for IL-1 β production ($r = 0.746$, $P = 0.005$). This technique also produced the newly described cytokine, IL-1 receptor antagonist. We conclude that the whole blood method is an acceptable alternative to isolated cell culture methods for measuring IL-1 β in situations that preclude the standard in vitro approach. *J. Leukoc. Biol.* 52: 687-692; 1992.

Key Words: interleukin-1 • tumor necrosis factor • interleukin-1 receptor antagonist • whole blood assay • cytokine

INTRODUCTION

The purpose of this investigation was to develop and standardize a practical method for measuring cytokine production by blood leukocytes from human subjects in situations that make the standard in vitro methods impractical, such as intensive care units or delivery rooms where samples need to be taken unexpectedly at any time of the day or night. The cytokines interleukin-1 α (IL-1 α), IL-1 β , and tumor necrosis factor α (TNF α) are produced by monocytes and other cells in response to substances such as endotoxin [1-3]. These cytokines mediate acute phase and inflammatory host responses that are thought to enhance host defense during infection. For example, pretreatment of mice with IL-1 β increased survival time after administration of lethal doses of *Pseudomonas aeruginosa* [4]. However, because IL-1 β and TNF α mediate degradation of cartilage and bone, inappropriate production of these factors has been implicated in degenerative diseases such as rheumatoid arthritis [5]. Elevated levels of circulating cytokines have been reported in patients with septic shock [1], meningococcal disease [6], and other diseases associated with fever, muscle wasting, hypotension, and disseminated intravascular coagulation. These

clinical signs can be induced by administration of cytokines to laboratory animals [7]. Furthermore, nutritional factors such as protein malnutrition [8] and dietary fatty acid intake [9] can influence the ability of the host to produce cytokines.

The naturally occurring, specific inhibitor of IL-1 [10, 11] has recently been cloned [12, 13] and renamed the IL-1 receptor antagonist (IL-1ra) [reviewed in refs 14, 15]. This member of the IL-1 family specifically blocks IL-1 receptors without triggering signal transduction. Administration of IL-1ra reduces the severity of shock and inflammation in a variety of animal models and is presently in clinical trials. The balance of production of IL-1 versus IL-1ra may be an important determinant in the outcome of several diseases where IL-1 is thought to have a role. Therefore, measuring the relative amounts of IL-1 and IL-1ra production is an important aspect of cytokine determinations from circulating leukocytes.

Human cytokine production is usually studied in vitro. In general, peripheral blood mononuclear cells (PBMCs) are separated by Ficoll-Hypaque gradient centrifugation, washed, counted, and incubated with lectins or bacterial toxins in an environment of controlled temperature, humidity, and CO₂ content. The process is laborious, requiring special attention to the preparation of reagents and culture vessels to prevent contamination by bacterial endotoxin. Blood volumes in excess of 10 ml are usually necessary to ensure sufficient cell yield, a requirement that can be prohibitive for some pediatric studies. This study examines the parameters of a whole blood stimulation procedure that does not require specialized equipment, has a low risk of contamination, and can be performed with 3 ml of blood.

MATERIALS AND METHODS

Whole Blood Cytokine Stimulation

Venous blood samples were collected from healthy male and female subjects between the ages of 20 and 40 years. Blood was collected aseptically into pyrogen-free syringes and transferred into previously prepared 3 cc-draw sterile vacuum tubes (Becton-Dickinson, Rutherford, NJ) contain-

Abbreviations: IL-1 α , interleukin-1 α ; TNF α , tumor necrosis factor α ; IL-1ra, IL-1 receptor antagonist; PBMCs, peripheral blood mononuclear cells; LPS, lipopolysaccharide *Escherichia coli* 055:B5; PHA, phytohemagglutinin P; ANOVA, analysis of variance.

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ing lyophilized 4.5 mg EDTA(Na_2) and 2.0 trypsin inhibitory units sterile aprotonin (0.67 TIU/ml final concentration, Sigma, St. Louis, MO). Control samples contained 100 μl endotoxin-free 0.154 M sodium chloride (saline) solution and stimulated samples contained 100 μl of appropriate stimulant. Some tubes had two stimulants added, i.e., endotoxin [lipopolysaccharide *Escherichia coli* 055:B5 (LPS) at 1, 10, or 100 ng/ml, Sigma, St. Louis, MO] and concanavalin A (Con A, 5 or 50 $\mu\text{g/ml}$, Sigma, St. Louis, MO) or phytohemagglutinin P (PHA, 3 or 30 $\mu\text{g/ml}$, Difco Laboratories, Detroit, MI). Stimulants were added through the alcohol-swabbed rubber stopper of the vacuum tube with a 27 gauge needle attached to a 1 cc tuberculin syringe containing the stimulant solution. Tubes were gently inverted once to mix and then incubated for up to 24 h at 37°C. In pilot studies, cytokine (IL-1 β) yield during stationary incubation or incubation on a rocking platform was compared.

After incubation, the tubes were centrifuged at 1000 $\times g$ for 30 min at 4°C. The rubber stoppers were removed and the resulting platelet-poor plasma was pipetted into sterile 1.5 ml microcentrifuge tubes. Samples were stored at -70°C until cytokine analysis was performed. To determine appropriate temperature of incubation, tubes containing whole blood were incubated in water baths or incubators at 17, 24, 27, 30, 34, 37, and 40°C.

Mononuclear cell viability in whole blood was assessed after 24 h of incubation. The cells were then separated by Ficoll-Hypaque isolation (see below), washed twice with saline, then resuspended in saline with 0.05% trypan blue. Viability, determined per 200 cells, was >90%.

For each subject, additional blood was collected into 3 ml draw EDTA vacuum tubes and analyzed for complete blood count and differential white blood cell count by the Clinical Hematology Laboratory at New England Medical Center Hospital in order to normalize cytokine production per 10^6 mononuclear cells (as presented in Table 1).

PBMC in vitro Stimulation

Standard in vitro cultures were prepared by established methods [16]. PBMCs were isolated from heparinized blood by Ficoll (Sigma, St. Louis, MO)-Hypaque (Winthrop Pharmaceutical, New York, NY) density gradient centrifugation and washed twice in saline. Mononuclear cells were suspended at a concentration of 5×10^6 cells/ml in ultrafiltered [17] RPMI-1640 culture medium (Whittaker M.A. Bioproducts, Walkersville, MD) supplemented with 2 mM glutamine, 100 U/ml of penicillin, 100 $\mu\text{g/ml}$ streptomycin, and 2% heat-inactivated human AB serum. Aliquots of 500 μl (2.5×10^6 cells) were added to 24-well, flat-bottomed microtiter plates. An equal volume of serum-free RPMI with or without LPS (10 ng/ml) and PHA (30 $\mu\text{g/ml}$) was added. The cells were incubated at 37°C for 24 h in a humidified, 5% CO_2 atmosphere. Supernatants were aspirated and centrifuged, then cell-free supernatants were frozen at -70°C until further analysis.

Cytokine assays

Plasma samples were analyzed by radioimmunoassay for IL-1 β [2,18,19], TNF α [20], and IL-1ra [21]. Plasma samples intended for IL-1 β assay were first extracted with chloroform [2]. Sensitivities of the radioimmunoassays for each cytokine were as follows: IL-1 β , 80 pg/ml; TNF α , 80 pg/ml; and IL-1ra, 156 pg/ml.

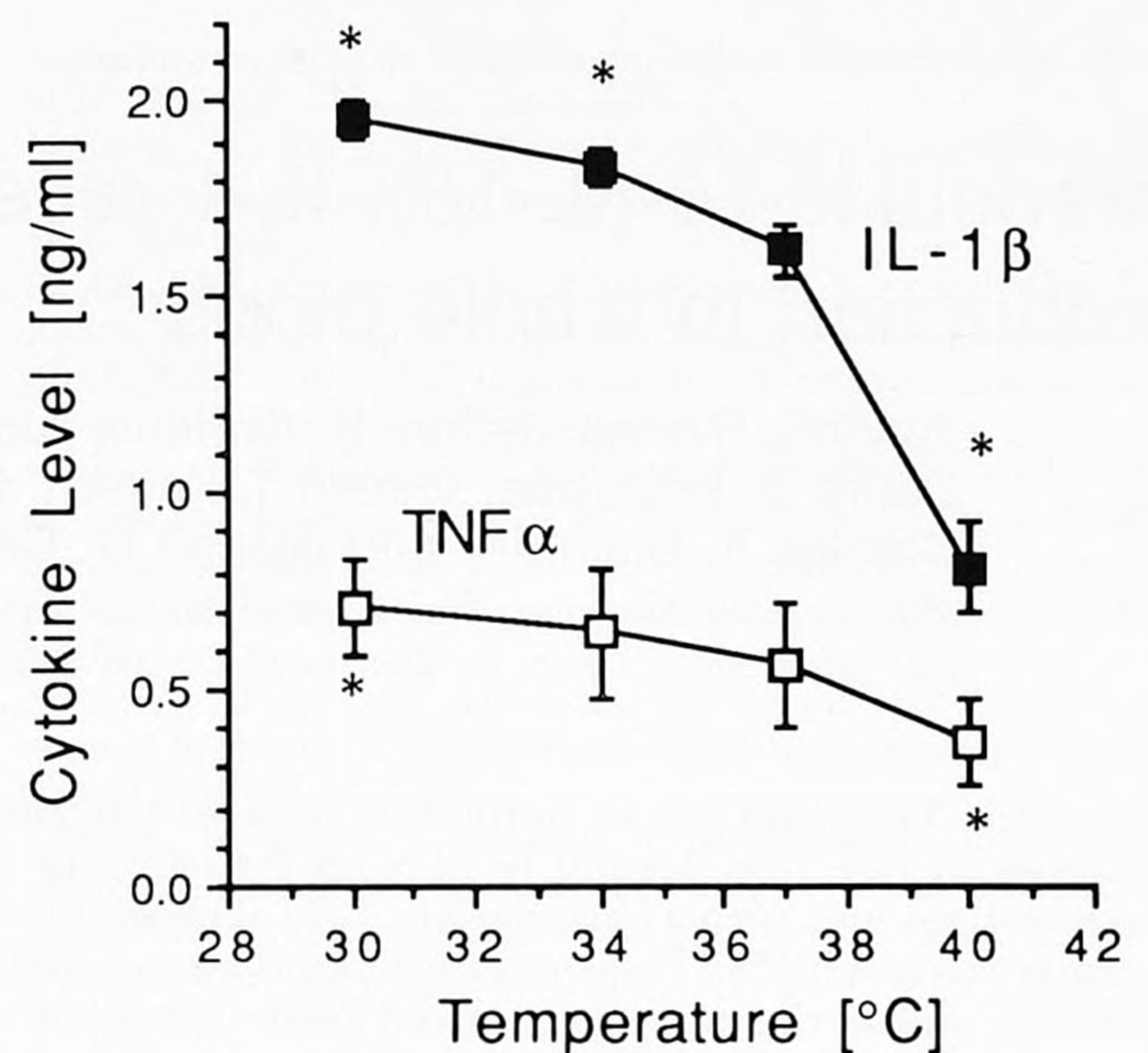


Fig. 1. IL-1 β and TNF α production by the whole blood method at different temperatures of incubation for 24 h. Whole blood was stimulated by LPS (10 ng/ml). The data represent the mean \pm SE of five subjects. Asterisk indicates significantly different from 37°C ($P < 0.05$ by ANOVA).

Statistics

Differences between means were analyzed using one-way and two-way analysis of variance (ANOVA). Pearson product-moment analysis was used to determine the correlation of coefficients using StatView software (Abacus Concepts, Inc., Calabasas CA) on a Macintosh SE/30 computer. Data were transformed logarithmically before ANOVA. Values are reported as means with standard error of the mean (SE).

RESULTS

Temperature Dependence

Incubation in the sealed vacuum-collection tubes eliminated the requirement of a humidified, 5% CO_2 atmosphere. To investigate the minimum equipment requirements necessary to obtain consistent results, we determined whether a controlled temperature was necessary. There was no IL-1 β or TNF α production detectable at 17°C. Whole blood cytokine production increased as temperature increased to 27–30°C. Over the physiological temperature range (30 to 40°C), maximal production of IL-1 β and TNF α after LPS stimulation of whole blood was observed at 30°C (skin temperature) (Fig. 1). Cytokine production was attenuated at febrile temperatures (40°C) as has been shown previously in PBMC cultures [22]. Because of the significant effect of temperature on IL-1 β and TNF α production, it was clear that the minimum equipment requirement included a constant-temperature water bath or air chamber. All subsequent whole blood studies were carried out at 37°C, nominal body core temperature in health.

Additionally, there was no difference in IL-1 β production between tubes that were rocking for 24 h compared with tubes incubated in a stationary position (data not shown).

Dose-Response to Single and Multiple Stimuli

LPS, over a range of 1 to 100 ng/ml, stimulated significant dose-related increases in the production of IL-1 β and TNF α

in whole blood ($P = 0.021$ and 0.017 , respectively). PHA induced production of both cytokines when used alone at a concentration of $30 \mu\text{g/ml}$. In conjunction with LPS, PHA tended to increase cytokine production over the levels induced by LPS alone, but the increases were statistically significant only for IL- 1β ($P = 0.003$). In this experiment, Con A (5 and $50 \mu\text{g/ml}$) was not a consistent stimulus for cytokine production, either alone or in combination with LPS (data not shown).

TABLE 1. Comparison of Cytokine Concentration (ng/ml per 10^6 cells) in Isolated Mononuclear Cell Cultures and in Whole Blood Preparations^a

Method	IL- 1β	TNF α
In vitro	3.78 ± 0.76	8.08 ± 1.08
Whole blood	$2.02 \pm 0.30^{b,c}$	0.22 ± 0.04^b

^aBoth isolated PBMCs (in vitro method) and stimulated whole blood were incubated for 24 h at 37°C . Samples from 12 subjects were tested simultaneously by each method.

^bSignificantly lower than in vitro method ($P < 0.01$).

^cIndicates significant correlation with in vitro method ($r = 0.746$, $P = 0.005$).

Comparison of Whole Blood and in vitro Methods

Blood samples from 12 subjects were evaluated simultaneously by the whole blood method and the in vitro method of isolated mononuclear cells. A combined stimulus of LPS (10 ng/ml) and PHA ($30 \mu\text{g/ml}$) was used because IL- 1β production was essentially maximal at these concentrations, as shown in **Figure 2**. To facilitate comparisons, the data in **Table 1** were normalized per 10^6 mononuclear cells. Mean IL- 1β concentrations measured in the whole blood method were about half of the concentrations determined by the in vitro method, but the relative intersubject amounts produced were maintained, i.e., whole blood IL- 1β measurements correlated with the results obtained by the in vitro method ($r = 0.746$, $P < 0.005$) (**Table 1**). TNF α concentrations measured in the whole blood system were much less than the concentrations measured in the in vitro system ($<15\%$). Furthermore, there was no relationship between whole blood concentrations and in vitro concentrations for TNF α ($r = 0.147$, $P > 0.2$).

The concentrations of TNF α in whole blood samples were inversely related to the neutrophil count ($r = -0.677$, $P = 0.016$). IL- 1β concentrations did not correlate with TNF α concentrations in either the whole blood or in vitro assay methods, consistent with previous results [23].

Time Course of Cytokine Production in the Whole Blood Method

Whole blood from 5 donors was stimulated with LPS (10 ng/ml) and PHA ($30 \mu\text{g/ml}$) and incubated from 1 to 24 h at 37°C (**Fig. 3**). TNF α and IL- 1β were detectable after 2 and 4 h of incubation respectively. IL- 1β reached a maximum of $5.8 \pm 2.3 \text{ ng/ml}$ after 8 h and TNF α a maximum of ($0.7 \pm 0.1 \text{ ng/ml}$) after 6 h of incubation. There was no significant difference between levels of IL- 1β after 8 or 24 h of incubation, but TNF α levels were significantly lower after 24 hours of incubation compared to 6 h ($P < 0.05$).

The capability to measure the newly described cytokine, IL-1 receptor antagonist (IL-1ra), prompted us to measure IL-1ra in this experiment as well. Whole blood was stimulated with LPS and PHA (as above). IL-1ra was detectable in plasma after 3 hours of incubation and reached maximal concentrations of $11.8 \pm 1.4 \text{ ng/ml}$ after 10 hours (**Fig. 3**).

DISCUSSION

The purpose of this investigation was to describe a simple, practical method for measuring cytokine production from human cells. The method we have characterized uses a minimum of equipment, requires less than 3 ml of blood per

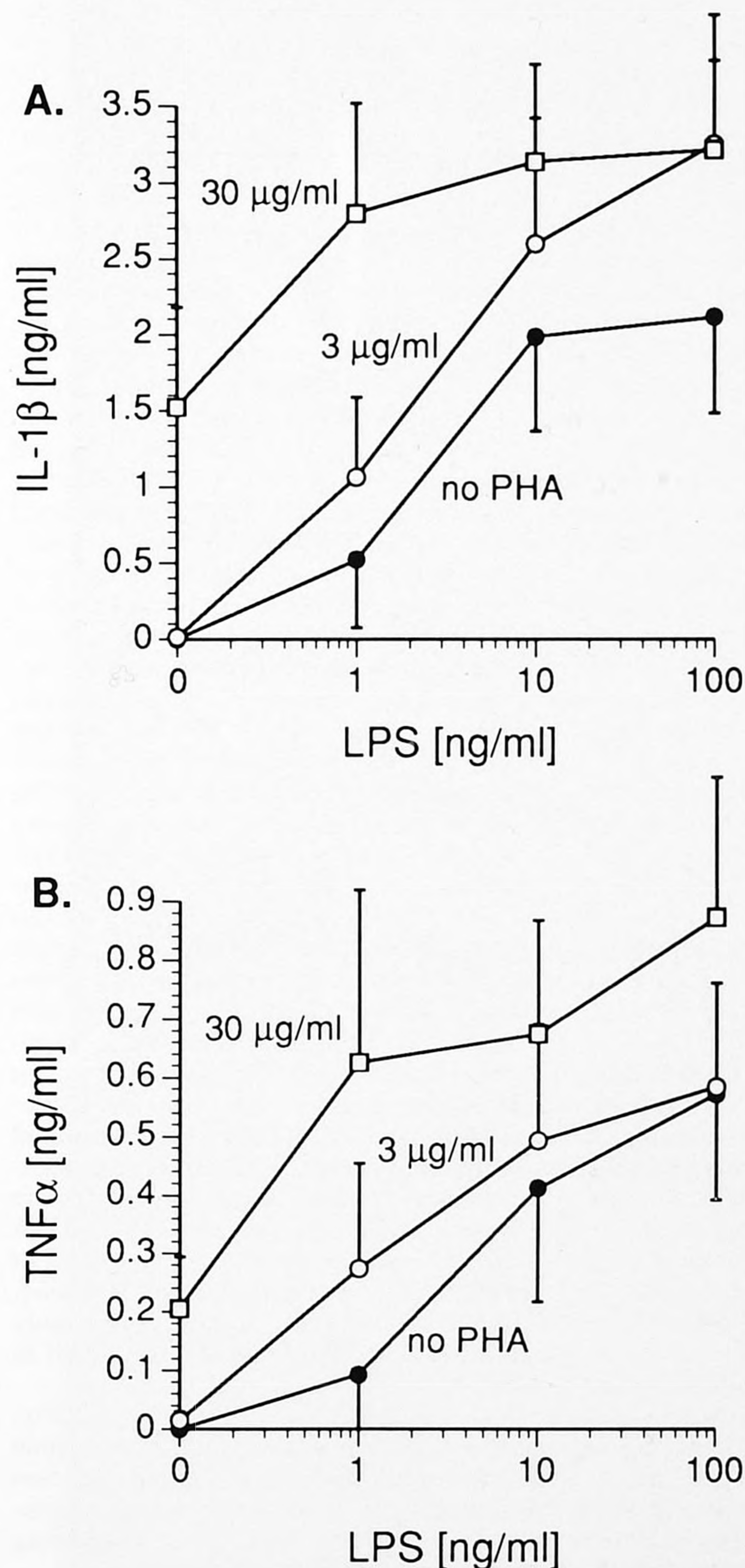


Fig. 2. Representation of dose-related response of (A) IL- 1β and (B) TNF α production by the whole blood method using combinations of LPS and PHA (incubated for 24 h at 37°C). The data represent the mean of four subjects. Note different vertical scales.

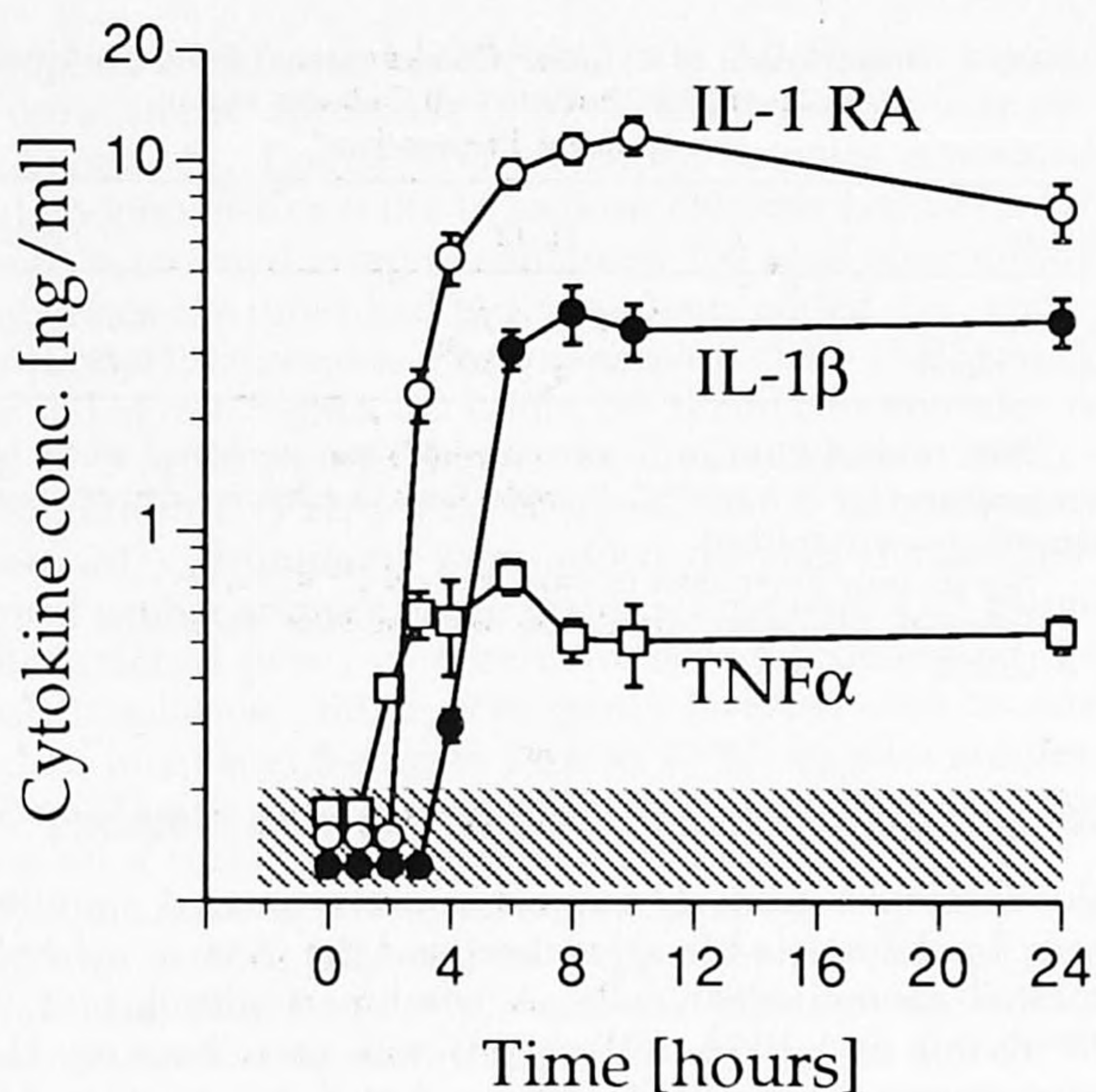


Fig. 3. Time course of IL-1 β , TNF α , and IL-1ra production in whole blood. Each time point represents the mean of 4 subjects except IL-1 β and TNF α at 6 h and 24 h where the values are the mean of 16 subjects.

person, and is reproducible. Two prevailing concerns in the development of this assay were to minimize sample manipulation (and therefore the possibility of contamination) and to carry out the procedure with the simplest possible equipment.

Eskola et al. first introduced the concept of examining leukocyte function in unfractionated blood samples [24]. These authors assessed lymphocyte proliferation measured by ¹²⁵uridine uptake in diluted blood mixtures stimulated with lectins in microtiter plates. Kirschner et al. [25] used a similar cell incubation arrangement to assess production of interferon. More recently, Desch et al. [26], Strieter et al. [27], and Schumann et al. [28] studied TNF production in undiluted blood preparations. Yachie et al. [29] studied IL-6, and Ham et al. [30] studied IL-8 production in similar preparations. These methods involved the sterile transfer of blood from the collection vessel to microtiter plates or other tubes requiring specialized incubators and/or environments. The present report characterizes production of IL-1 β and its naturally-occurring counter-regulator, IL-1ra. The whole blood assay method has been modified to allow incubations to be carried out in virtually any surrounding environment that can be maintained at a constant 37°C, with no requirement for control of humidity or CO₂.

As shown in Figure 2, PHA and LPS together resulted in greater stimulation of IL-1 β production than either stimulant alone. At LPS concentrations greater than 10 ng/ml, there was no additional cytokine production due to PHA. The original intent for including a lectin was to induce IL-2 and GM-CSF production as well. These cytokines were not detected, but this could have been due to an inability of the immunoassays to detect these cytokines in plasma, rather than a lack of production. To assess IL-1 β or TNF α production, LPS alone from 1 to 10 ng/ml is a sufficient stimulus. IL-1 β and TNF α in control samples incubated with saline only were uniformly nondetectable or very low, suggesting negligible contamination under these conditions.

In preliminary studies with the whole blood method, the coefficient of variation for cytokine production from the same individual on different days was 30% [31]. This is simi-

lar to the intraindividual daily variation previously reported for peripheral blood mononuclear cells [32].

In vitro cultures yielded 2- and 36-fold higher concentrations than whole blood preparations for IL-1 β and TNF α , respectively (Table 1). There are several reasons for this. First, red blood cells present in whole blood preparations bind lectins, thus reducing the effective concentration of the stimulant. Second, isolation of mononuclear cells removes circulating plasma factors, such as cortisol, that are physiological modulators of cytokine secretion [33]. Other circulating factors, i.e., proteases, soluble receptors [34, 35], or nonspecific binding proteins, could potentially interfere with cytokine measurements. These factors could bind the cytokines in the plasma and facilitate their internalization via cell-surface receptors. Soluble receptors may also bind the cytokines with affinities higher than that of the antibodies used in the RIAs. Third, the whole blood preparation contains neutrophils that are not present in the in vitro cultures. Neutrophils can influence cytokine production by secreting inhibitors [36], they may accelerate cytokine breakdown by releasing proteases, or they may promote clearance by binding and internalizing cytokines. The inverse correlation ($r = -0.677$, $P = 0.016$) between neutrophil counts in the whole blood and TNF concentrations after 24 h of incubation supports the concept that neutrophils may be an important determinant of TNF α concentrations in the whole blood preparation. IL-1 β concentrations in the whole blood method correlated significantly with the IL-1 β concentrations in the in vitro cultures, indicating that the whole blood method may be best applied to the study of this cytokine.

As a result of the recent discovery and characterization of IL-1ra, it is clear that a more complete assessment of IL-1 status now requires the measurement of this antagonist. As shown in Figure 3, the whole blood method yielded significant quantities of IL-1ra, similar to the magnitudes reported for PBMC cultures [21]. IL-1ra production was first detectable after 3 h of incubation but was not maximal until 10 h. This time course varies from that of IL-1 β , indicating differential regulation of these proteins. Although IL-1ra competes with IL-1 β at the cell surface receptor, there is no cross-reactivity between IL-1 β and IL-1ra in the RIA measurements and the IL-1ra does not bind to IL-1 β [21]. It has been demonstrated recently that the addition of 1% serum to isolated mononuclear cell cultures increased IL-1ra production sevenfold, but had no effect on IL-1 β production [21]. Likewise, pooled human IgG (for intravenous use) induced IL-1ra production from isolated mononuclear cells at concentrations as low as 1 μ g/ml. However, under these conditions, IL-1 β was not produced. Therefore, the whole blood method, which contains the physiologically correct concentration of serum factors and IgG may be a more appropriate approach for the study of IL-1ra. We speculate that EDTA in the whole blood incubations prevents IgG stimulation of IL-1ra but allows LPS and PHA induction of this cytokine. Thus, the whole blood stimulation of IL-1ra more closely reflects the in vivo production of this cytokine, compared to cultured mononuclear cells.

Significant near maximal concentrations of IL-1 β , TNF α , and IL-1ra were observed by 6 h indicating that the whole blood method has the potential for use as a rapid (i.e., less than 1 day) method. On the other hand, the change in concentration over the next 18 h was not significant, indicating that overnight cultures can be done for convenience.

In summary, the whole blood method is a simple, reliable alternative to standard cell culture techniques for determining cytokine production by stimulated leukocytes. It is ideal for

field work in that little blood is required for measurement, it is not labor-intensive, samples remain sterile, and no specialized equipment or facilities are required. The potential value of this assay method has been demonstrated recently by Miller et al. [37]. Using the methodology we had previously reported in abstract form [38], these authors were able to demonstrate differential IL-1 β production in infants with perinatal infectious complications, compared to healthy infants.

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