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# *In vitro* production of IL 1β, IL 1α, TNF and IL 2 in healthy subjects: distribution, effect of cyclooxygenase inhibition and evidence of independent gene regulation*

Numerous studies have reported altered *in vitro* cytokine production in various diseases. In the present study we used specific immunoassays to quantitate production of interleukin 1β (IL 1β), IL 1α, tumor necrosis factor (TNF) and IL 2 from human peripheral blood mononuclear cells (PBMC). The distribution of cell-associated and secreted cytokines was studied in PBMC of 21 individuals; in response to lipopolysaccharide (LPS) the proportion of cell-associated IL 1β ranged from 13% to 56%, for IL 1α 29% to 98%, and for TNF 2% to 17%. In a larger cohort of 32 subjects, the total amount of immunoreactive cytokines produced in response to LPS or phytohemagglutinin was normally distributed within the study group. Mean production of IL 1α in response to LPS was 10.1 ng/ml and exceeded production of IL 1β (5.6 ng/ml) and TNF (2.2 ng/ml). The distribution pattern was characterized by high intersubject variability extending over two orders of magnitude and the presence of high and low "producers". Production of IL 1α and IL 1β correlated ($R = 0.69$). In contrast, production of IL 1β did not correlate with production of TNF or IL 2. Indomethacin present during stimulation of PBMC increased the amount of IL 1β producd and showed a high correlation ($R = 0.83$) compared to cultures without indomethacin. Thus, low production of IL 1β in certain subjects appears not to be due to inhibitable levels of cyclooxygenase products. In a retrospective study, PBMC from 12 subjects who had taken oral cyclooxygenase inhibitors during the preceding 7 days produced 43% more IL 1β than subjects who did not take these drugs ($p < 0.05$). These studies demonstrate that the amount of cytokine synthesized by PBMC (a) is regulated independently for IL 1, TNF and IL 2; (b) correlates for IL 1β and IL 1α; (c) is intrinsic for low and high "producers", and (d) production of IL 1β increases with the use of oral cyclooxygenase inhibitors.

## 1 Introduction

IL 1 and TNF participate in the host's response to acute and chronic injury, infection, immunologic challenge or malignant disease. A widely used method to help elucidate the role of these cytokines in the pathogenesis of different diseases involves measuring cytokine production from stimulated blood leukocytes. Altered production of IL 1 *in vitro* has been reported in several diseases [1]. Increased production of TNF *in vitro* occurs in some cancer patients [2]. Reduced IL 2 production *in vitro* has been observed in patients with AIDS [3], some forms of cancer [4], rheumatoid arthritis [5], SLE [6] and type I diabetes mellitus [7].

Because of these reports on altered levels of *in vitro* production of IL 1, TNF or IL 2 in disease, the present study was designed to define normal ranges and the distribution patterns of production of those cytokines *in vitro* in a cohort of normal volunteers. The cytokines were quantified by specific RIA or ELISA to avoid confounding factors introduced by the use of bioassays. For the different cytokines we found a varying degree of correlation within the study group, indicating independent regulation of their production. Furthermore, we demonstrated an enhancing effect of either *in vivo* or *in vitro* cyclooxygenase inhibition of cytokine production.

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## 2 Materials and methods

### 2.1 Study population

The study was approved by the Human Investigation Review Committee of the New England Medical Center Hospitals. Healthy volunteers were recruited from laboratory and hospital personnel. Each volunteer completed a standardized questionnaire on medical history, medication, smoking habits and diet. Blood was obtained during the morning hours.

### 2.2 *In vitro* PBMC stimulation

Twenty milliliters of venous blood was drawn into heparinized (20 U/ml) syringes. The PBMC fraction was obtained

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by centrifugation of diluted blood (1 part blood to 2 parts pyrogen-free normal saline) over Ficoll-Hypaque (Ficoll Type 400; Sigma, St. Louis, MO; and Hypaque-M, 90%; Winthrop-Breon Lab., New York, NY) which was diluted in ultrafiltered water. PBMC were washed twice in 0.15 M NaCl and slides were prepared by cytocentrifugation. One hundred cell differential counts were performed in a blind fashion on coded slides after staining with Wright's stain. In addition, some PBMC preparations were stained for nonspecific esterase (Sigma) and 200 cells were counted. RPMI 1640 culture medium (Whittaker M.A. Bioproducts, Walkersville, MD), supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, was ultrafiltered (hollow fiber filter U2000; Gambro, Hechingen, FRG) in order to remove LPS and other microbial products as previously described [8]. The Ficoll-Hypaque and RPMI media were LPS negative by the Limulus amebocyte lysate assay with a detection limit of 20 pg/ml (Associates of Cape Cod, Woods Hole, MA). PBMC were suspended at $5 \times 10^6$/ml in RPMI with 2% heat-inactivated human serum from a single AB donor, and 100 µl was aliquoted in each well of 96-well flat-bottom microtiter plates. An equal volume of either RPMI (control) or RPMI containing various stimulants was then added. The cells were stimulated with LPS (from *E. coli* O55:B5; Sigma) or with PHA-P (Difco, Detroit, MI) at indicated concentrations. LPS concentrations ranging between 0.5 and 10 ng/ml were chosen because they more closely approximate clinical situations than concentrations up to 50 µg/ml used in some other studies. Stimulus aliquots were stored at 1 µg/ml stock concentration for LPS and at 600 µg/ml for PHA, and were diluted in RPMI immediately before use. All stimuli were added to duplicate wells. In part of the study cells were incubated with or without 1 µg/ml indomethacin. Crystalline indomethacin (Sigma) was diluted in 95% ethanol at 100 mg/ml. This stock solution was diluted with RPMI to 2 mg/ml and stored in aliquots at −70 °C. The aliquots were diluted 1000-fold immediately before use.

After 24 h at 37 °C in 5% $CO_2$, SN in the wells were replaced by medium for some of the subjects. The microtiter plates containing the cells were frozen at −70 °C. At the end of each study all plates were simultaneously thawed and exposed to two more freeze-thaw cycles to lyse the cells. The contents of duplicate wells, consisting of cell lysates and SN, were pooled, spun at 13000 × g for 1 min and the SN was refrozen at −70 °C. Approximately one month later, the samples were diluted in 0.01 M phosphate-buffered saline containing 0.25% BSA and 0.05% sodium azide (BSA buffer) for cytokine assays. Dilutions of the cell SN or lysates were adjusted to obtain measurements in the sensitive range of the standard curves of each cytokine assay.

### 2.3 Cytokine determinations

IL 1β [9, 10], IL 1α and TNF [12] were determined by specific RIA as previously described. These RIA employ polyclonal antisera raised in rabbits against human recombinant IL 1β, IL 1α or TNF. Briefly, samples were incubated with specific antibody overnight, and radioiodinated cytokine tracer was added and allowed to equilibrate during another overnight incubation. Finally, bound radioactivity was precipitated by sheep anti-rabbit IgG antiserum and the antibody-antigen complexes were precipitated by centrifugation. Radioactivity in the pellet was determined in a gamma counter and expressed as percent binding of a zero standard. Cytokine concentration was read from a standard curve obtained with serial dilutions of known standards. Recombinant cytokines for iodination and standard curves were kindly provided by Dr. Alan Shaw, Glaxo Institute of Molecular Biology, Geneva, Switzerland. IL 2 was determined by ELISA (Genzyme Corp., Boston, MA). Statistical analysis was performed using Stat-View software (Abacus Concepts Inc., Calabasas, CA) on a Macintosh SE computer (Apple Computer Inc, Cupertino, CA) and on a Clinfo software system (Bolt, Beranek and Newman, Cambridge, MA).

## 3 Results

### 3.1 Cell-associated *vs.* secreted cytokine

Cytokine levels were quantified separately in the cell-associated and secreted compartments. PBMC from 21 donors were stimulated with 10 ng/ml of LPS for 24 h. SN was withdrawn and replaced with medium. Cells in the medium were lysed by three freeze-thaw cycles. Cytokine concentrations were determined by RIA in the SN (secreted compartment) and in the lysate (cell-associated compartment) (Fig. 1). IL 1α remains mostly cell associated (median 72%), slightly more IL 1β is secreted than remains cell associated (median 39%) and very little TNF remains cell associated (median 10%). These results confirm previous studies on the distribution of cytokines between the cell-associated and the secreted compartments [13, 14]. Furthermore, for a given cytokine there is wide interindividual variation in the amount of cell-associated cytokine, ranging from 13% to 56% for IL 1β, from 29% to 98% for IL 1α and from 2% to 17% for TNF.

Because of the considerable proportion of cell-associated cytokine, the differences in this proportion for different cytokines and the wide interindividual variation, determination of the combined cell-associated and secreted com-

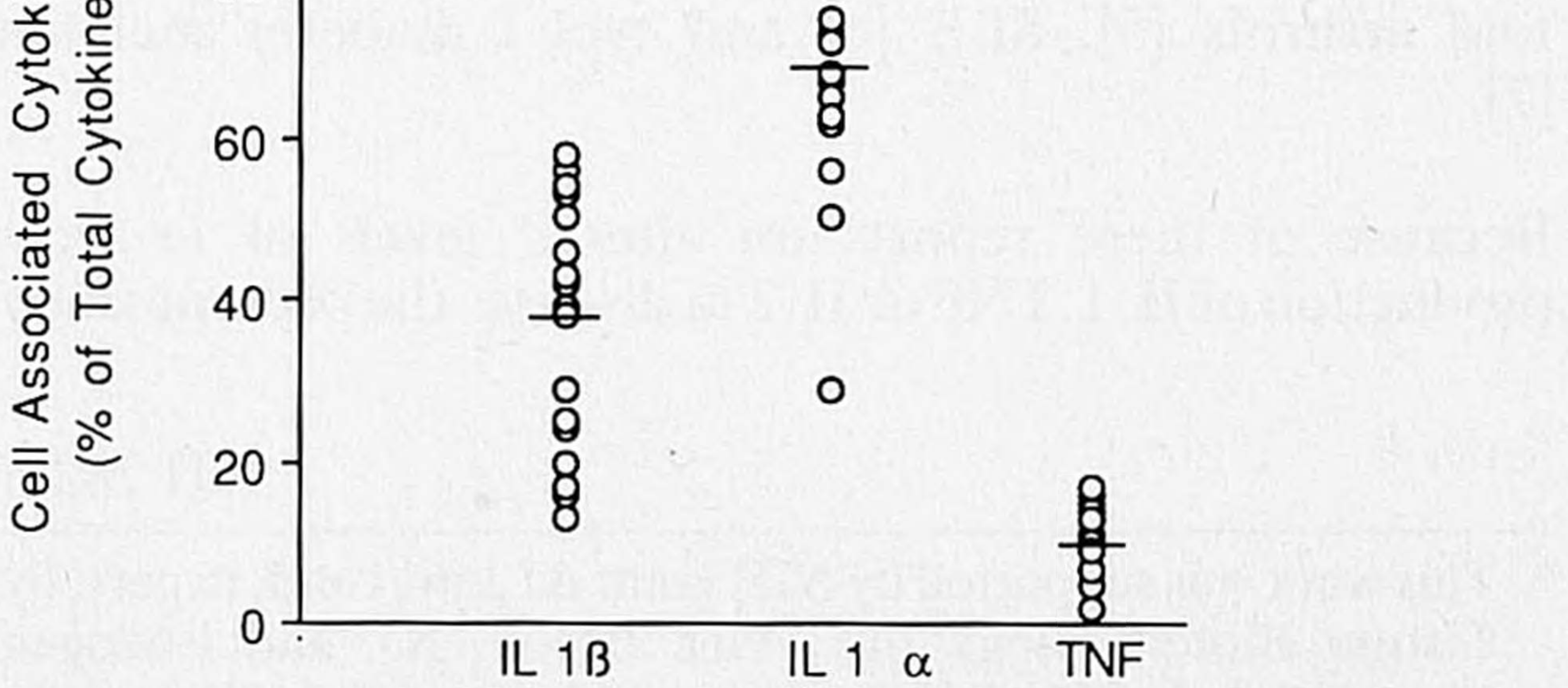


*Figure 1.* Relative proportion of cell-associated *vs.* total cytokine production. PBMC from 21 subjects were stimulated with 10 ng/ml of LPS. Cytokine concentrations were determined by RIA in the SN (secreted) and the lysate (cell associated). Horizontal bars indicate the mean proportion of cell-associated compartment for each cytokine. IL 1α concentrations were available for 18 of the 21 subjects.

partment was necessary in order to compare the amount of cytokine synthesized between IL 1β, IL 1α and TNF.

### 3.2 Normal distribution of cytokine production

Of the group of 44 volunteers, 32 of the subjects indicated that they had not taken any medication for at least 1 week, whereas 12 volunteers had taken cyclooxygenase inhibitors during the past week (5 subjects Aspirin, 5 subjects Ibuprofen, 2 subjects Naproxen). Age and sex distribution of the two cohorts are summarized in Table 1. The mean response to 0.5 ng/ml of LPS was 10.1 ng/ml of IL 1α, 5.6 ng/ml of IL 1β and 2.2 ng/ml of TNF (Table 2) for PBMC from the study cohort without medication. Previous studies have shown that 0.5 ng/ml of LPS does not stimulate IL 2 production from PBMC *in vitro*. Therefore, we used PHA as a stimulus for IL 2 production. *In vitro* production of IL 2 in response to 30 μg/ml of PHA averaged 9.4 ng/ml. The distribution of *in vitro* cytokine production in this study group is depicted in Fig. 2. There was a normal distribution for all four cytokines as calculated by the Wilk-Shapiro test. The largest SD (6.9 ng/ml) was seen for IL 2 production, indicating high intersubject variability.

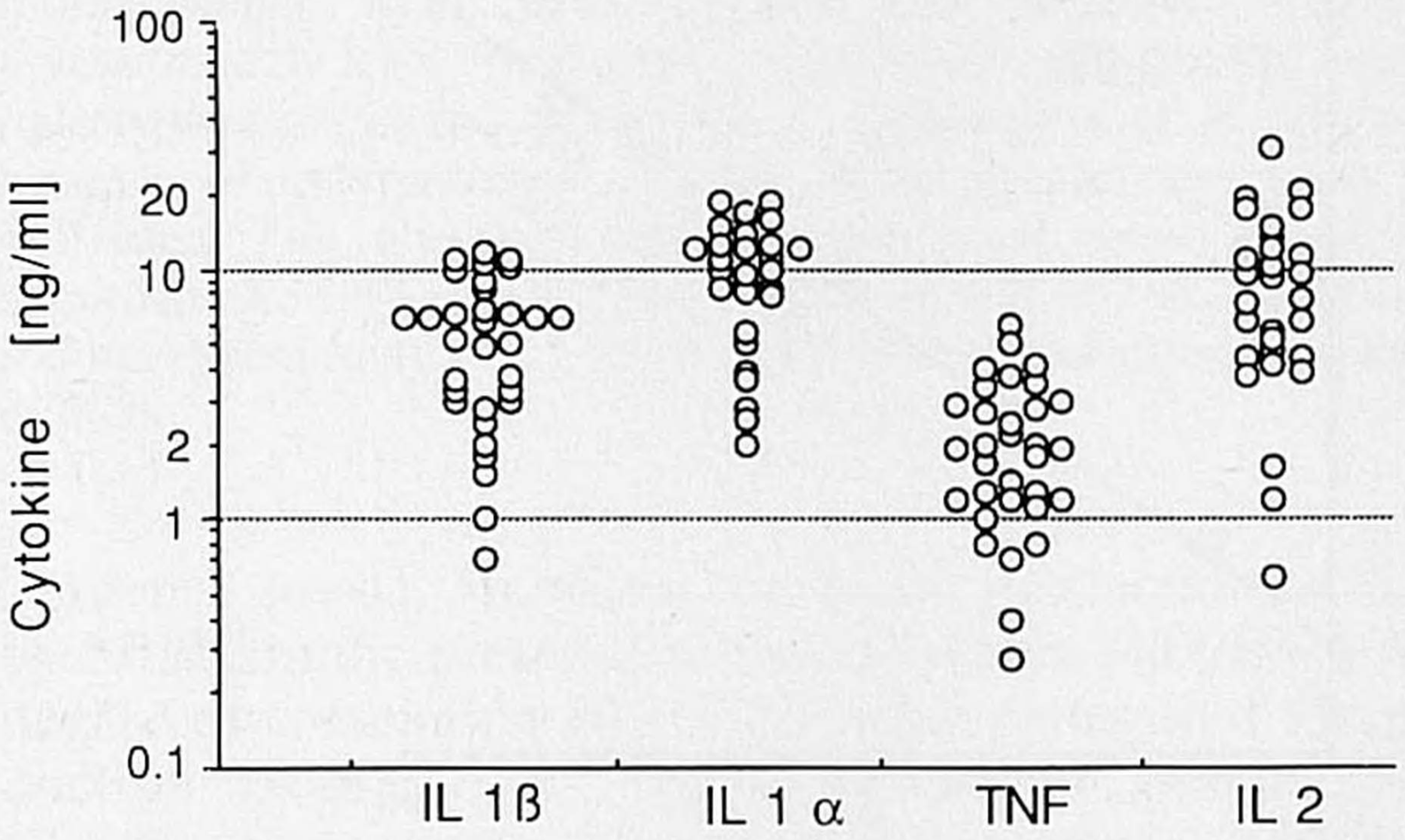


*Figure 2.* Interindividual distribution of cytokine production. PBMC from 32 subjects were stimulated with 0.5 ng/ml LPS for induction of IL 1β, IL 1α and TNF, and with 30 μg/ml PHA for induction of IL 2. Total (cell-associated plus secreted) cytokine was determined by RIA or ELISA.

**Table 1.** Characteristics of study cohort

| Medication[a)] | No | Yes |
|---|---|---|
| Males | 19 | 4 |
| Females | 19 | 8 |
| Age (years) | | |
| Median | 27 | 30 |
| Mean | 29.1 | 30.4 |
| Range | 20 -42 | 23 -43 |

a) Cyclooxygenase inhibitors.

### 3.3 Covariation of cytokines

Fig. 2 shows that cytokine production from the cells of different donors vary up to two orders of magnitude. Correlation analysis was next performed to determine whether an individual with high production of one cytokine also exhibits high production of other cytokines in response to a given stimulus. Production of IL 1 and TNF using LPS as a stimulus was compared. Fig. 3A depicts the production of TNF *vs.* the production of IL 1β. Each square represents the data obtained from a single individual's PBMC culture sitmulated with 0.5 ng/ml of LPS. The quadrants of the graph, defined by the median production of each cytokine, distribute the subjects according to their cytokine response: low TNF and low IL 1β producers, high TNF and high IL 1β producers, and the two groups with mixed responses. The subjects are equally distributed in the four quadrants, indicating independent variation of production. This is reflected by a low correlation coefficient of $R = 0.18$. The correlation of IL 1α to TNF production is also low ($R = 0.44$; data not shown).

In contrast, there is a high correlation of IL 1β production with production of IL 1α. The majority of subjects fall into the group of low IL 1α and low IL 1β producers or high IL 1α and high IL 1β producers (Fig. 3B). The correlation coefficient is $R = 0.69$. Using 30 μg/ml of PHA as a stimulus, we found no correlation between production of IL 2 and IL 1β ($R = -0.10$) or IL 2 and TNF ($R = -0.27$) measured in the same PBMC samples.

### 3.4 Comparison of *in vitro* cytokine production with the proportion of monocytes in the population of PBMC

For the group of 32 subjects the mean percentage of monocytes in the PBMC cultures was 19.7% (± 10.5% SD) as determined morphologically. There was no correlation between the percentage of monocytes in the PBMC population and the amount of LPS-induced IL 1β (correlation coefficient $R = 0.08$), IL 1α ($R = 0.15$) or TNF ($R = 0.07$) produced. Correlation of IL 1β production and

**Table 2.** Distribution of cytokine production for 32 subjects without medication[a)]

| Cytokine:<br>Stimulus: | IL 1β<br>LPS<br>0.5 ng/ml | IL 1α<br>LPS<br>0.5 ng/ml | TNF<br>LPS<br>0.5 ng/ml | IL 2<br>PHA<br>30 μg/ml |
|---|---|---|---|---|
| Mean cytokine production | 5.6 ng/ml | 10.1 ng/ml | 2.2 ng/ml | 9.4 ng/ml |
| SD | ± 3.2 | ± 4.7 | ± 1.4 | ± 6.9 |
| Percentile (10th–90th)[b)] | (1.8–10.5) | 3.6–16.1) | (0.8–4.0) | (3.7–17.6) |

a) Calculated for the data illustrated in Fig. 2.
b) Tenth percentile indicates concentrations below which samples from 10% of the subjects fall. Ninetieth percentile indicates concentration below which samples from 90% of the subjects fall. Data are not available for IL 1α production in 1 and for IL 2 production in 3 of the 32 subjects.

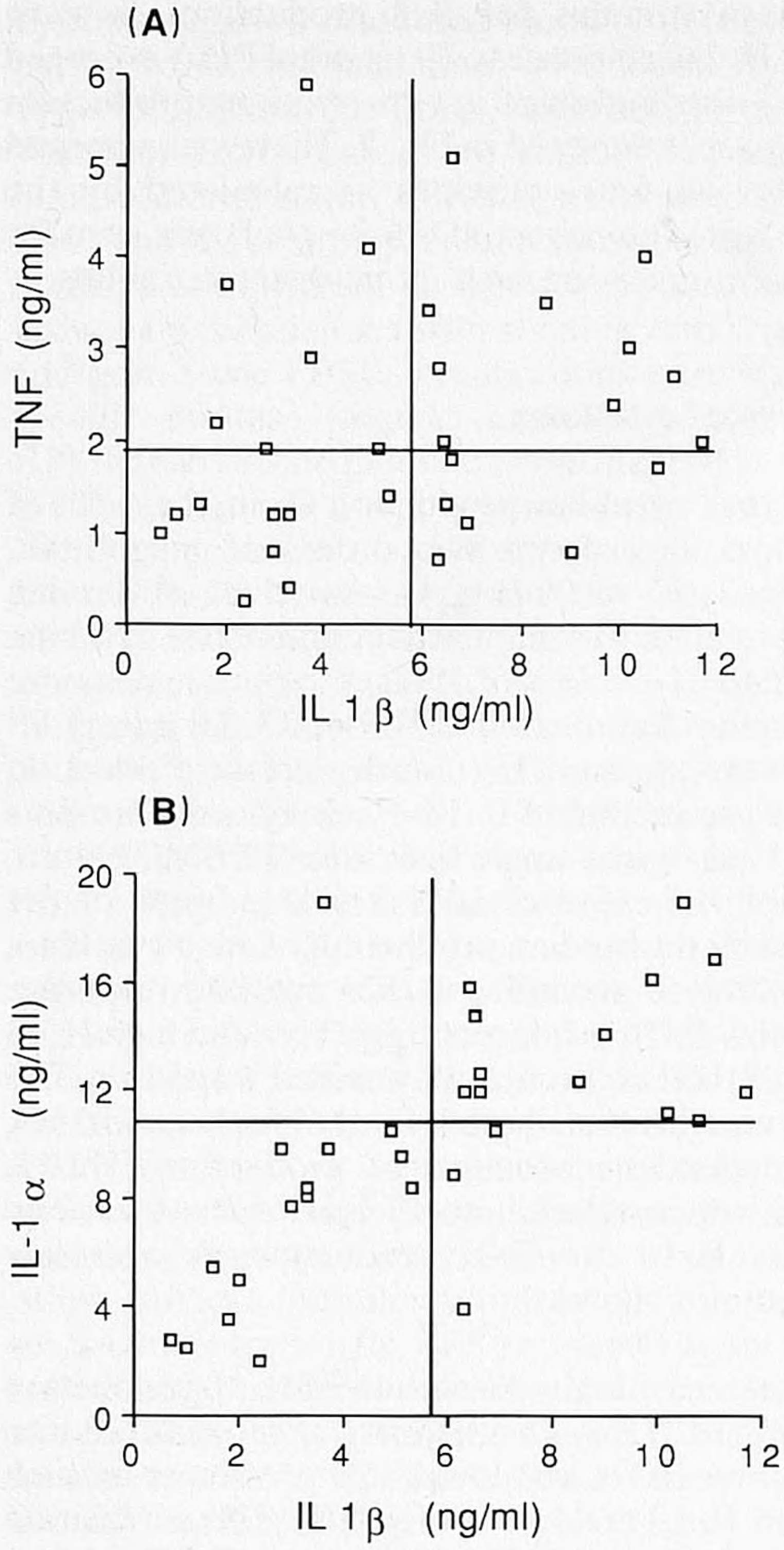


*Figure 3.* Covariation of IL 1β, IL 1α and TNF production. (A) Concentration of TNF *vs.* concentration of IL 1β is plotted for respective subjects (correlation coefficient $R = 0.18$). (B) Concentration of IL 1α *vs.* concentration of IL 1β ($R = 0.69$). PBMC from 32 subjects were stimulated with 0.5 ng/ml LPS, and total production of IL 1β, IL 1α and TNF was assayed by RIA. Each square represents one subject. The vertical lines indicate the median conentration for IL 1β (5.9 ng/ml); the horizontal lines indicate the median concentrations of TNF (1.9 ng/ml) and of IL 1α (10.5 ng/ml). (A) has 31 entries since the sample from one of the subjects was not assayed for IL 1α.

proportion of esterase-positive cells in the PBMC population was studied in a group of 7 subjects. In this group PBMC were isolated and stimulated on two different days, two days apart for each individual. There was no correlation ($R = 0.09$) between the mean esterase-positive cell proportion and mean production of IL 1β, both determined as the mean of two different days (Fig. 4A). This lack of correlation is not due to lack of precision in determination of either cytokine concentration or esterase-positive cell proportion, since both these values are reproducible for a given subject on two different days ($R = 0.87$ for IL 1β data in Fig. 4B; $R = 0.65$ for esterase-positive cell proportion, data not in figure).

Furthermore, we examined whether for a given individual the day to day variaton of *in vitro* IL 1β production correlated with day-to-day variation in the proportion of esterase-positive cells in the PBMC population (intraindividual correlation). If the absolute concentration of IL 1β is corrected for the proportion of esterase-positive cells (ng IL 1β per $2.5 \times 10^6$ esterase-positive cells), the day-to-day reproducibility is reduced ($R = 0.59$) when compared to the day-to-day reproducibility of either absolute IL 1β concentration ($R = 0.87$, panel B) or esterase-positive cell proportin ($R = 0.65$). Thus, we found absolute production of IL 1β (*e.g.* per $2.5 \times 10^6$ PBMC) to be the parameter that is the most reproducible in a given individual. This is probably due to the fact that mononuclear cells other than monocytes, *e.g.* lymphocytes, make IL 1 and TNF [13] and affect each other's ability to synthesize these cytokines.

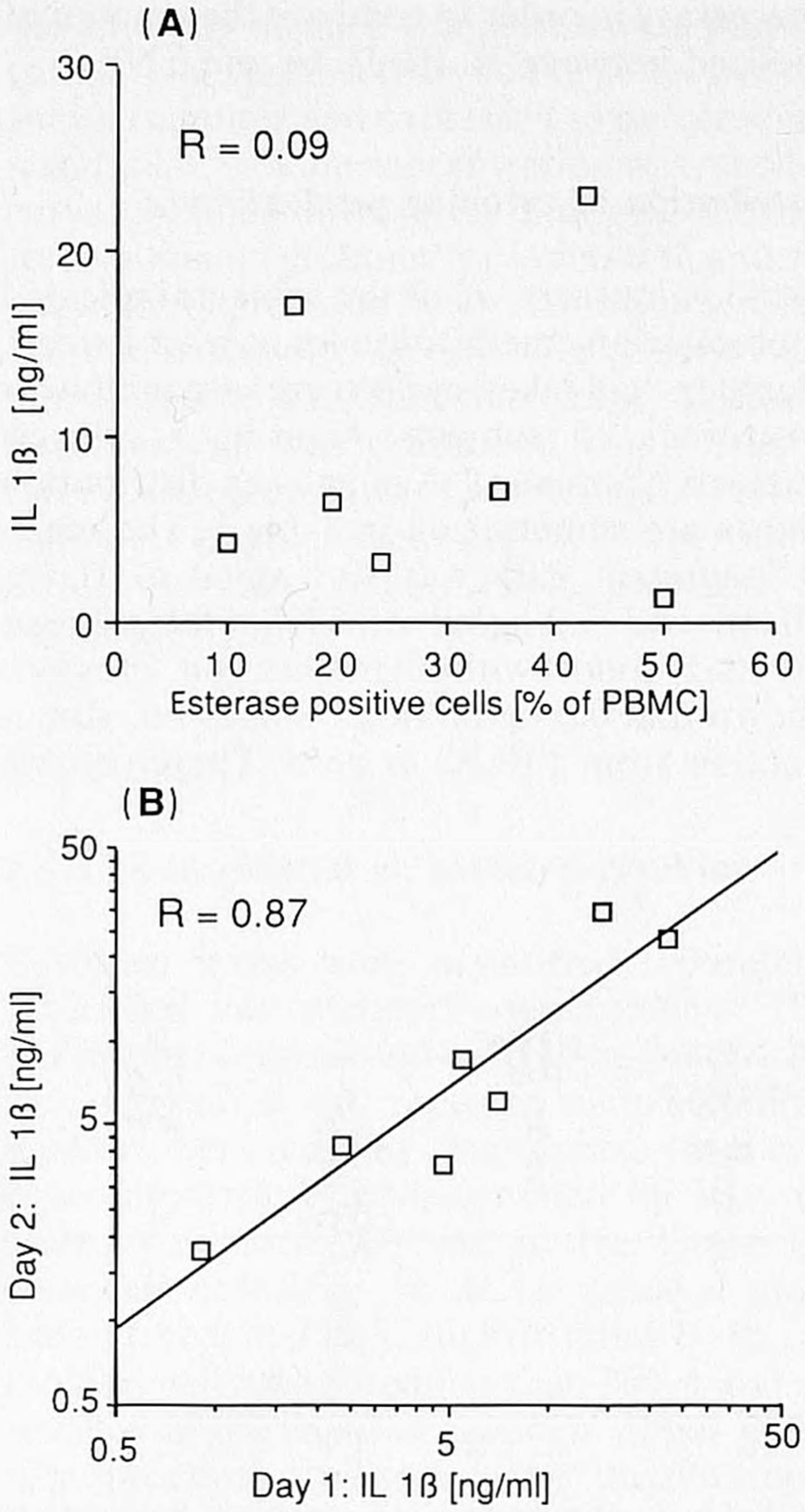


*Figure 4.* Production of IL 1β *vs.* proportion of esterase-positive cells in the PBMC population. PBMC from seven subjects were stimulated with 5 ng/ml of LPS on two different days two days apart. Total (associated plus secreted) cytokine was determined by RIA. (A) Lack of correlation between production of IL 1β and proportion of esterase-positive cells for different individuals. Production of IL 1β (mean of two different days) is plotted *vs.* mean proportion of esterase-positive cells. (B) Intraindividual reproducibility of absolute production of IL 1β determined on two different days.

### 3.5 Effect of cyclooxygenase inhibition *in vitro*

Addition of indomethacin to the cultures increased IL 1β production in the study group without medication by an average of 27%. In this cohort of subjects, this increase did not reach significance ($p = 0.055$ by one-tailed paired

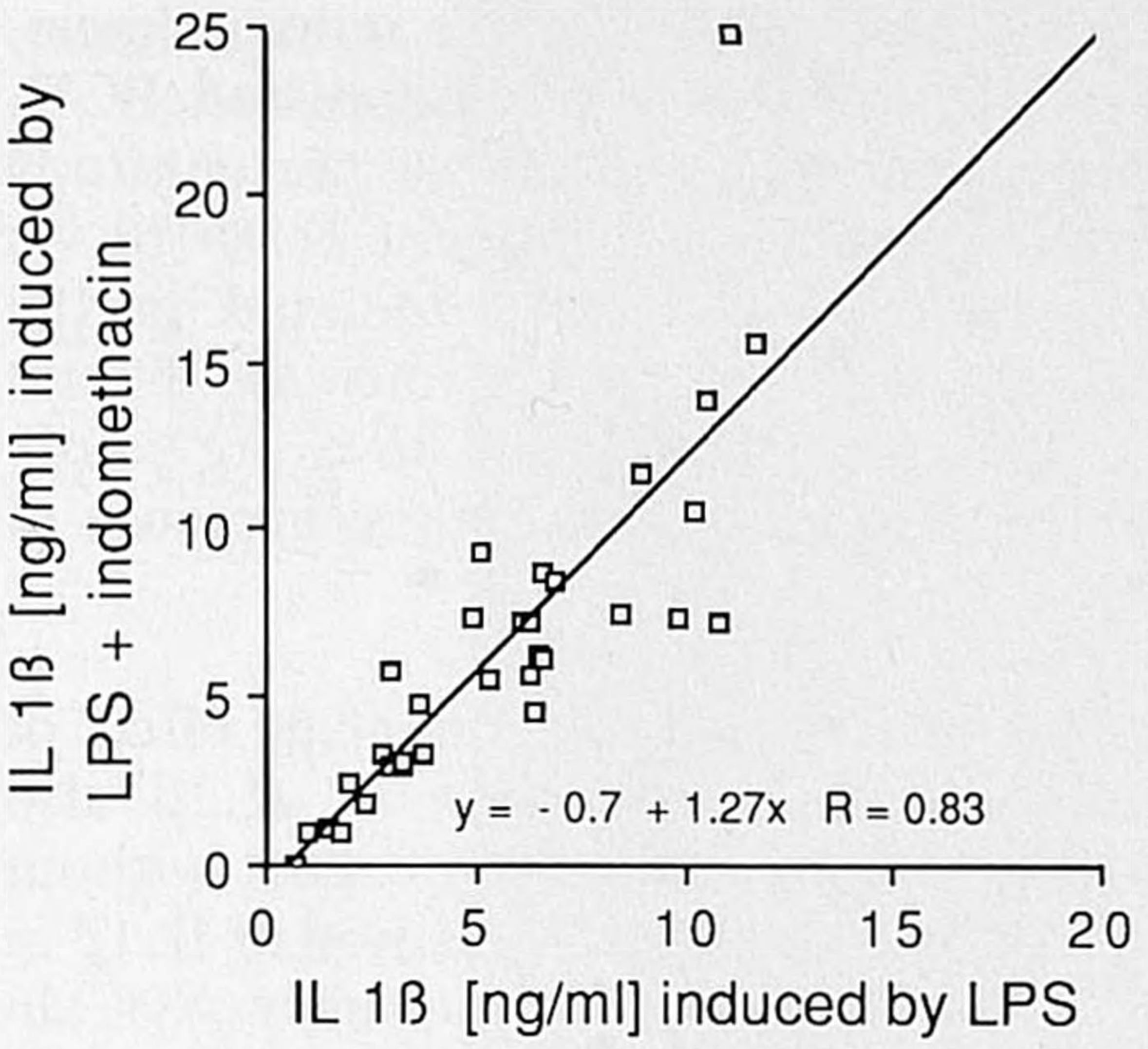


*Figure 5.* Correlation of IL 1β production *in vitro* with and without addition of indomethacin. PBMC from 32 donors were stimulated with 0.5 ng/ml LPS in the presence or absence of 1 µg/ml indomethacin. Total (cell-associated plus secreted) IL 1β was determined by RIA. Production of IL 1β in the presence of 1 µg/ml indomethacin (y-axis) is plotted *vs.* production of IL 1β in the absence of indomethacin (x-axis). Each square represents one individual. The (diagonal) regression line is calculated by the least square method. The correlation coefficient is $R = 0.83$. Addition of indomethacin increases LPS-induced IL 1β production by a mean of 27%.

Student's *t*-test). However, increased production of IL 1β by PBMC in the presence of indomethacin did show a high degree of correlation ($R = 0.83$) when compared the production from PBMC cultured without indomethacin (Fig. 5). Low production of IL 1β in certain individuals could have been due to the effect of high levels of cyclooxygenase products which suppress production of IL 1β *in vitro* at the level of translation [15]. As apparent from Fig. 5 and reflected in the high correlation coefficient, however, "low producers" for IL 1β production without the addition of indomethacin were also "low producers" when cyclooxygenase was inhibited by indomethacin. Thus, the observed intersubject variability in cytokine production is not due to differences in cyclooxygenase products. Rather,

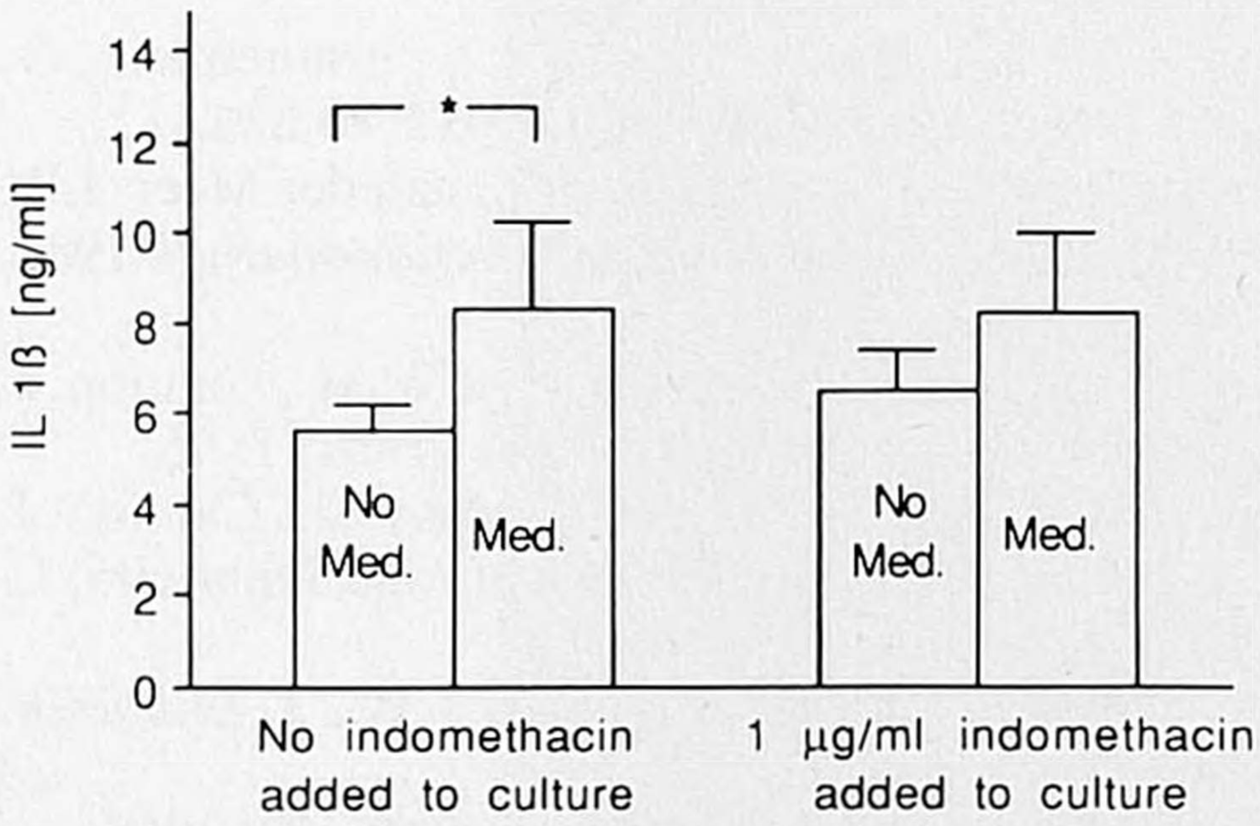


*Figure 6.* Effect of cyclooxygenase inhibition *in vivo* and *in vitro* on LPS-induced IL 1β production. PBMC from 44 donors were stimulated with 0.5 ng/ml LPS with or without addition of 1 µg/ml indomethacin. The bars to the left compare IL 1β production in the absence of *in vitro* indomethacin between the groups without ($n = 32$) or with ($n = 12$) medication (cyclooxygenase inhibitors). The bars to the right compare production of IL 1β from the same groups when stimulated in the presence of *in vitro* indomethacin. Error bars indicate standard error of the mean. * Denotes significant difference between the two subject groups by nonpaired, one-tailed Student's *t*-test.

it appears to reflect the genuine regulation of IL 1β production at the pretranslational level in individual subjects.

### 3.6 Effect on cyclooxygenase inhibition *in vivo*

Production of IL 1β was significantly increased in the group who had taken a cyclooxygenase inhibitor (8.3 ng/ml; $n = 12$) as compared the the group without medication (5.7 ng/ml; $n = 32$, $p = 0.048$ by one-tailed unpaired Student's *t*-test; Fig. 6). When PBMC from both groups were stimulated in the presence of indomethacin, the production of IL 1β was increased in the group without medication but was statistically unchanged in the group taking cyclooxygenase inhibitors.

## 4 Discussion

In the present study we measured the total synthesis of IL 1β, IL 1α, TNF and IL 2 by PBMC stimulated *in vitro*. We used this procedure to define the range and distribution pattern for the production of these cytokines in a cohort of healthy volunteers. We measured total (cell-associated plus secreted) cytokine concentrations in order to assess overall cytokine synthesis during 24 h, independent of release kinetics. This approximates the *in vivo* condition since both IL 1 [16] and TNF [17] may be cell associated and exhibit biological activity in that compartment. Furthermore, we showed that the proportion of cell-associated *vs.* secreted cytokine differs for the two forms of IL 1 and TNF and, moreover, exhibits wide intraindividual variation. This latter finding is an important consideration in performing gene linkage studies in health or disease.

We chose to stimulate the entire population of PBMC rather than enriched subpopulations. Although most of the IL 1 and TNF are produced by monocytes, it has been shown that Tcells [18], B cells [19] and NK cells [13, 20] also synthesize IL 1 and TNF. Thus, we believe that for the purpose of this study the response of the whole PBMC population is biologically more relevant than that of enriched subpopulations. Mononuclear cells other than monocytes may contribute to IL 1 and TNF production through cell contact or through formation of other cytokines, such as IFN-γ. We demonstrate that the proportion of monocytes and production of IL 1β do not correlate between individuals. For a given individual, correction of IL 1β production for the monocyte proportion in the population of stimulated PBMC actually reduces the reproducibility of this parameter.

During enrichment procedures variability due to differences in monocyte adherence and recovery may interfere. Monocyte adherence can be altered in disease [21] and in subjects taking medication with cyclooxygenase inhibitory activity [22]. Finally, stimulating the whole PBMC population allows measurement of cytokines originating predominantly from monocytes (IL 1β, IL 1α and TNF) or lymphocytes (IL 2 and TNF) in the same sample employing the same stimulus. This allowed us to examine whether IL 1 or TNF production correlated with IL 2 production in the samples stimulated with PHA.

We report here that 0.5 ng/ml of LPS stimulated more IL 1α than IL 1β or TNF (IL 1α > IL 1β > TNF). This is somewhat surprising given the fact that transcription of IL 1β in PBMC exceeds that of IL 1 α by 20- to 50-fold [23]. Since our results are based on measuring immunoreactive IL 1α and IL 1β, this discrepancy cannot be explained by inhibitors which may confound bioassays. The lower level of IL 1β in response to Fig. 4A is probably not a reflection of preferential degradation during incubation and freeze thawing since, in another study using a different stimulus (heat-killed *S. epidermidis*), we found production of IL 1β to exceed production of IL 1α [10].

There appears to exist a normal distribution for cytokine production *in vitro*, as judged from the pattern in Fig. 2 and as calculated by the Wilk-Shapiro test. Normal distribution of a parameter is a prerequisite for performing standard statistical procedures such as Student's *t*-test on sample groups. Within the normal distribution the production of the four cytokines is spread over a wide range,indicating high intersubject variability. There appear to be individuals with high and with low production of particular cytokines *in vitro*.

We have previously examined the intrasubject reproducibility of *in vitro* cytokine production [10]. We demonstrated that *in vitro* production of IL 1β, IL 1α and TNF is a reproducible parameter when assayed in a given individual on different occasions, several days apart. Furthermore, when PBMC from healthy subjects were stimulated in different experiments as far as 25 weeks apart, PBMC from the majority of individuals produced similar amounts of IL 1β when compared between the two time points. Taken together with the results of the present study, there appear to be a few subjects who produce consisently low or consistently high amounts of a particular cytokine within a cohort of healthy subjects. This finding supports the concept of high and low producers in response to particular stimulants and the concept of genetic factors controlling cytokine synthesis. There is a parallel in the mouse model, where certain inbred strains, which are resistant to either Gram-negative or Gram-positive toxins, produce less IL 1 and TNF in response to these toxins. This is an inherited trait that appears to be controlled at the level of translation rather than transcription [24, 25].

Correlation analysis reveals a high correlation between production of IL 1β and IL 1α contrasting a lack of correlation between IL 1β and TNF production within the study cohort. This suggests linked gene regulation for IL 1β and IL 1α, and independent gene regulation for IL 1β and TNF. There was no correlation between IL 2 production and production of either IL 1β or TNF within the study cohort.

There is ample evidence that arachidonic acid metabolites are endogenous regulators of IL 1 production. Macrophage IL 1 production *in vitro* is suppressed by addition of exogenous $PGE_2$ or $PGI_2$ and is augmented by inhibition of the cyclooxygenase pathway [26]. In humans, we have shown that dietary supplementation with eicosapentaenoic acid results in decreased production of IL 1β, IL 1α and TNF [27]. $PGE_2$ appears to reduce expression of IL 1 at the post-transcriptional level by inducing elevated levels of cyclic AMP [15]. When monocytes are stimulated in the presence of cyclooxygenase inhibitors like indomethacin, an enhancement of IL 1 synthesis via suppressed $PGE_2$ production has been observed [15, 16]. In the present study we have observed increased production of IL 1β by an average of 27% when PBMC were stimulated in the presence of 1 μg/ml of indomethacin. This increase did not quite reach significance ($p = 0.055$). We have previously reported increased production of TNF in the presence of indomethacin [12].

We were able to demonstrate that the enhancing effect of cyclooxygenase inhibitors on production of IL 1β also functions *in vivo*. When compared to the cohort without medication, we found that the mean production of IL 1β *in vitro* by stimulated PBMC was significantly higher (48%) in the group taking cyclooxygenase inhibitors.

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