Measurement of Immunoreactive Interleukin-1β from Human Mononuclear Cells: Optimization of Recovery, Intrasubject Consistency, and Comparison with Interleukin-1α and Tumor Necrosis Factor[1]

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Numerous studies have reported altered levels of *in vitro* production of the cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNF) from blood leukocytes in various human disease states. Most of these studies have used bioassays which are vulnerable to inhibitors produced by these cells. Furthermore *in vitro* cytokine production is often assessed on a single occasion. The present study was designed to standardize stimulation conditions for *in vitro* IL-1β production and to employ a competitive radioimmunoassay (RIA) to demonstrate reproducibility and long-term variation of *in vitro* cytokine production in a cohort of healthy human subjects. We also examined relative amounts of immunoreactive IL-1β, IL-1α, and TNF induced by the stimuli endotoxin, phytohemagglutinin, or *Staphylococcus epidermidis*. We show that the RIA can reliably detect IL-1β produced from mononuclear cells in concentrations as low as 115 pg/ml. Lysing cells by repeated freeze-thawing yields maximal recovery of total (i.e., secreted plus cell-associated) immunoreactive IL-1β, when compared to extraction with the detergent CHAPS or addition of protease inhibitors. Repeated measurement of *in vitro* cytokine production on different days within 1 week shows good reproducibility for a given individual and a given stimulus (variation coefficient 20 to 30%). Over a long time period (6 months) *in vitro* cytokine production is stable in some individuals but changes considerably in others. The soluble stimulus endotoxin induces twofold more IL-1α than IL-1β or TNF; in contrast the phagocytic stimulus heat-killed *S. epidermidis* induces fourfold more IL-1β and TNF than IL-1α. This distinct pattern of cytokine response indicates differential stimulation of the mononuclear cells by different stimuli. The results form the basis for studying *in vitro* cytokine production in different human disease states. © 1988 Academic Press, Inc.

INTRODUCTION

Interleukin-1β (IL-1β), interleukin-1α (IL-1α), and tumor necrosis factor (TNF) are polypeptides produced by monocytes and other cells. These cytokines participate at several levels of the immune response and mediate many of the acute phase changes in response to injury, infection, immunologic, or malignant disease (1, 2).

Numerous studies have investigated whether the amount of *in vitro* IL-1 or TNF

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production from mononuclear cells is altered in various human disease states. Decreased IL-1 production has been reported in patients with systemic lupus erythematosus (3, 4), pemphigus vulgaris (5), scleroderma (6), malnutrition (7, 8), fatal sepsis (9), as well as in patients with duodenal ulcer on cimetidine therapy (10). In vitro production of IL-1 appears to be increased in patients with rheumatoid arthritis (11, 12), idiopathic osteoporosis (13), chronic liver disease (14), major burns (15), Hodgkin's disease (16), or tuberculosis (17). For some disease states a "normal level" of *in vitro* IL-1 production when compared to healthy controls has been reported. These include ankylosing spondylitis (18), asthma (19), multiple sclerosis (20), chronic lymphocytosis (21), graft-versus-host disease after allogenic bone marrow transplantation (22), and leprosy (23). Alterations of *in vitro* IL-1 production in patients with human immunodeficiency virus infection have been reported to differ dependent on stage of disease; increased levels of production were found in patients with generalized lymphadenopathy (24), whereas production appeared to be decreased (25) or unchanged (26, 27) in patients with fully developed acquired immunodeficiency syndrome. Increased *in vitro* production of TNF has been reported in some cancer patients (28).

The above-described studies primarily used bioassays to determine the amount of cytokine production following stimulation of blood mononuclear cells. Furthermore, cytokine production was determined for a given individual on a single occasion most often to a single stimulus, usually endotoxin.

The present study pursued the following goals: (i) to define the optimal conditions for *in vitro* cytokine synthesis by human mononuclear cells (MNC); (ii) to optimize recovery of immunoreactive IL-1β from MNC; (iii) to employ a sensitive and specific radioimmunoassay (RIA) that is less vulnerable to inhibitory and potentiating factors of bioassays; and (iv) to determine the reproducibility of *in vitro* IL-1 and TNF production for a given individual at different time points (intrasubject variation). Moreover we compared the relative amount of immunoreactive IL-1β, IL-1α, and TNF produced by MNC in response to different stimuli. These studies form the basis for methods to quantitate IL-1β, IL-1α, and TNF production from human MNC *in vitro* in order to assess elevated or decreased production as part of a disease process.

MATERIALS AND METHODS

Preparation of Anti-IL-1β Antiserum

New Zealand white rabbits (Pine Acres, Burlington, VT) were immunized with 100 μg recombinant human interleukin-1β (hrIL-1β) in complete Freund's adjuvant by intradermal injections as previously described (29). Human rIL-1β was obtained from Cistron (Pine Brook, NJ) and is the 17.5 kDa protein representing the carboxy-terminus of the IL-1β precursor molecule. It is >99% pure by SDS–polyacrylamide gel electrophoresis, has a specific activity of 2×10^6 U/mg, and has alanine N-terminus homogeneity (position 112). At monthly intervals the rabbits received booster immunizations by intramuscular injection of 25 μg hrIL-1β in incomplete Freund's adjuvant. After 29 weeks the rabbits were bled, and the unfractionated serum was pooled and used as anti-IL-1β antiserum.

Radiolabeling of hrIL-1β

Human rIL-1β was labeled as previously described (30) using the chloramine-T method (31). Ten micrograms of hrIL-1β (1 μg/μl, kindly provided by Dr. Alan Shaw, Glaxo Institute of Molecular Biology, Geneva, Switzerland) was mixed with 0.5 mCi Na^{125}I (100 mCi/ml, New England Nuclear, Boston, MA) and was added to 10 μl of 0.5 M sodium phosphate buffer (pH 7.4) in a 500-μl polypropylene tube. Next, 10 μg chloramine-T (Sigma Chemical Co., St Louis, MO; 2.5 mg/ml in 0.25 M sodium phosphate buffer, pH 7.4) was added and mixed by pipetting for exactly 10 sec. The reaction was terminated by adding 20 μl of the reducing agent sodium metabisulfite (Sigma; 5 mg/ml in 0.25 M sodium phosphate buffer, pH 7.4). Finally 300 μl of bovine serum albumin (BSA) buffer containing 0.01 M phosphate-buffered saline, 0.25% BSA (Sigma), and 0.05% sodium azide was added.

The radiolabeled material was immediately chromatographed on a Sephadex G-50 (fine) (Pharmacia, Piscataway, NJ) column (0.8 × 30 cm) which separated the radiolabeled protein from the free ^{125}I. BSA buffer was used to equilibrate the column. Forty fractions (0.5 ml) were collected. The radioactivity of 100 μl of a 1:100 dilution of these fractions was determined in a gamma counter. One hundred microliters of a 1:100 dilution of each fraction was incubated with a 1:100 dilution of the anti-IL-1β antiserum for 18 hr at room temperature, and then was precipitated by adding 500 μl of 6% (w/v) polyethylene glycol 8000 (PEG; Fisher Scientific Company, Fair Lawn, NJ) and 2% sheep anti-rabbit IgG (Cambridge Medical Diagnostics, Billerica, MA). After centrifugation (15 min, 1500g) and decanting, the precipitated radioactivity was counted in a gamma counter. Those fractions in which anti-hrIL-1αβ antiserum precipitated more than 85% of the total counts were pooled and used as the tracer in the RIA. The incorporated radioactivity in this pool was derived by multiplying the total radioactivity used in the labeling reaction by the proportion of bound iodine (area under peak of precipitable iodine) as the percentage of total iodine (combined area of peaks for free and precipitable iodine). The specific activity of the pool was calculated by dividing the incorporated activity by the amount of protein used in the labeling reaction. It ranged between 25 and 40 μCi/μg in several iodinations. This is similar to the specific activity for radioiodinated IL-1β previously reported (30).

The homogeneity of the ^{125}I-hrIL-1β was demonstrated by SDS–polyacrylamide gel electrophoresis (32) and autoradiography. A single band at 17 kDa was observed. ^{125}I-hrIL-1β was biologically active as determined by a comitogenic assay using the murine-cloned T cell line D.10G4.1 (29).

Titration of the Anti-IL-1β Antiserum

[^{125}I]hrIL-1β (10,000 cpm) in 100 μl BSA buffer was incubated with serial twofold dilutions of the anti-IL-1β antiserum for 18 hr at room temperature and then precipitated with 500 μl of 6% PEG and 2% sheep anti-rabbit IgG (Fig. 1). The dilution of anti-IL-1β antiserum that precipitates about 35% of ^{125}I-hrIL-1β (i.e., 1:6400) was chosen for use in the RIA.
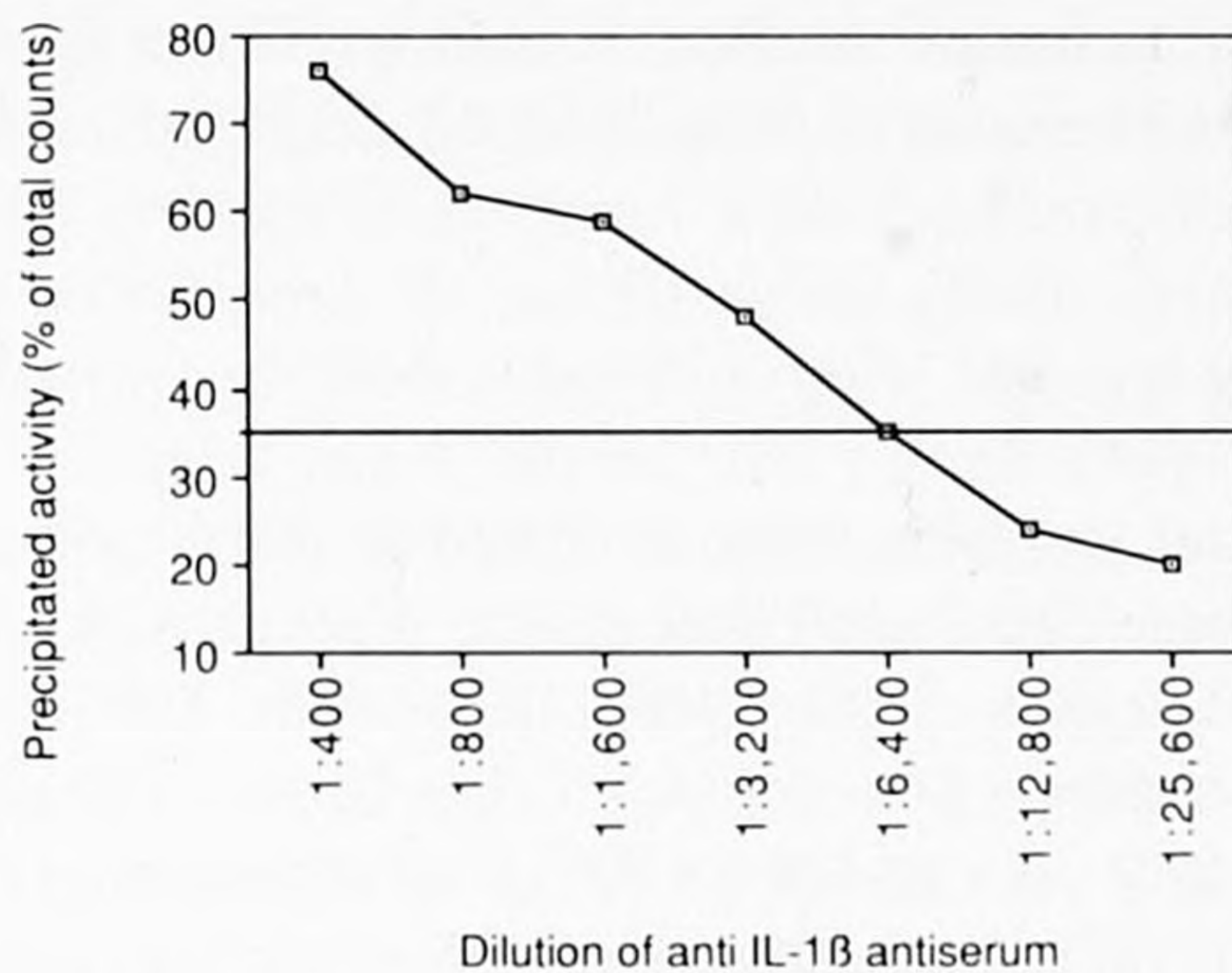
Dilution of anti IL-1β antiserum

FIG. 1. Titration of anti IL-1β antiserum. Serial twofold dilutions of anti IL-1β antiserum were incubated overnight with ^{125}I-hrIL-1β and precipitated with sheep anti-rabbit IgG in PEG. Precipitated γ activity is expressed as the percentage of total counts. The dilution (1:6400) that precipitates about 35% of ^{125}I-hrIL-1β (indicated by the horizontal line) was chosen for use in the RIA.

Separation of Bound from Free ^{125}I-hrIL-1β

To separate bound from free ^{125}I-hrIL-1β, antigen–antibody complexes were precipitated with a sheep anti-rabbit IgG antiserum. Precipitate formation was enhanced by adding normal rabbit serum and polyethylene glycol. To optimize precipitation conditions, 10,000 cpm of ^{125}I-hrIL-1β in 100 μl BSA buffer, 100 μl of anti-IL-1β antiserum, and 300 μl of either 1:300 or 1:600 dilution of NRS were incubated overnight. Bound label was precipitated by adding 500 μl of sheep anti-rabbit IgG antiserum at serial dilutions in 6% or 10% (w/v) PEG amounting to final concentrations of 2.7 or 4.5% (w/v) PEG, respectively (Fig. 2). Maximal precipitation was achieved with a 1:600 dilution of NRS and a 1:50 dilution of sheep anti-rabbit IgG in 6% PEG. These conditions were subsequently used for the RIA. They differ from the previously described procedure (30) by the addition of 300 μl

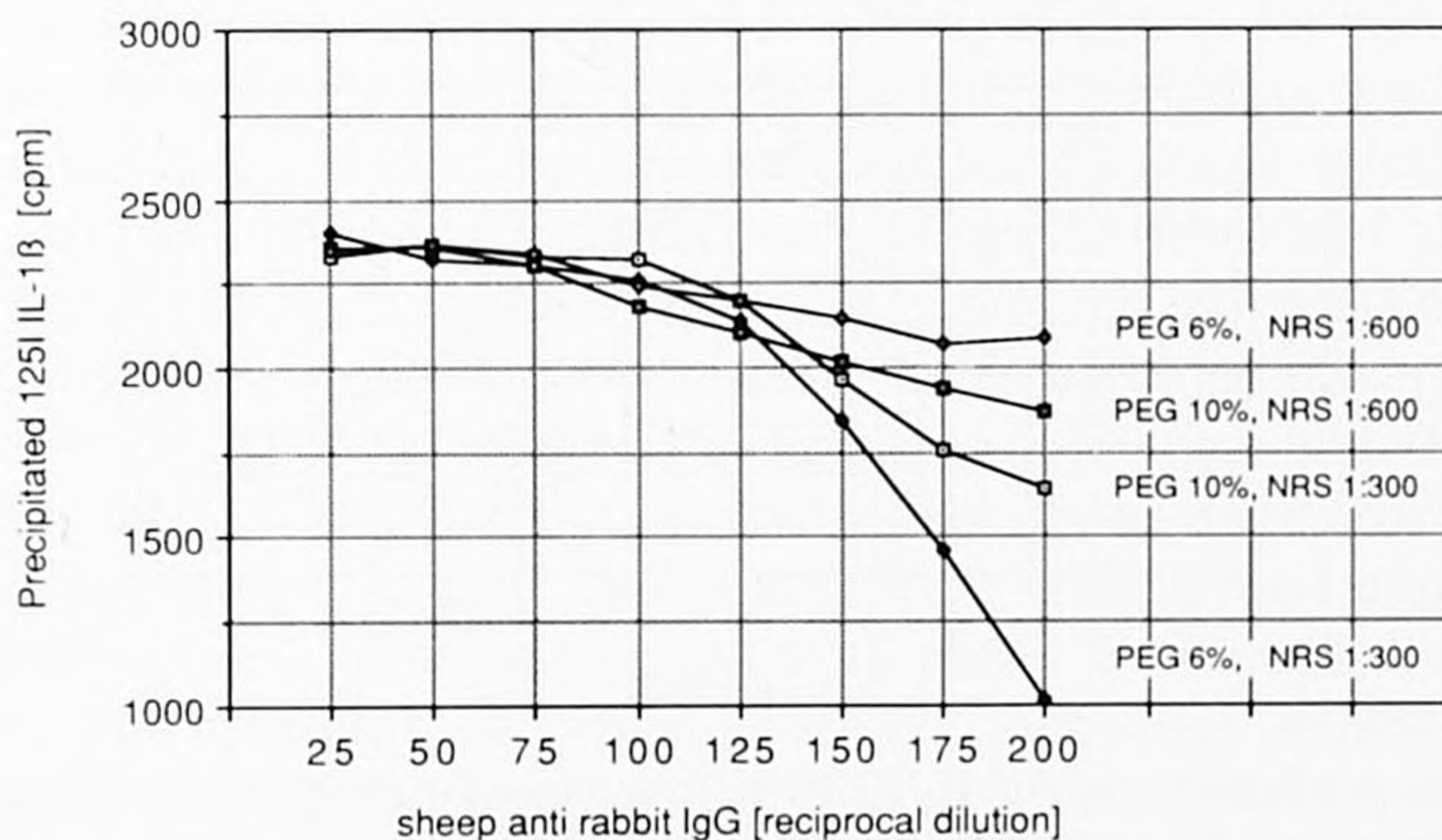sheep anti rabbit IgG [reciprocal dilution]

FIG. 2. Dose response for sheep anti-rabbit IgG antiserum. One hundred microliters of ^{125}I-hrIL-1β, 100 μl of 1:6400 diluted anti IL-1β antiserum and 300 μl of either a 1:600 or a 1:300 dilution of NRS were incubated overnight. Bound label was precipitated by adding 500 μl of sheep anti-rabbit IgG antiserum at serial dilutions in 6 or 10% (w/v) PEG (final dilutions (2.7 or 4.8%, respectively).

RIA buffer as "carrier volume" at Day 1, and by lower final concentrations of sheep anti-rabbit IgG antiserum (0.9 instead of 2%) and of PEG (2.7 instead of 4.8% w/v).

Radioimmunoassay for IL-1β

Known standards and samples were assayed in duplicate or triplicate in 10 × 75-mm polystyrene tubes (Stockwell Scientific, Walnut, CA). In every assay, nine standards of hrIL-1β (10,000, 5000, 2500, 1250, 625, 313, 157, 79, 40, and zero pg/ml) diluted in BSA buffer were employed. On Day 1, 100 μl of the anti-hrIL-1β antiserum (diluted 1:6400) was added to 100 μl of the standards or to 100 μl of the samples. To one pair of tubes 100 μl of BSA buffer was added instead of anti-IL-1β antiserum in order to determine nonspecific binding (NSB) of the tracer. BSA buffer (300 μl) containing a 1:600 dilution of heat-inactivated normal rabbit serum was added to each tube. The tubes were vortexed and incubated at room temperature overnight. On Day 2, 100 μl of BSA buffer containing 10,000 cpm of ^{125}I-hrIL-1β was added to each tube; after vortexing the tubes were again incubated at room temperature overnight. On Day 3, 500 μl of BSA buffer containing 6% of PEG and 2.0% sheep anti-rabbit IgG were added. The tubes were vortexed and centrifuged at 1500g for 15 min in room temperature. The supernatants were decanted, and the tubes were kept inverted and allowed to drain on absorbent paper for 15 min before being counted for radioactivity.

The mean γ activity (cpm) of duplicate or triplicate standards was calculated and the counts of the NSB were subtracted. Counts of all standards and samples were expressed as the percentage of the standard containing no IL-1β (zero standard = 100% binding). A standard curve was created by plotting the values (in percentage) of the standard samples against the concentration, on logit vs logarithmic graph paper (Team Papers, Tamworth, NH) (Fig. 3). Detection limit of the
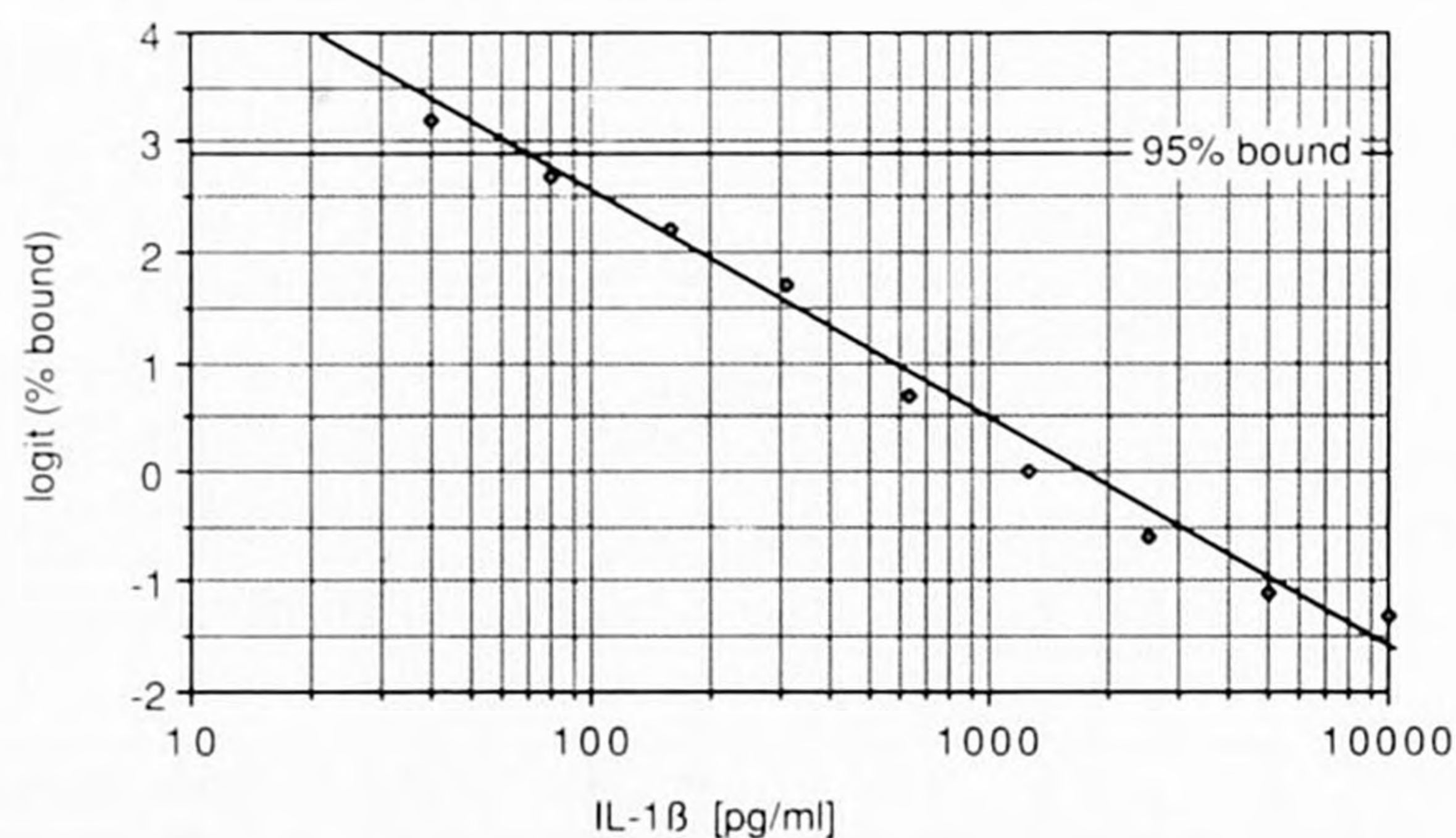

FIG. 3. Typical standard curve for IL-1β RIA. Precipitated activity (cpm) of standards was expressed as the percentage of activity precipitated by anti IL-1β in the absence of cold IL-1β (zero standard = 100% binding). A standard curve was created by plotting the values in the percentage of the standard samples against the known concentration on logit vs logarithmic graph paper. The logit function (logit b = ln $[b/(100-b)]$) transforms a sigmoid to a linear-binding curve. The horizontal line indicates the detection limit which was set at 95% of zero standard.

assay was set at binding ≤95% of zero standard. In one series of 13 consecutive RIA's the median detection limit was 115 pg/ml (range 35 to 250 pg/ml).

The RIA for hrIL-1β did not cross-react with the human recombinant proteins at the indicated concentrations: 10 or 100 ng/ml IL-1α (kindly provided by Dr. Alan Shaw, Glaxo Institute of Molecular Biology), 100 or 1000 U/ml interleukin-2 (spec act 10^6 U/mg, Cetus Corp.), 10 or 100 ng/ml TNF (kindly provided by Biogen, Cambridge, MA), 100 or 1000 U/ml interferon-α (spec act 10^7 U/mg, Schering Corp., Kenilworth, NJ), 100 or 1000 U/ml of interferon-γ (spec act 10^7 U/mg, Schering Corp.), 0.5 or 5 ng/ml granulocyte monocyte colony-stimulating factor (Genetics Institute, Cambridge, MA), and 50 or 100 ng/ml of complement component C5a des arg (kindly provided by Dr. K. B. Yancey, Uniformed Services University of the Health Sciences, Bethesda, MD).

Radioimmunoassays for human IL-1α (33) and TNF (34) were performed as described.

In Vitro Mononuclear Cell Stimulation

Venous blood was drawn into heparinized (20 U/ml) syringes. At the same time, a sample of unheparinized blood was taken in order to prepare heat-inactivated (56°C, 40 min) serum. The MNC fraction was obtained by density centrifugation of diluted heparinized blood (one part blood to two parts pyrogen-free normal saline) over Ficoll–Hypaque (spec wt 1.077 g/ml). This was prepared by dissolving Ficoll (Type 400, Sigma) with Hypaque (Hypaque-M, 90%; Winthrop–Breon Lab., New York, NY) in ultrafiltered water. MNC were washed twice in 0.15 M NaCl, slides were prepared by cytocentrifugation, and 100 cell differential counts were performed by light microscopy on Wright stained slides. 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 subjected to ultrafiltration in order to remove endotoxins as previously described (35). MNC were suspended at 5 × 10^6 MNC/ml in RPMI with 2% heat-inactivated autologous serum and 100 μl were aliquoted in 96-well flat-bottom microtiter plates. An equal volume of either RPMI or RPMI containing various stimulants was added. The cells were stimulated with endotoxin (lipopolysaccharide *Escherichia coli* 055:B5, Sigma L-2880; aliquots stored at 1 μg/ml stock concentration) at 1 and 10 ng/ml; phytohemagglutinin (PHA-P; Difco, Detroit, MI; aliquots stored at 600 μg/ml) at 3 μg/ml; or heat-killed, opsonized (with 2.5% fresh autologous serum) *Staphylococcus epidermidis* at 20 bacteria:MNC. Each stimulus was added to triplicate wells. After 24 hr at 37°C in 5% CO_2, the microtiter plates were frozen at −70°C until the end of the study. At that time plates were thawed and exposed to two more freeze–thaw cycles, simultaneously for all plates from each donor, to complete cell lysis. The contents of triplicate wells, consisting of cell lysates and supernatants, were pooled and refrozen for IL-1 and TNF determinations. Alternatively, the cells were treated with the detergent 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), aprotinin, or phenylmethylsulfonyl fluoride (PMSF; all from Sigma) during the freeze–thaw cycles.

RESULTS

Solubilization of IL-1β

We examined different procedures to prepare total (i.e., secreted plus cell-associated) immunoreactive IL-1β from MNC at the termination of the incubation period. Commonly, MNC cultures are lysed by freeze–thawing and the lysates are directly assayed in the RIA. Several issues regarding this procedure were addressed: (i) Does removal of cell debris from the lysates by short, high-speed centrifugation affect the recovery of IL-1β? (ii) Can more IL-1β be solubilized by adding the detergent CHAPS to the lysates as compared to repeated freeze–thawing? and (iii) Does addition of the protease inhibitors aprotinin and PMSF increase recovery of IL-1β by reducing proteolytic degradation?

MNC from three healthy donors were stimulated in two microtiter plates as described above. The cells were stimulated identically with 10 ng/ml of endotoxin. After 24 hr the incubation was stopped by freezing at $-70°C$. Thereafter the wells were treated according to three different protocols.

(i) "FT" (freeze–thaw): The plate was subjected to two more cycles of freezing (60 min, $-70°C$) and thawing (60 min; 37°C); after the last thaw the wells were mixed and the content of two wells (200 µl) was transferred into a 1.5-ml microfuge tube; one of the tubes was kept for direct assay by RIA; the other tube was spun in a microcentrifuge (13,000g 5 min) and 180 µl of the supernatant was transferred into a new tube for later assay by RIA.

(ii) "FT/AP" (freeze–thaw, aprotinin, PMSF): After thawing, 22 µl of aprotinin (5 trypsin inhibitory units [TIU]/ml) and PMSF (10 mM) in RPMI was added to each well and the plate was subjected to the same procedures described for FT above;

(iii) "CH/AP" (CHAPS, aprotinin, PMSF): After thawing the plate was placed on ice; 22 µl of CHAPS (90 mM), aprotinin (5 TIU/ml), and PMSF (10 mM) in RPMI were added to each well; after 30-min incubation on ice 200 µl from each of two wells was transferred into one microfuge tube containing 200 µl of ice-cold BSA buffer. From one of the tubes cell debris was removed as for FT wells.

Triplicate wells were prepared by each of the three protocols for each donor. Samples were measured for immunoreactive IL-1β in duplicate by RIA. Preparation of IL-1 standards in medium containing the same final concentrations of CHAPS (9 mM), PMSF (0.5 mM), and aprotinin (0.5 TIU/ml) as the samples did not significantly change the standard curve of the RIA (data not shown).

As shown in Fig. 4, for all three donors maximal immunoreactive IL-1β was recovered when the MNC samples were subjected to three freeze–thaw cycles without removing cell debris. Removing cell debris reduced measurable IL-1β, probably due to IL-1β associated with fragments of cell membranes. Even though this membrane-associated IL-1β is not solubilized, its epitopes probably bind with the polyclonal antiserum in the RIA. In the presence of protease inhibitors, improved recovery of IL-1β was not observed. In fact, the presence of these inhibitors reduced the amount of measurable IL-1β in the samples where cell debris
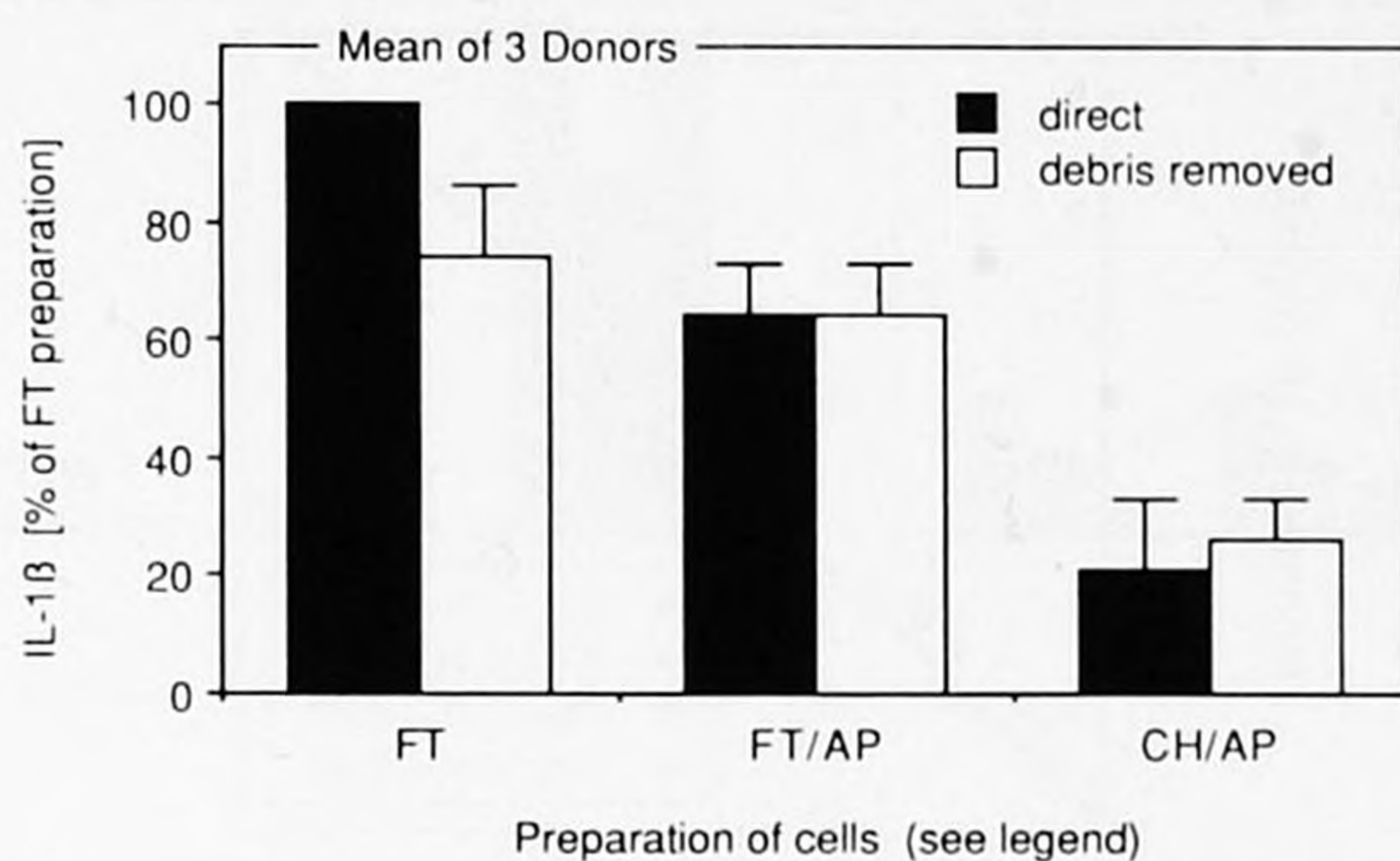
FIG. 4. Recovery of immunoreactive IL-1β for different preparations of mononuclear cells after incubation. MNC were stimulated with 10 ng/ml endotoxin for 24 hr. At the end of the incubation period cells were prepared according to different protocols (for details, see text): "FT", three freeze–thaw cycles; "FT/AP", addition of aprotinin and PMSF after first of three freeze–thaw cycles; "CH/AP", addition of the detergent CHAPS, aprotinin, and PMSF. All samples were assayed directly or after removing cell debris by centrifugation (13,000g, 5 min). Bars indicate mean ± SEM for three donors expressed as the percentage of maximal recovery (FT preparation).

had not been removed. This may indicate that the action of proteases actually increases the recovery of immunoreactive IL-1β from samples which contain cell debris. It has previously been shown that trypsin can liberate IL-1 from membranes (36). Surprisingly, the lowest recovery occurred with the addition of CHAPS detergent. This agent has been shown to liberate IL-1 from membranes (36). However, it may also lead to partial denaturation of the IL-1β molecule as indicated by reduced bioactivity (37). The low values measured in the presence of CHAPS are not due to interference of the detergent with the antigen–antibody binding in the RIA since we did not observe a shift of the standard curve in the presence of 9 mM CHAPS. In our subsequent studies, the protocol using three freeze–thaw cycles was employed for preparing MNC for total IL-1β measurement.

Interassay Reproducibility

To determine interassay reproducibility, MNC from one donor were stimulated with endotoxin, PHA, or heat-killed *S. epidermidis* at several time points over a period of 6 months. At the end of 6 months, samples were aliquoted in duplicate tubes and stored at −70°C. IL-1β was measured by RIA at two time periods: 1 or 4 months later. In the first assay, samples for all stimulation conditions were assayed in a single RIA. In the second assay, 3 months later, the samples were assayed in four different RIAs, separate for samples from each stimulation condition. Each of these latter RIAs were compared to the results of the RIA performed during the first month (Fig. 5) and a correlation coefficient was calculated by the least squares method (Cricket Graph 1.0, Cricket Software, Philadelphia, PA). The individual correlation coefficients between the first RIA and the four RIAs 3 months later are depicted in Fig. 5. The correlation coefficient for the pooled data pairs is $R = 0.97$. The average ratio of concentrations measured at Month 4 vs Month 1 was 0.94. Thus, the RIA yields highly reproducible results.
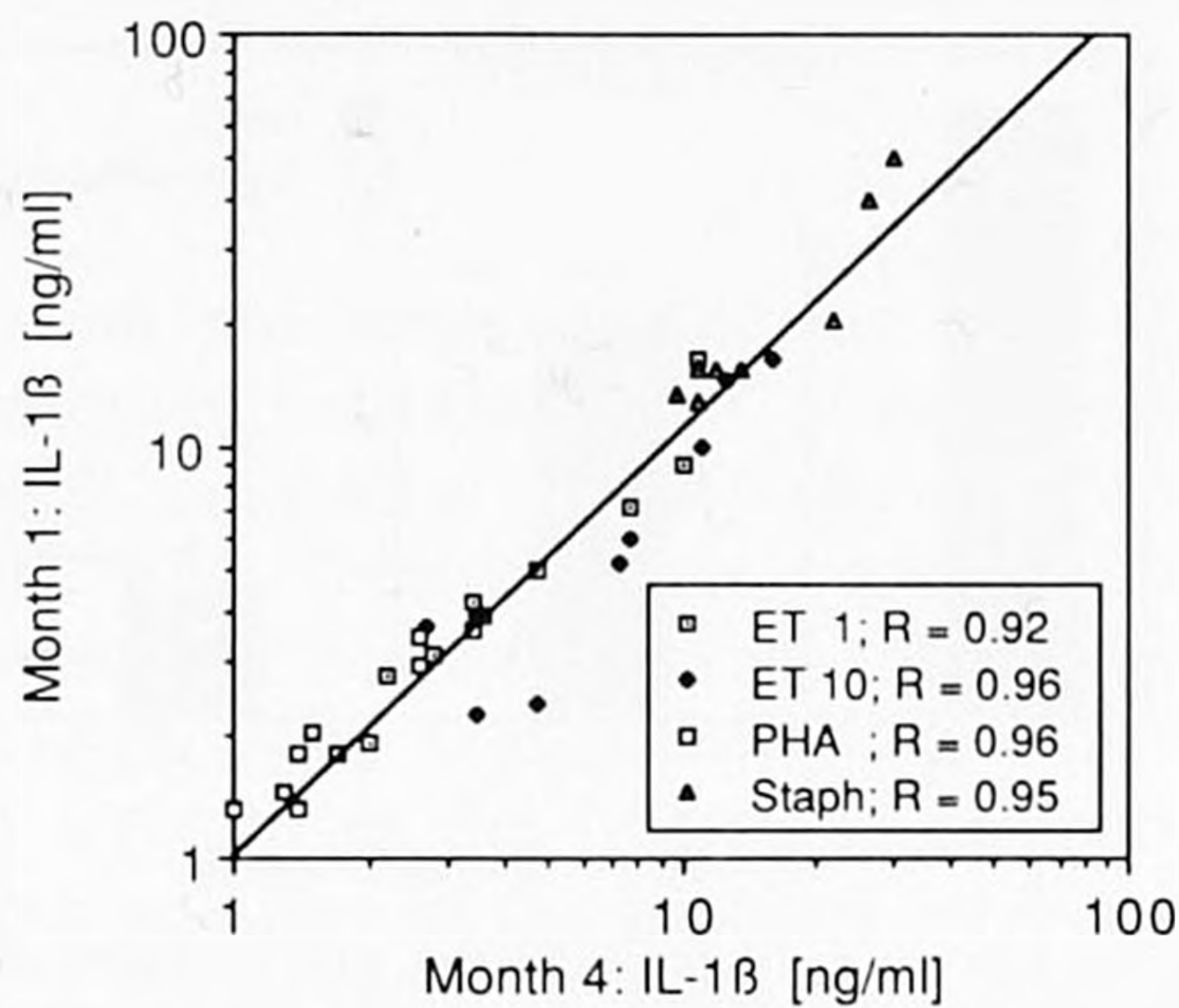
FIG. 5. Interassay reproducibility and stability of natural IL-1β at −70°C. Thirty-six samples of MNC (stimulated with four different stimuli at different times) from one donor were stored in duplicate tubes at −70°C and assayed at two different occasions 3 months apart. Result of assay during Month 1 versus result of four different assays (one for each stimulus) during Month 4 is plotted for each sample. The correlation coefficients between the assay during Month 1 and the individual assays during Month 4 are indicated. The combined correlation coefficient for all data points is 0.97. The slope of the diagonal line indicates the mean ratio (0.94) of measurement during Month 4 over measurement during Month 1.

The data also demonstrate that samples can be stored at −70°C for at least 3 months without appreciable loss of recovery.

Intrasubject Consistency

To assess intrasubject consistency, MNC from six healthy donors were stimulated on 3 different days, each 2 to 3 days apart. Four different stimulation conditions were used (endotoxin, 10 and 1 ng/ml; PHA, 30 μg/ml; and heat-killed *S. epidermidis*). Immunoreactive IL-1β, IL-1α, and TNF were measured. Undiluted samples from unstimulated (control) MNC consistently contained cytokines below the detection limit of 115 pg/ml (IL-1β), 40 pg/ml (IL-1α), and 20 pg/ml (TNF). The levels of IL-1β for the six different donors are depicted in Fig. 6. The levels are consistent for the repeated determinations on different days; the average ($n = 24$) coefficient of variation (SD/mean) is 29%. Production of IL-1α and TNF was equally consistent (individual data not shown). The average coefficient of variation was 20% for TNF and 30% for IL-1α. The average proportion of monocytes in the MNC population (after Ficoll–Hypaque separation), assessed by light microscopy, was 21%. We found no correlation between the proportion of monocytes in the MNC population and the level of IL-1β produced. This supports the recent findings that in addition to monocytes, natural killer cells (38, 39), and B cells (40) produce IL-1 and TNF (41) in response to endotoxin or PHA.

MNC from nine donors were again stimulated 26 weeks later, again on 3 different days, 2 to 3 days apart. Concentrations of IL-1β induced by 1 ng/ml endotoxin are plotted in Fig. 7 (mean ± SEM for determination on 3 different days). Six of the donors remained in the same range of low, intermediate, or high pro-
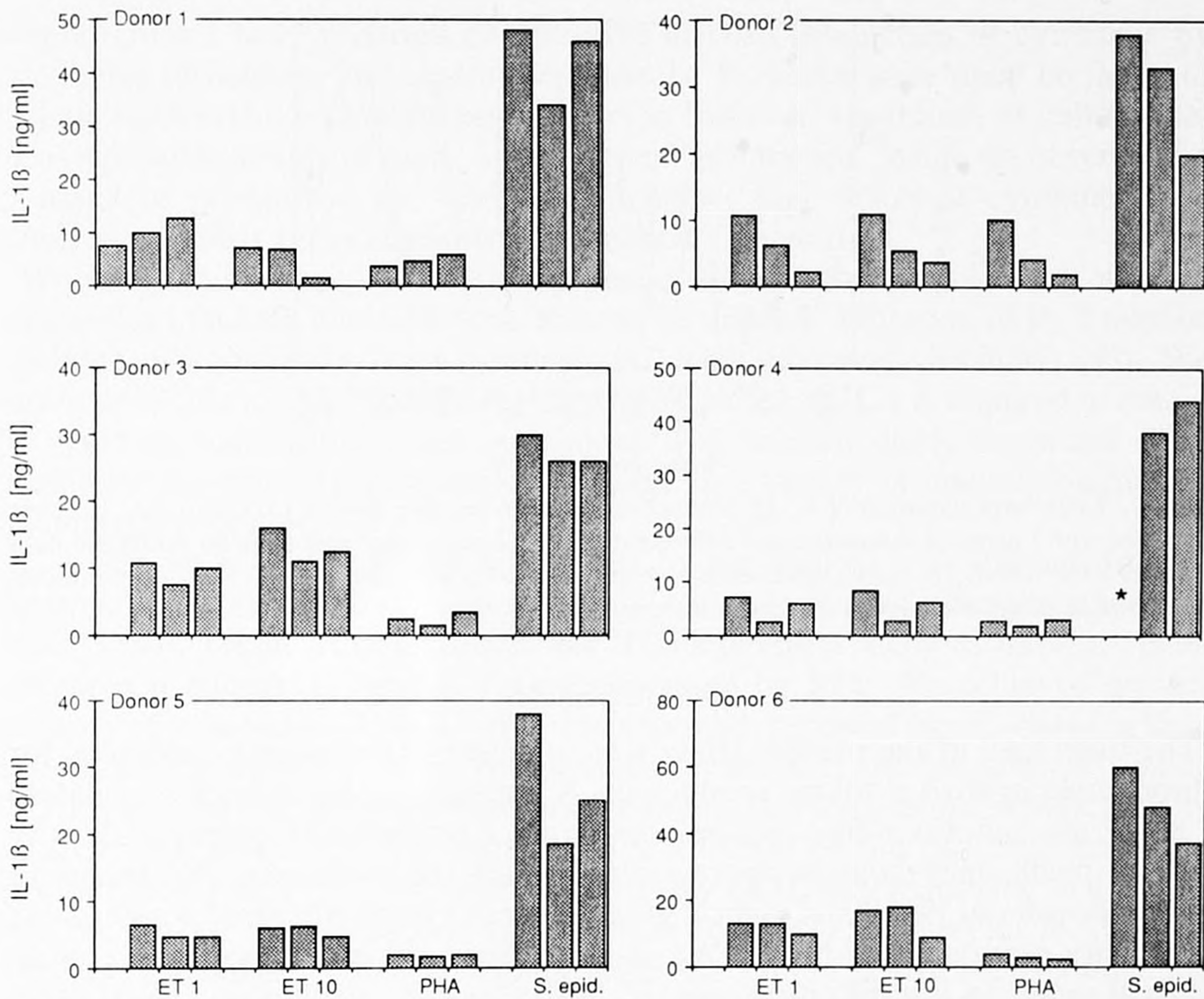
FIG. 6. Intrasubject consistency for *in vitro* production of IL-1β. MNC from six healthy donors were stimulated on 3 different days, 2 to 3 days apart. Four different stimulation conditions were used: 1 ng/ml of endotoxin (ET 1), 10 ng/ml of endotoxin (ET 10), 3 μg/ml of PHA, and heat-killed *S. epidermidis* (20 organisms/MNC). Total, i.e., cell-associated plus secreted, IL-1β was measured by RIA. * Denotes sample not tested for donor 4.

ducer, respectively, after this time period. MNC from donors 4 and 9 produced more IL-1β at the end of the study period, while the cells from donor 6 showed a decreased response when compared to the baseline determination.

Relative Amounts of Cytokines

Figure 8 compares induction of cytokine production by the different stimuli employed. Each value represent the mean of 18 measurements (six donors on 3 different days each). There appears to be a strikingly discrete pattern for each stimulation condition. Endotoxin induces twofold more IL-1α than IL-1β or TNF, which are produced in equal amounts. When PHA is used as a stimulus, concentrations of IL-1α and TNF are comparable to endotoxin stimulation, while only half as much IL-1β is produced. The phagocytic stimulus heat-killed *S. epidermidis* induces markedly higher levels of total cytokine, with a fourfold prevalence of both IL-1β and TNF over IL-1α.
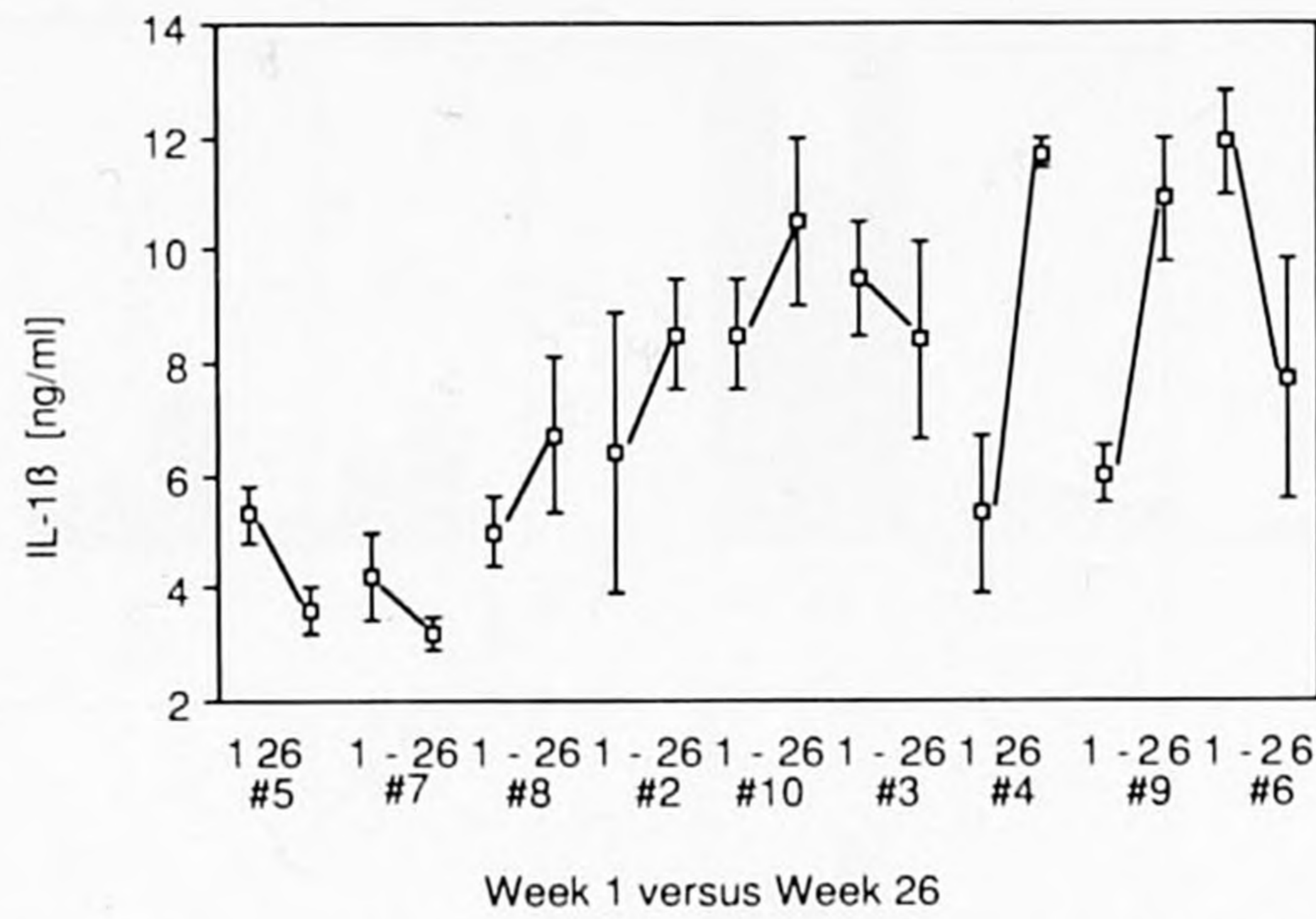
FIG. 7. Long-term variation of IL-1β production. MNC from nine donors (No. 2 to No. 10) were stimulated with 1 ng/ml of endotoxin on 3 different days, 2 to 3 days apart, and again on 3 different days 25 weeks later. Total, i.e., cell-associated plus secreted, IL-1β was measured by RIA. Bars indicate mean ± SEM of determinations on three different days.

DISCUSSION

The main goal of the present study was to define experimental conditions for reproducible *in vitro* cytokine production. A prerequisite for defined stimulation of MNC are endotoxin-free culture conditions. Commercially prepared density gradient media may contain appreciable amounts of endotoxin. We therefore prepare powdered Ficoll and clinical grade Hypaque in ultrafiltrated water to use for density separation of MNC. We also routinely subject commercial culture media through an ultrafiltration unit (U 2000, Gambro, Hechingen, FRG). This removes endotoxin both by molecular weight exclusion (cut-off 30,000 D) and by adsorption to the large polyamide surface (35). Since fetal calf serum is also a possible source of endotoxin we use heat-inactivated autologous or human AB serum as a serum supplement. These measures enable us to culture MNC without significant activation of the cells. Cytokine production in control wells without addition of a stimulus was consistently below the detection limit of the RIAs.
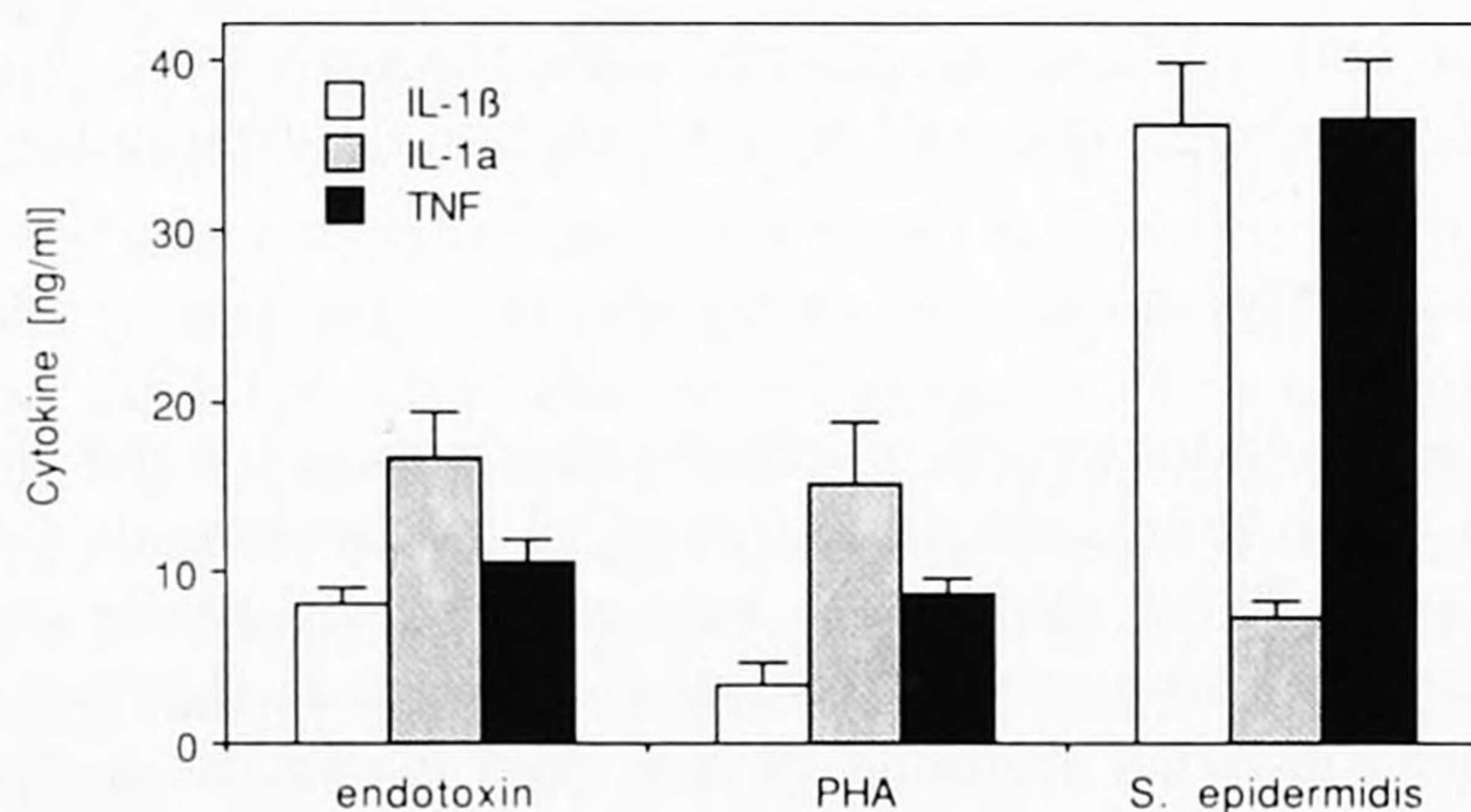

FIG. 8. Relative amounts of IL-1β, IL-1α, and TNF in response to different stimuli. MNC were stimulated with endotoxin 10 ng/ml, PHA 3 µg/ml, or heat-killed *S. epidermidis* (20 organisms/MNC) for 24 hr and total, i.e., cell-associated plus secreted, cytokine production was assayed by RIA. Each value represents the mean of 18 measurements (six donors on 3 different days each).

Several studies have reported constituitive *in vitro* production of cytokines by monocytes of patients and healthy individuals. Particular care must be taken to exclude inadvertent endotoxin contamination from cell separation or culture media as a possible source of such "spontaneous" production. While we observed no spontaneous production we were able to induce high levels of cytokine (1–10 ng/ml) by minimal concentrations of endotoxin (1 ng/ml).

With the availability of RIAs, the optimal conditions for the preparing of MNC cultures for cytokine measurements have to be defined. Secretion of IL-1 may be regulated independently from synthesis (42) and selectively inhibited (43). We thus believe that total (i.e., cell-associated plus secreted) IL-1 is required to assess the synthetic capacity of a cell population. This is particularly important since membrane-bound IL-1β is biologically active in a variety of immunological and inflammatory models.

IL-1 can be solubilized from intact cells by mechanical lysis through freeze–thawing, sonication (42), or homogenizing (36) or by use of detergents such as CHAPS (36), Triton X, or digitonin (44). These different methods have not been compared in preparing samples for measurement by RIA. We achieved greater recovery of immunoreactive IL-1β by lysis through repeated freeze–thawing than by solubilizing using CHAPS. The reduced levels of IL-1β we observed using CHAPS may be due to partial denaturation of the IL-1β molecule as indicated by reduced bioactivity (42). Furthermore, the addition of the protease inhibitors aprotinin and PMSF did not improve recovery of IL-1β. Removing cell debris from the lysates by high-speed centrifugation decreased recovery of IL-1β.

Using the conditions that achieve maximal recovery of IL-1β, we examined the reproducibility of *in vitro* cytokine production for individual subjects on different days, 1 or 2 days apart. We found consistently reproducible concentrations of IL-1β, IL-1α, and TNF. The average coefficient of variation for repeated measurements was 29% for IL-1β, 30% for IL-1α, and 20% for TNF. These values comprise the cumulative variation from two sources: first, the temporal variation due to measurement on different days; second, the variation due to measurement error in repeated measurements. From the format of the study we cannot dissect what relative share of the variability stems from either of these two sources. However, we can conclude that neither of the two sources of variation amounts to more than the observed level. This implies that *in vitro* cytokine production is a biological parameter that is both technically reproducible and stable within one subject over the time span of several days. Technical reproducibility and temporal stability are required to investigate altered cytokine production in different disease states. We found reproducible levels of induced cytokines for separate stimulations as far as 6 months apart (Fig. 7). This indicates the preserved potency of the stimuli used when stored in sufficiently high concentrations at −70°C.

In this study we show that each of the cytokines are produced at different levels in response to different stimuli. For the soluble stimuli, endotoxin and PHA, a roughly similar pattern was found (Fig. 8). Surprisingly, using these stimuli, twice as much IL-1α as IL-1β was produced despite the well-established abundance of IL-1β mRNA over that of IL-1α mRNA in MNC (45, 46). Heat-killed *S. epi-dermidis* as a phagocytic stimulus induced a distinctly different pattern of cyto-

kine production. IL-1β and TNF production were fivefold higher than IL-1α production. This finding supports the concept of differential cytokine gene expression in MNC in response to different stimuli. It has been previously reported that relatively more IL-1 remains cell associated upon stimulation with endotoxin as compared to a phagocytic stimulus (42). On the other hand, the cell-associated compartment appears to be mainly IL-1α, while secreted IL-1 is mostly the IL-1β form as determined by physicochemical characterization (37) and by immunoreactivity (33, 39, 47).

For different stimulation conditions, IL-1β and TNF production thus appears to be correlated while IL-1α production changes independently. This is in contrast to the intersubject correlation of cytokines: in another study (48) we have examined *in vitro* IL-1β, IL-1α, and TNF production in a large cohort of normal volunteers. From individual to individual IL-1β and IL-1α production appeared to be linked, whereas TNF production showed no correlation with either IL-1β or IL-1α.

In summary, we observed a parallel variation of IL-1β and TNF production for different stimuli, and a parallel variation of IL-1β and IL-1α production for different individuals. The gene for IL-1β is localized on chromosome 2 of the human genome (49); in the murine genome the genes for IL-1β and IL-1α are tightly linked on chromosome 2 (50). Correlation of cytokine production suggests dependent regulation of the respective genes. Whether this linkage of regulation occurs at the transcriptional or the translational level remains to be investigated.

The finding of consistently high and consistently low cytokine production for some subjects supports the concept of high and low responders to particular stimulants and the concept of genetic factors controlling cytokine gene expression. Recently, the human TNF gene has been located in the HLA region on the short arm of chromosome 6, probably closely linked to the HLA-B locus (51). This chromosomal location raises the possibility of a linkage disequilibrium between different HLA-haplotypes and polymorphisms of the TNF gene. One can speculate on high and low TNF producer phenotypes as a possible link between HLA alleles and some HLA haplotype-associated diseases, such as ankylosing spondylitis and insulin-dependent diabetes mellitus (52).

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