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A Radioimmunoassay for Human Interleukin-1α: Measurement of IL-1α Produced In Vitro by Human Blood Mononuclear Cells Stimulated with Endotoxin

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ABSTRACT

A specific radioimmunoassay (RIA) for human interleukin-1α (IL-1α) which detects less than 25-50 pg/ml IL-1α is described. Although human IL-1α shares structural homology, receptors and multiple biological properties with IL-1β, this RIA does not detect human IL-13 or other human cytokines. We recovered nearly 100% of IL-1α added to fresh human heparinized blood or freshly voided urine; in contrast, using a specific RIA for IL-1β, recovery of IL-1β added to fresh blood is approximately 50% reduced by nonspecific factors. In the present study, we employed this RIA to measure the amount of total (extracellular and cell-associated) immunoreactive IL-1α produced by human blood mononuclear cells stimulated in vitro by different concentrations of endotoxin. Using ultrafiltered culture medium to reduce endotoxin content, there was no detectable (less than 50 pg/ml) IL-1α produced after 24 hours. Endotoxin (0.5 ng/ml) induced a mean concentration of 900 pg/ml (range 180-1660 pg/ml). At higher-concentrations of endotoxin (500 ng/ml), a mean of 6,990 pg/ml (range 415-11,900 pg/ml) was produced. These levels were comparable to the amount of IL-1β produced under similar culture conditions. The results indicate that IL-1α can be measured independently of IL-1β in human body fluids and from human mononuclear cells.

INTRODUCTION

Polypeptide products of mononuclear macrophages are important mediators of the host responses to infection, inflammation or immunological challenge. Cell culture assays and animal models
demonstrate many overlapping biological activities of these cytokines including fever, T cell activation, induction of liver acute phase protein synthesis, osteoclast activation, chemotaxis, catabolic effects on muscles and connective tissue remodelling (reviewed in 1). Because of these multiple effects, the proteins were named according to their predominant activity: for example, endogenous pyrogen, leukocyte endogenous mediator, lymphocyte activating factor, osteoclast activating factor catabolin and hemopoietin-1 (2). Active cytokines had a molecular weight of 12-17 kD and further purification suggested that the various effects were caused by a small group of related proteins rather than many different molecules. Today these include tumor necrosis factor (TNF) and interleukin-6.

In 1979 the term interleukin-1 (IL-1) was given to a family of polypeptides (3). Two different IL-1 molecules were cloned, an acidic form (IL-1α; isoelectric focussing point pl 5)(4), and a neutral form (IL-1β; pl 7)(5). Although IL-1α and IL-1β share biological activities, they are different proteins transcribed and translated from two different genes with an amino acid homology of only 28%. Several studies indicate that IL-1β predominates at the RNA level and that expression of IL-1α can be two orders of magnitude less than IL-1β. In fact, in some cells, gene expression of IL-1α requires protein synthesis inhibitors. Biological assays using non cross-reacting anti-human IL-1α and IL-1β antibodies indicate that the β-form is the predominant IL-1 in humans (6). However, in order to better understand the role of the two IL-1's in various diseases it is necessary to quantitatively differentiate the amount of IL-1α and IL-1β produced by mononuclear phagocytes. A radioimmunoassay (RIA) for IL-1β has been reported (7). We here report the development and use of a highly specific and sensitive radioimmunoassay for IL-1α. A rabbit anti-human recombinant IL-1α (hrIL-1α) antiserum was used. This antiserum does not cross-react with hrIL-1β (8). The sensitivity of the assay is 25-50 pg/ml hrIL-1α with a confidence limit of 95%.

MATERIALS AND METHODS

Preparation of rabbit anti-human recombinant IL-1α (hrIL-1α) antiserum. New Zealand white rabbits were immunized with 100 μg (hrIL-1α) in incomplete Freund's adjuvant. Rabbits received booster immunizations in incomplete Freund's adjuvant every 4 weeks. hrIL-1α used in these studies was obtained from 4 sources: Genzyme Corp., Boston, MA; Danippon Co., Osaka, Japan; Hofmann-LaRoche, Nutley, NJ and Biogen, Geneva, Switzerland. After 29 weeks the animals were bled and the serum was used. This antiserum did not crossreact with hrIL-1β (Cistron Technology Inc., Pine Brook, NJ), interleukin-2 (Cetus Corp., Emoryville, CA), TNF (Genentech Inc., South San Francisco, CA), hr-α-interferon, hr-γ-interferon (both from Schering Corp., Kenilworth, NJ), hr-granulocyte-macrophage colony stimulating factor (Genetics Institute, Cambridge, MA) or complement component C5a des arg (kindly provided by Dr. K. B. Yancey, Uniformed Services University of the Health Sciences, Bethesda, MD).
Fractions

Figure 1. G-50 gel-filtration of labeled hrIL-1α. An aliquot from each fraction was incubated with rabbit anti-human IL-1α and then precipitated with goat anti-rabbit IgG. Open squares represent cpm's in 5 μl of each fraction. Black diamonds represent the cpm's of each fraction which were precipitated by anti IL-1α antiserum.

Iodination and purification of labeled hrIL-1α. hrIL-1α was labeled by the chloramine T method (9). 5 μg hrIL-1α and 0.5 mCi 125Iiodine (100 mCi/ml, New England Nuclear) were added to 10 μl 0.5 M sodium phosphate buffer (pH 7.4) and mixed. 10 μg chloramine T (Sigma) were added and manually mixed for exactly 20-30 seconds. The reaction was terminated by adding 100 μg of the reducing agent sodium metabisulfite (Sigma).

The 125I IL-1α sample was chromatographed on a Sephadex G50 column (1 x 30 cm) equilibrated and run in BSA buffer (0.25% bovine serum albumin, BSA, Sigma) in 0.01 M phosphate buffered saline containing 0.05% sodium azide) to separate bound from unbound 125I. BSA-buffer was used to equilibrate the column and to run the sample. 25 fractions (0.7 ml) were collected and counted in a gamma counter. Two peaks of 125I activity were detected (Fig. 1). The first peak contains the 125I labeled IL-1α and the second peak the free iodine. A volume of each fraction containing approximately 25,000 cpm was incubated with an excess amount of rabbit anti-hrIL-1α antiserum to determine the percentage of specific binding to the antiserum. After 24 hours, each fraction was precipitated with sheep anti-rabbit IgG (Sigma) in 6% polyethylene glycol. Fractions in which the antiserum precipitated more than 85% of the total counts were pooled (Fig. 1). The specific activity following each labelling procedure ranged from 15 to 30 μCi/μg. To confirm the homogeneity of 125I IL-1α, SDS-polyacrylamide gel electrophoresis and autoradiography were performed (Fig. 2).

Anti hrIL-1α antiserum titration. Samples containing approximately 15,000 cpm of 125I IL-1α in 100 μl BSA-buffer were incubated for 18 hours at room temperature with serial dilutions of rabbit anti hrIL-1α.
Figure 2. Homogeneity of \(^{125}\text{I}}\)-1α: Sodium-dodecyl-sulfate polyacrylamide gel-electrophoresis (15%) of the pooled fractions of \(^{125}\text{I}}\)-1α shown in Fig. 1.

antiserum followed by precipitation with sheep anti-rabbit antiserum. Fig. 3 shows the titration curve. The 1:25,600 dilution of anti IL-1α antiserum bound 45% of the total cpm present in the incubation. We used a 1:22,500 dilution of the anti IL-1α antiserum in the RIA.

Figure 3. Rabbit anti human IL-1α anti serum titration curve. Data points represent the amount of \(^{125}\text{I}}\)-IL-1α precipitated by anti IL-1α. Results are expressed in per cent of total cpm.

RIA for hIL-1α. All standards and samples were assayed in duplicates in 10 x 75 mm polystyrene tubes. Eleven standards of hIL-1α containing 10, 5, 2.5, 1.25, 0.625, 0.313, 0.156, 0.078, 0.039, 0.020 and zero ng/ml were measured in each assay. On day 1, 100 µl of standards in BSA-buffer, heparinized plasma, or urine and 100 µl of samples were incubated with
100 µl of 1:22,500 dilution of anti IL-1α antiserum in BSA-buffer. Each tube also contained 300 µl of BSA-buffer containing 0.3% heat-inactivated normal rabbit serum (NRS). This mixture was vortexed and incubated for 18 hours at room temperature. On day 2, 100 µl of 125I IL-1α solution containing approximately 10,000 cpm were added to each sample, the mixtures were vortexed and incubated another 24 hours at room temperature. On day 3, 700 µl of BSA-buffer containing 9% polyethylene glycol and a 1:70 dilution of sheep anti-rabbit IgG (Sigma) were added. The samples were vortexed and centrifuged at 1500 g for 15 min in room temperature. Thereafter, the fluid phase of each sample was decanted, the tubes were kept inverted for 15 min and drained on absorbent paper. Samples were counted in a gamma counter. The mean of duplicates was calculated and the value for non-specific binding (without anti IL-1α antiserum) was subtracted. The binding percentage was converted using the logit transformation (logit x = ln [x/(100 - x)]). The logit values on the x axis were plotted against the standard concentrations of IL-1α in pg/ml on the logarithmic y axis (Fig. 4); cpm from unknown samples were equally transformed and IL-1α concentration was read off the standard curve. Values ≤ 95% zero standard (logit x ≤ 2.94) were accepted, corresponding to 25-50 pg/ml hIL-1α.

Figure 4. Logit plot of hIL-1α in either BSA-buffer, human plasma or urine. The line indicates the sensitivity of the RIA at the 95% confidence level (logit 2.94).

Preparation and incubation of human mononuclear cells (MNC). Heparinized donor blood (10 U/ml) was diluted 1:3 in 0.15 M saline and MNC were separated on Ficoll-Hypaque density gradients. MNC were washed three times in saline and suspended in ultrafiltered (10) RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 10 mM HEPES (all from Microbiological Ass., Walkersville, MD) containing 2% heat inactivated AB serum. 100 µl of MNC (5 x 10⁶ cells/ml) were added to flat-bottom microtiter plates (A/S Nunc, Roskilde, Denmark). An equal volume of endotoxin (from E. coli B:55,
Sigma) at various concentrations was added in serum-free RPMI. After 18 hours incubation at 37°C in humidified air with 5% CO₂ plates were exposed to 3 freeze-thaw cycles. The samples, containing both cell lysates and supernatants were centrifuged for 1 min at 13,000 g and the supernatants were frozen at -70°C until assayed.

**Spiking of fresh human blood with monocyte supernatants.** Human MNC were separated as described above and incubated in glass bottles at 4 x 10⁵ cells/cm² for 1.5 hours at 37°C. The bottles were shaken, non-adherent cells were decanted and replaced by 20 ml of fresh tissue culture medium containing 1% fresh human AB serum and heat-killed *Staphylococcus epidermidis* at a bacteria/MNC ratio of 10:1. Cells were incubated for 36 hours at 37°C and the supernatants obtained after centrifugation at 5,000 g for 30 min. The crude supernatant was used to spike freshly obtained heparinized blood or urine. Spiked blood and urine samples were incubated for 2 hours at 37°C on a rotating rack, centrifuged for 10 min at 1500 g and supernatants were assayed for IL-1α and IL-1β in the specific RIAs. As a control, the crude preparation was incubated for 2 hours in RPMI. After incubation all samples were diluted 5- and 50-fold in BSA-buffer and assayed.

**RESULTS**

**Recovery of human monocyte derived IL-1 from urine and plasma.** When corrected for the dilution factor, the original preparation of crude monocyte supernatant used in these experiments contained 15 ng/ml IL-1β and 4 ng/ml IL-1α (left hand side of Fig. 5). 100% of both IL-1α and IL-1β were recovered in the urine samples (middle part of Fig. 5). In contrast, when heparinized blood was spiked and plasma tested for IL-1,
100% of IL-1α was recovered but only 50% of IL-1β. Similar results were obtained in blood and urine from 3 human subjects.

Production of IL-1α from MNC of 8 human subjects. We obtained blood from 8 human subjects and stimulated the MNC with various concentrations of endotoxin during a single day. After 24 hours, IL-1α production (extracellular and cell-associated) was assayed in a single RIA. As shown in Fig. 6, there was no detectable IL-1α in the RPMI medium control (all donors MNC produced amounts of IL-1α less than the detection limit (50 pg/ml). In seven of the eight donors, MNC IL-1α production was elevated when a concentration of 5 ng/ml was employed. The amount of IL-1α increased with higher concentrations of endotoxin in six of the eight donors; however, in two donors, there was clearly less IL-produced at 5, 50 and even 500 ng/ml of endotoxin.

![Graph showing IL-1α levels measured in the MNC of eight human subjects incubated with endotoxin for 24 hours.](image)

Figure 6. IL-1α levels measured in the MNC of eight human subjects incubated with endotoxin for 24 hours. Total IL-1α (extracellular and cell-associated) is shown on the vertical axis.

DISCUSSION

In the present study, we report a sensitive and specific RIA for human IL-1α. This assay detects 25-50 pg/ml IL-1α in BSA buffer or tissue culture medium and the binding of labeled IL-1α in this RIA was not influenced by the presence of a variety of unlabeled human cytokines. The detection of human recombinant human IL-1α is also not significantly influenced by normal human heparinized plasma or freshly voided urine since we observed nearly identical standard curves when the recombinant IL-1α was diluted in plasma, urine, or BSA buffer. We also examined the recovery of natural IL-1α present in crude human monocyte supernates. The recovery of natural IL-1β from freshly obtained human heparinized blood was only 50%, unlike the 100% recovery of IL-1α. These results support the concept that the IL-1β molecule itself seems to be more
vulnerable to the action of unidentified agents present in plasma than IL-1α.

We used the RIA to measure the amount of IL-1α produced by human MNC in vitro. We employed endotoxin as a stimulant and measured the total IL-1α content of the incubation mixture. In all donors, the MNC did not produce detectable levels of IL-1α when cultured in RPMI which had been subjected to ultrafiltration to remove small amounts of endotoxins which often contaminate tissue culture media. We also used a single source of human AB serum which we prepared with pyrogen-free conditions. Therefore, we believe that under these conditions, human blood MNC do not produce more than 50 pg/ml of IL-1α. This methodology could be used to study the "spontaneous" production of IL-1α in human subjects with various diseases or undergoing physiologic stresses.

The production of IL-1α was observed in 3 donors at the lowest concentration of endotoxin tested (0.5 ng/ml) and increased in all donors in a dose-response fashion. However, two of the eight donors showed markedly less IL-1α at the higher concentrations of endotoxin. Since the stimulation and RIA were performed on all samples at one time, these results do not reflect differences in the preparation of the MNC, the potency of the endotoxin, nor the sensitivity of the RIA. One interpretation is that some human subjects are low responders to endotoxin. Since we do not have data on the IL-1β levels in these individuals, we cannot ascertain whether these donors also produce proportionately less IL-1β. Studies relevant to this question are currently under investigation.

In the present studies, we measured both cell-associated as well as extracellular IL-1α. Since a considerable amount of IL-1 remains cell-associated following stimulation with endotoxin (11), we measured total IL-1α. We did not separate the membrane fraction from the cytosolic compartment using ultracentrifugation in these studies and so the precise location of the IL-1α remains unclear. There has been evidence that both forms of IL-1 can be isolated or visualized on human monocyte plasma membranes (12, 13). Mouse peritoneal macrophage membranes contain membrane IL-1α (14, 15) but since there are no available antibodies to mouse IL-1β, it is unknown whether this form of IL-1 also exists on mouse cells.

Although the amount of IL-1α produced by the MNC of the eight human subjects in the present study are lower than those reported from this laboratory for IL-1β (7), we cannot make any conclusions since these samples were not assayed for IL-1β. Nevertheless, it appears that approximately 10-fold greater endotoxin is required to induce the production of comparable amount of IL-1α. If these findings are confirmed in subsequent studies, then the amount of IL-1α produced from human MNC may be greater than one would predict knowing the paucity of IL-1α mRNA in human MC stimulated with endotoxin in vitro (6). It may be possible to speculate at this time that gene expression is not linked to translation in
IL-1α as it is in IL-1β. Alternatively, translation of IL-1α may be dependent on endotoxin concentrations different from that of IL-1β. Finally, the differential stability of IL-1α compared to that of IL-1β, demonstrated in this paper, may also contribute to the amount of IL-1α measured by RIA compared to IL-1β. These and similar questions can now be studied employing the RIA for IL-1α.

REFERENCES


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