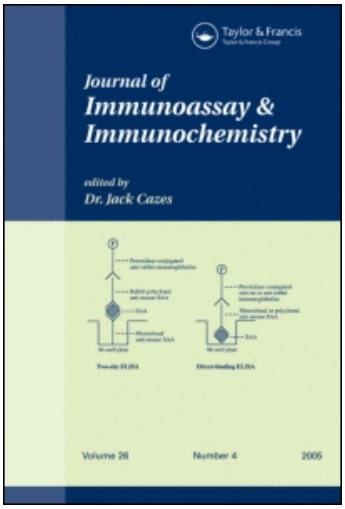

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A Method for Correcting for the Variability of Inhibitory Effects of Soluble Human Interleukin 1 Receptor II Measured by Different Elisas

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A METHOD FOR CORRECTING FOR THE VARIABILITY OF INHIBITORY EFFECTS OF SOLUBLE HUMAN INTERLEUKIN 1 RECEPTOR II MEASURED BY DIFFERENT ELISAS

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ABSTRACT

Seven ELISAs were developed by using several combinations of anti-human IL-1ß antibodies for detecting interleukin 1ß (IL-1ß) in cell culture supernatants. These ELISAs have different sensitivities in detecting standard preparations of recombinant human IL-1ß (WHO reference standard) compared with conventional preparations of IL-1ß produced by stimulated human peripheral blood mononuclear cells. The observed differences were attributed to differences in epitope specificity of the various monoclonal antibodies used and the heterogeneity of IL-1ß secreted into culture supernatants. The presence of soluble IL-1 receptor type I did not alter the levels of IL-1ß detected by these ELISAs. However, soluble IL-1 receptor type II interfered with the detection of IL-1ß to different degrees in these ELISAs. A method involving standarization by means of separate measurement of the amount of receptor and its inhibitory effect in the IL-1ß ELISA, yields consistent estimates of the correct IL-1ß levels.

(KEY WORDS: Interleukin 1ß, ELISA, soluble IL-1 receptor, standardization)

INTRODUCTION

Interleukin-1 (IL-1) is a pleiotropic cytokine involved in the host response to infection and injury (reviewed in 1, 2). IL-1 promotes immunologic responses by enhancing the growth and

differentiation of both B and T lymphocytes. Many of the biological effects of IL-1 are mediated by the induction of a cascade of other cytokines, such as tumor necrosis factor α (TNFα), colony-stimulating factors, IL-1 itself, IL-2, IL-6, IL-8, and homologous members of the chemokine family. The IL-1 family of proteins consists of three distinct but structurally related molecules: IL-1α and IL-1ß, and a specific receptor antagonist, IL-1 receptor antagonist (IL-1ra) (3). IL-1α is predominantly membrane-bound while IL-1ß is secreted after proteolytic cleavage. Two distinct IL-1 receptors, IL-1 receptor types I and II (IL-1RI and IL-1RII), and a receptor accessory protein, the signal-transducing subunit of the IL-1 receptor complex, have been identified and characterized (4, 5). The biological effects of IL-1 are mediated through the interaction of IL-1α or IL-1ß with IL-1RI on target cells. Type II IL-1 receptor acts as "decoy" receptor, as it binds and sequesters IL-1 with high affinity without signal transduction (6).

IL-1 is produced by mononuclear phagocytes and other cells in response to infectious agents, endotoxin, bacterial cell wall products and superantigens, immune complexes, and endogenous cytokines: IL-1 and TNFα. Overproduction of IL-1 is associated with a variety of inflammatory diseases and autoimmune disorders (1). Thus the analysis of the production of IL-1 is important for understanding the physiological responses and signalling mechanisms during immune stimulation. With the commercial availability of ELISA kits for IL-1ß, ELISAs are commonly used to measure IL-1ß present in sera or in cell culture supernants (7-9). In-house ELISAs using anti-IL-1ß specific antibodies are also employed in the detection of IL-1ß. The proliferation of different ELISAs makes it difficult to compare results from

different laboratories. A recent WHO International collaborative study of TNFα assays indicates substantial differences in results obtained with different commercial and in-house assays (10). Moreover, the presence of soluble (s) IL-1RI and sIL-1RII interferes differently with the IL-1ß measurement by ELISA (7, 9, 11).

We previously reported that the superantigen, staphylococcal enterotoxin B (SEB), stimulates peripheral blood mononuclear cells (PBMC) to produce moderate levels of IL-1ß (12). Unexpectedly, the anti-IL-1ß antibodies used in that study were discontinued by the manufacturer and another source of anti-IL-1ß antibody was used in subsequent studies. However, the results from the new antibodies were poor; IL-1ß was not detected or was barely detectable in supernantants of SEB-stimulated cells. Anti-IL-1ß antibodies from several other commerical sources were tested in a search for a reliable assay for IL-1ß.

The major goal of this study was to characterize different ELISAs for assaying IL-1ß and to develop a method to correct for the widely varying results. The variability of ELISA results and the method for correcting for it were evaluated in a study of substances that potentially interfere with IL-1ß, the two IL-1 receptors.

MATERIALS AND METHODS

Materials

Two different preparations of recombinant human (rh) IL-1ß were obtained; one from Dupont (Glenolden, PA), referred hereafter as rhIL-1ß (Du), and another from the NIH (Biological Response Modifiers Program, Frederick, MD). The NIH rIL-1ß is

accepted as the interim reference WHO standard. Recombinant hIL-1α was provided by Dainippon (Osaka, Japan). Seven different anti-hIL-1ß antibodies were obtained from four different sources as follows. Monoclonal antibodies (mab) were purchased from Genzyme (Cambridge, MA), Boehringer-Mannheim (Indianapolis, IN) and R&D Systems (Minneapolis, MN). Rabbit anti-hIL-1ß was purchased from Endogen (Woburn, MA) and Genzyme. Biotinylated anti-hIL-1ß, goat anti-IL-1ß, goat anti-IL-1RII, IL-1ra, rhsIL-IRI, and rhsIL-1RII were obtained from R&D Systems. Monoclonal antibody to shIL-1RII was from Genzyme. Peroxidase-labeled streptavidin was purchased from Kirkegaard and Perry (Gaithersburg, MD). Peroxidase-conjugated, anti-rabbit IgG and anti-goat IgG were obtained from Boehringer-Mannheim. SEB was obtained from Toxin Technology (Sarasota, FL).

ELISA for IL-1ß

Seven different IL-1ß ELISAs were formatted with the three mab for coating 96-well Nunc plates and the rabbit anti-hIL-1ß or goat anti-hIL-1ß or the biotinylated anti-hIL-1ß as detecting antibodies. Preliminary experiments were performed to optimize the combination and the amounts of antibodies used per well as follows. ELISA A, mab (0.1 μg) and goat anti-IL-1ß (20 ng) from R&D. ELISA B, mab (0.25 μg) from Genzyme and goat anti-IL-1ß (20 ng) from R&D. ELISA C, mab (0.2 μg) from Boehringer-Mannheim and goat anti-IL-1ß (25 ng) from R&D. ELISA D, mab (0.17 μg) from R&D and rabbit anti-IL-1ß (0.69 μg) from Endogen. ELISA E, mab (0.33 μg) from Genzyme and rabbit anti-IL-1ß (0.5 μg) from Endogen. ELISA F, mab (0.13 μg) from Boehringer-Mannheim and rabbit anti-IL-1ß (0.69 μg) from Endogen. ELISA G, mab (50 ng) and biotinylated anti-IL-1ß (5 ng) from R&D. Plates were coated

with mab at 4°C for 18 h. Plates were washed with wash buffer (PBS containing 0.05% Tween 20) and blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at 37°C. Plates were then incubated with 100 μl of standards or samples in duplicate for 2 h at 37°C. Plates were washed three times with wash buffer. Rabbit anti-hIL-1ß antibody was added and plates were incubated at 37°C for 100 min, then washed four times with wash buffer. Peroxidase-conjugated, anti-rabbit IgG at 1:120,000 dilution in 1% BSA was added and plates were incubated at 37°C for 20 min. Plates were washed four times with wash buffer. The same procedure was used when goat-anti-IL-1ß was used and anti-goat IgG was used at 1/2000 dilution in 1% BSA. Alternatively, after the sample incubation step, plates were incubated sequentially with biotinylated anti-hIL-1ß, washed, and incubated with peroxidase-labeled streptavidin for 35 min at 37°C. Bound enzyme was detected at 450 nm after incubating with the substrate 3,3',5,5'-tetramethylbenzidine (TMB) (Kirkegaard and Perry). Recombinant hIL-1ß (Du) (20-500 pg/ml) was used to construct a standard curve for each ELISA. The amount of IL-1ß present in each sample was calculated by comparing it with the rhIL-1ß (Du) standard by regression analysis (Stata, Stata Corp, College Station, TX). In addition, the WHO IL-1ß reference standard was used to calibrate these ELISAs against each other. The detection limit of these assays was 20 pg/ml. All seven ELISAs were specific for hIL-1ß as other cytokines (IL-1α, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, TNFα, and IFNγ) tested at 1 ng/ml were negative in these assays.

ELISA for sIL-1RII

A sandwich ELISA for sIL-1RII was devised using procedures similar to those used above for hIL-1ß. Coating and detecting

antibodies were used at 0.3 μg and 0.2 μg, respectively. Recombinant sIL-1RII was used to construct a standard curve for each assay. The detection limit of this ELISA was 50 pg/ml. IL-1ß present in preparations (described below) did not interfere with sIL-1RII in this ELISA.

Preparation of cell culture supernatants

PBMC were isolated by ficoll-hypaque density gradient centrifugation of heparinized blood from normal human donors. PBMC were plated at 1 x 10^6 cells/ml in 24-well plates in RPMI 1640 cell culture medium containing 10% heat-inactivated fetal bovine serum. Cells were stimulated with SEB at 100 ng/mL for 16 h. Supernatants from stimulated PBMC were used as samples containing IL-1ß in the different ELISAs.

Preparation of samples containing rhIL-1ß and rhsIL-1R

Samples were prepared to contain a known concentration of IL-1ß (Du) and different molar ratios of IL-1ß/sIL-1RI combinations to examine the effect of sIL-1RI in these assays. Constant amounts of IL-1ß (450 pg/ml) were incubated for 1 h at room temperature with increasing amounts of sIL-1RI. These tubes contained first the ligand without any sIL-1RI, then IL-1ß:sIL-1RI molar ratios of 1:1, 1:5, 1:10, 1:50, and 1:100. Aliquots of these mixtures were examined for detectable levels of IL-1ß in the different ELISAs performed in duplicate. The data were expressed as the mean level of IL-1ß detected vs the molar ratio of sIL-1RI to IL-1ß.

Samples containing a constant concentration of IL-1ß (Du) (450 pg/ml) and varying molar ratios of IL-1ß/sIL-1RII were prepared as described above to evaluate the effect of the non-

signal-transducing receptor, IL-1RII. Tubes containing IL-1ß:sIL-1RII molar ratios of 1:1, 1:5, 1:10, 1:50, and 1:100 were incubated for 1 h at room temperature and assayed by the different ELISAs.

Statistics

Data are presented as the mean ± SEM. Significant difference was determined by the Student's t-test with Stata (Stata Corp).

RESULTS

An unknown sample of IL-1ß, prepared as the supernantant from PBMC stimulated with SEB, was tested by each ELISA. The antibodies that failed to detect hIL-1ß from this supernatant were not investigated further. Preliminary experiments were then performed to establish the optimal conditions for each of the ELISAs using effective anti-IL-1ß antibodies from a number of commercial sources. Molecules that bind IL-1ß, such as the two types of sIL-1R, were also examined to determine their effects in these ELISAs.

Different ELISAs detect different levels of IL-1ß in cell culture supernatants

The comparisons of the antibodies that yielded reproducible standard curves with the recombinant human (rh) IL-1ß(Du) are summarized in Table 1. A WHO reference standard (200 pg/ml) was used to evaluate the performance of these ELISAs (second column). ELISA A, E, and G were comparable and had the highest sensitivity for this reference standard, in contrast with the lower sensitivity of ELISA D and F. To simplify comparison of the

TABLE 1

Comparison of the results of seven different ELISAs for hIL-1ß using combinations of six different antibodies.

ELISA code	Mean IL-1ß[a] of WHO Std 200 pg/ml	Ratio[b]	Mean IL-1ß[a] of Supernatant from PBMC	Ratio[b]	Mean IL-1ß[a] of (Du) Std 250 pg/ml
A	330 ± 14	1	364 ± 18	1	272 ± 8
B	309 ± 11	0.94	221 ± 27	0.61	265 ± 8
C	297 ± 17	0.90	257 ± 25	0.71	253 ± 9
D	260 ± 16	0.79	226 ± 17	0.62	258 ± 9
E	351 ± 28	1.06	580 ± 16	1.59	268 ± 10
F	256 ± 17	0.77	164 ± 6	0.45	259 ± 10
G	352 ± 40	1.07	359 ± 11	0.99	263 ± 10

a. Values are in pg/ml.

b. "Ratio" represents the mean of each measurement of WHO or Du standard divided by mean of ELISA A.
Results were from the averages of 4-5 experiments.

assays, a ratio of the "measured" mean of each ELISA to that of ELISA A was used. This ratio underscored the differences among these ELISAs in the detection of IL-1ß in SEB-stimulated PBMC supernatants (fourth column). ELISA E reported the highest level of IL-1ß in the supernatant, reporting as much as 3.4-fold higher IL-1ß than that in ELISA F (580 pg/ml vs 164 pg/ml). Although ELISA B was "close" in its measurement of the WHO rIL-1ß standard to that by ELISA E (309 pg/ml vs 351 pg/ml), ELISA B reported far less IL-1ß in stimulated PBMC supernatants than that measured by ELISA E (221 pg/ml vs 580 pg/ml, $P < 0.001$). Thus there was a discrepancy in the levels of IL-1ß detected in the recombinant

standard compared with that in cell culture supernatants. One possible explanation for these different sensitivities might be the heterogeneity of IL-1ß forms in cell culture supernatants due to posttranslational modification. Thus, antibodies used in ELISA B may not recognize natural IL-1ß as well as the recombinant standard IL-1ß. Another explanation for these discrepancies might have been the presence of other substances interfering with the detection of IL-1ß in these ELISAs.

Lack of influence in ELISAs due to soluble IL-1RI

Soluble cytokine receptors, including the sIL-1RI and sIL-1RII, are found in the supernatants of stimulated cells and their release from cell membranes is regulated by various pro-inflammatory and anti-inflammatory agents (13, 14). Soluble IL-1Rs, which can bind IL-1ß and mask sites on the IL-1ß molecule, can prevent anti-IL-1ß antibodies from interacting with these sites. Moreover the different epitope specificities of these antibodies suggest that sIL-1Rs may influence the IL-1ß measurement differentially. The effect of different concentrations of sIL-1RI on these ELISAs was therefore examined. Varying concentrations of sIL-1RI in the presence of a fixed amount of IL-1ß did not affect these assays (Figure 1). There was no statistically significant difference ($P > 0.05$) between IL-1ß alone or IL-1ß plus IL-1α or IL-1ra or both in the presence of any concentration of sIL-1RI in all seven ELISAs (data not shown).

Soluble IL-1RII interfered with ELISAs to different degrees

Previous reports indicate that sIL-1RII interferes with the detection of IL-1ß (7, 9, 11). The effect of sIL-1RII in the

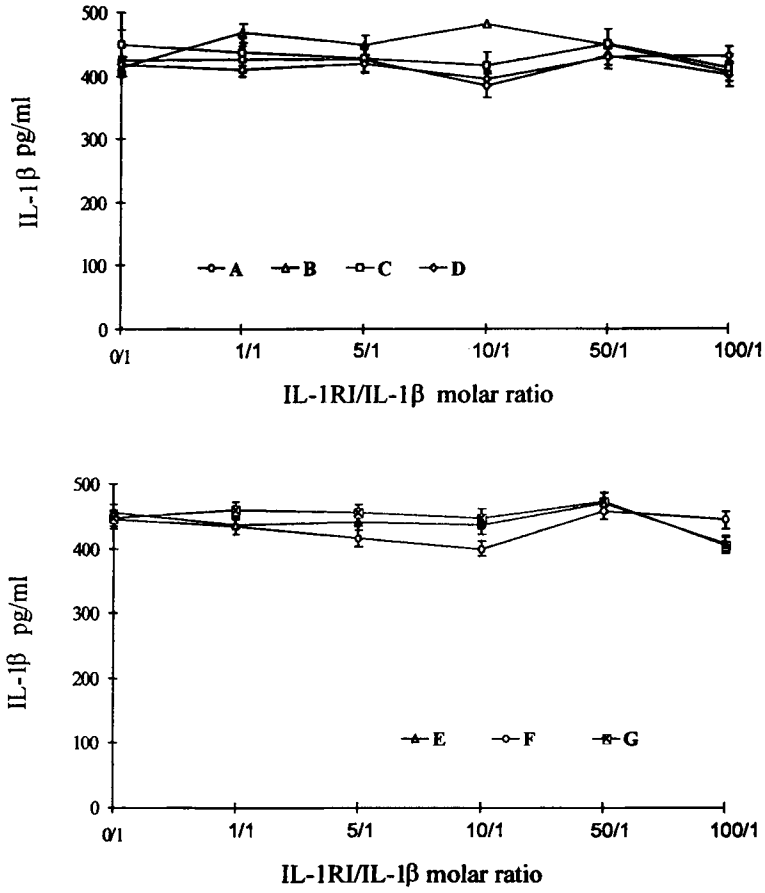

FIGURE 1. Effect of varying molar ratios of sIL-1RI/IL-1ß on the detection of IL-1ß in seven different ELISAs. Different antibody pairs were used in these ELISAs as described in detail in Materials and Methods. Data points are mean ± SEM of duplicate samples and results are representative of three experiments.

seven ELISAs was also examined to determine if sIL-1RII affected these ELISAs equally. Figure 2 shows the levels of IL-1ß detected at a wide range of sIL-1RII/IL-1ß ratios. All ELISAs detected lower levels of IL-1ß when IL-1RII was present at 10-

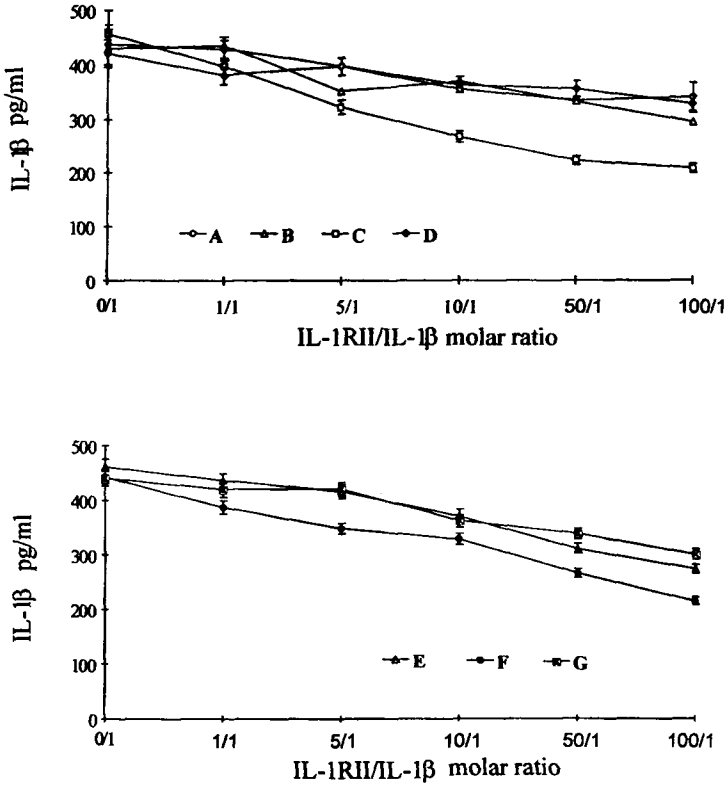

FIGURE 2. Effect of varying molar ratios of sIL-1RII/IL-1ß on the detection of IL-1ß in seven different ELISAs. Different antibody pairs were used in these ELISAs as described in detail in Materials and Methods. Data points are mean ± SEM of duplicate samples and results are representative of three experiments.

fold higher concentration than IL-1ß. At 100:1 (sIL-1RII:IL-1ß), the IL-1ß detected in ELISA C and F were reduced by >50% (P >0.001). Soluble IL-1RII reduced detectable IL-1ß by 22% in ELISA A and D. Similar results were obtained with IL-1ß plus IL-1α or IL-1ra or both in the presence of varying concentrations of sIL-1RII in these seven ELISAs (data not shown).

Estimation of sIL-1RII in samples before measurement of IL-1ß by ELISAs

Methods were then devised to determine the "true" concentrations of IL-1ß in the presence of sIL-1RII. Antibodies to sIL-1RII were used to compete for sIL-1RII present in the above prepared solutions (used in Figure 2) containing varying concentrations of sIL-1RII in the presence of a fixed amount of IL-1ß. Neither soluble nor plate-bound anti-IL-1RII antibodies corrected for the reduction of IL-1ß present in these samples (data not shown).

However an estimation of the "true" IL-1ß present can be performed by using the inhibition curves generated (Figure 2) for each ELISA. Soluble IL-1RII present in the prepared mixtures of IL-1ß plus sIL-1RII were measured first by an ELISA specific for sIL-1RII. An estimate of the amount of IL-1ß present in these preparations was made using any of the seven ELISAs described. Based on the sIL-1RII/IL-1ß molar ratios generated from ELISAs for sIL-1RII and IL-1ß, compensation for the reduction of IL-1ß levels in these preparations due to inhibitory sIL-1RII was deduced from the inhibition curves for each IL-1ß ELISA shown in Figure 2. Table 2 shows the results of this method with ELISA A and C for measuring IL-1ß present in three different preparations (IL-1ß alone at 450 pg/ml, IL-1ß with 10-fold molar excess of sIL-1RII, IL-1ß with 100-fold molar excess of sIL-1RII). Preparation 2 containing IL-1ß (0.45 ng/ml) and sIL-1RII (10.58 ng/ml) had a reduction in detectable IL-1ß by 21% in ELISA A and 50% in ELISA C. The measured IL-1ß with ELISA A and ELISA C was 0.36 ng/ml and 0.27 ng/ml, respectively. The "calculated" value for IL-1ß, correcting for the interference by sIL-1RII, was 0.45 ng/ml for both ELISA A and C.

TABLE 2

Determination of IL-1ß in samples containing sIL-1RII.

	Samples		Step 1	Step 2	Step 3	Step 4
	IL-1ß + sIL-1RII ng/ml		sIL-1RII measured by ELISA	IL-1ß detected by ELISA A	IL-1ß detected by ELISA C	estimated value
1.	0.45	0	0	0.42 ±0.02	0.43 ±0.09	0.45
2.	0.45	10.58	10.01 ±0.17	0.36 ±0.03	0.27 ±0.02	0.45[a]
3.	0.45	105.75	101.09 ±0.09	0.33 ±0.03	0.21 ±0.02	0.50[b]

a. Step 1 indicates sIL-1RII was present at approximately 10-fold molar higher concentration than IL-1ß detected by both ELISA A and C (step 2 and 3). The IL-1ß estimate of 0.45 ng/ml was based on the average IL-1ß deduced from ELISA A (0.45) and ELISA C (0.45) at 10/1 ratio of sIL-1RII/IL-1ß from Fig. 2. ELISA A and ELISA C reduced IL-1ß present by 21% and 40%, respectively.

b. Step 1 indicates sIL-1RII was present at approximately 100-fold molar higher concentration than IL-1ß detected by both ELISA A and C. The IL-1ß estimate of 0.50 ng/ml was based on the average IL-1ß deduced from ELISA A (0.55 ng/ml) and ELISA C (0.45 ng/ml) at 100/1 ratio of sIL-1RII/IL-1ß from Fig. 2. ELISA A and ELISA C reduced IL-1ß present by 24% and 53%, respectively.

DISCUSSION

Assays of multifunctional cytokines are increasingly important as cytokines regulate immune responses and play key roles in host defense. The most widely used method of detecting cytokines is by using an ELISA, as commerical kits are available and ELISAs are generally sensitive, specific, and easy to use. However, as shown in a recent collaborative evaluation of assays of TNFα, discrepancies with the same samples arose among

laboratories even when commercial, standardized ELISA kits were used (10).

In this study, seven different ELISAs were developed and compared for their ability to detect and measure IL-1ß in a number of samples containing IL-1ß. We found discrepancies in the detection of IL-1ß in standard samples (reference standard) and those from stimulated cell culture by some ELISAs. The observed variability in these ELISA assays may have been caused by the epitope specificities of the monoclonal antibodies used in these assays. These epitope specificities arise from different recognition sites on IL-1ß. Heterogeneity of IL-1ß proteins in culture supernatants and serum is known to occur due to posttranslational modification (2). These include myristoylation, and phosphorylation. Some of these forms may be cell- or tissue-specific depending on the stimulant used. An ELISA optimal for standard rIL-1ß may not be ideal for measuring IL-1ß from a different source containing natural heterogeneous forms of IL-1ß. To achieve better standardization of these ELISAs in different laboratories, additional preparations of IL-1ß containing natural IL-1ß besides the WHO reference standard should be included.

We also evaluated the effects of exogenously added sIL-1RI and sIL-1RII on these ELISAs. The presence of sIL-1RII influences the measurement of IL-1ß differentially in these ELISAs. Thus, when agents known to induce the release of sIL-1RII are used, the IL-1ß detected may be underestimated substantially in some ELISAs. However, by measuring the level of sIL-1RII in cell supernatants or unknown samples containing IL-1ß, a more accurate estimate of IL-1ß can be achieved.

In summary, we observed important differences among the seven ELISAs in the assay of natural IL-1ß present in supernatants of

stimulated cells and the recombinant WHO reference standard. Soluble IL-1RII moderately lowered the levels of IL-1ß detected by some ELISAs while reducing the measured levels substantially, by as much as 50%, in two of these ELISAs. However, by properly standardizing the assays by means of separate measurement of the amount of the receptor in the assay, the magnitude of its inhibitory effect can, nevertheless, be correctly estimated. Because of the discrepancies in the detection of natural IL-1ß in cell supernatants and rIL-1ß standard, new reagents and kits must be evaluated carefully to assure reproducible and relevant results.

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