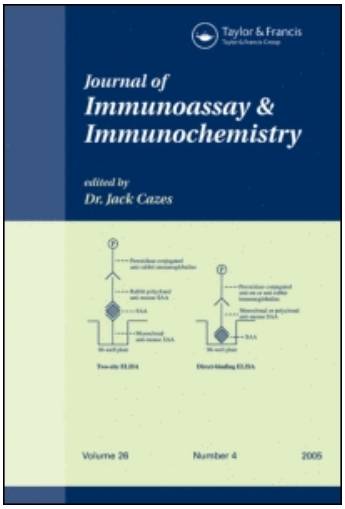

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Preparation and Application of Polyclonal Antibodies to Recombinant Human Interleukin-1α and Interleukin-1β

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PREPARATION AND APPLICATION OF POLYCLONAL ANTIBODIES TO RECOMBINANT HUMAN INTERLEUKIN-1α AND INTERLEUKIN-1β

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ABSTRACT

We report the preparation and application of polyclonal antisera to the analysis and quantitation of human interleukin-1α (IL-1α) and human interleukin-1β (IL-1β). The anti IL-1α antibodies specifically react with the alpha form of IL-1 and do not cross react (<0.1%) with the β form of IL-1 and vice versa. Data reported here demonstrate that detection of human IL-1α or β by a radioimmunoassay technique is sensitive enough to measure picogram levels of these lymphokines. The practical application of using these highly specific antisera for radioimmunoassays was established by measuring exogenously added IL-1α or IL-1β to human plasma. Potential benefits of these reagents and the radioimmunoassay procedures described herein are discussed in relation to the biological assays which cannot distinguish between human IL-1α and human IL-1β.

KEYWORDS: IL-1α, IL-1β, polyclonal antibodies, radioimmunoassay.

INTRODUCTION

Human interleukin-1 (IL-1) activity has a wide range of *in vivo* effects both at the site of production by macrophages and also in distinct tissues (1-5). It may also be involved in several pathological states. For example, it has been shown to be present in the synovial

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fluid of patients suffering from arthritis (6). In addition it has been found to be active as a bone resorption inducing agent (7).

IL-1 activity has been shared by two structurally different proteins derived from human monocytes (8-10). These proteins are called IL-1α and IL-1β. They compete with one another for binding to IL-1 receptor (11-13) and are known to mediate similar biological activities (8-10). The IL-1 gene has been cloned (14) and both the DNA (15) and protein sequence have been determined (8,9). Pure recombinant human IL-1α (rhIL-1α) and IL-1β (rhIL-1β) have been obtained and well characterized (10,14,15).

IL1α and IL1β are encoded by two different genes, show only 26% amino acid homology, and have pI values of 5 and 7, respectively (9). Although there is no clear evidence of a functional (biological) difference between the two classes of IL-1, the potential for such a difference still exists. However, there is some evidence which suggest that expression of one or the other type of IL-1 may be a function of the cell type and its activated state (16). Therefore, identification and quantitation of the type of IL-1 may have both biological and clinical significance. Bioassays (17-19) have a major drawback since there is more than one IL-1 (8-10). In addition other growth factors such as IL-2 or effector molecules can influence assay results (20). Although monoclonal antibodies to IL-1α or IL-1β have been reported (21, 22) there appears to be no report in the literature on polyclonal antibodies specific for both IL-1α and IL-1β. We report here the preparation of highly specific antiserum to rhIL-1α and rhIL-1β and their usefulness in the differentiation and quantitation of these IL-1 variants (15) by radioimmunoassay (RIA) techniques.

MATERIALS AND METHODS

The Bolton Hunter reagent (monoiods) for protein iodination was purchased from Amersham (Arlington Heights, IL). ^{125}I labeled rhIL-1β was from Dupont (Wilmington, DE). IL-1 standards (IL-1α, 86/632; IL-1β, 86/552) were obtained from the National Institute for Biological Standards and Control, Holly Hill, Hampstead, London, NW3 6RB. ^{14}C-labelled high molecular weight markers were from Bethesda Research Laboratories. ^{125}I-Protein-A, immunology grade, was from New England Nuclear. The antigen rhIL-1α was obtained from Dainippon Pharmaceutical Company, Ltd. (Japan). It was produced in *E. coli* by recombinant DNA technology (23), and purified and characterized in Research Laboratories of the supplier. Production and purification of rhIL-1β are described elsewhere (15). This antigen has three additional amino acids at its N-terminal compared with the natural IL-1β and is sometimes referred to as IL-1β$^+$ (15).

Preparation of Antibodies to rhIL-1α or rhIL-1β

Antibodies to rhIL-1α or rh-ILβ were prepared in New Zealand white female rabbits. Preimmune serum was obtained from each animal before immunization. An initial dose of 8 ug of rhIL-1α or 25 ug of rhIL-1β was emulsified in complete Freund's adjuvant (1 ml) and injected into each animal. This was given interdermally at multiple sites along the back and flank. Booster injections (5 ug/ml) in incomplete Freund's adjuvant were given subcutaneously at

approximately one month intervals for 4 additional months. Rabbits were bled from the ear vein and serum was separated from the other blood products by centrifugation and stored at -70°C.

Preparation of ^{125}I IL-1α and ^{125}I IL-1β

For comparison with the commercially available ^{125}I IL-1β a radiolabel (^{125}I) IL-1β was prepared using the Bolton-Hunter reagent. Coupling was done essentially the same way as described in the technical specification sheet number 11437 from Amersham. Our attempts to prepare ^{125}I IL-1α using the Bolten-Hunter procedure resulted in preparations which were unsuitable, presumably due to low specific activity. Therefore, rhIL-1α (Dainippon Pharmaceutical Co.) was iodinated using the Chloramine T method.

Western Blot Analysis

For Western blot analysis, samples were first analyzed by 17% SDS polyacrylamide gel electrophoresis (24). Following electrophoresis, proteins were electroblotted onto nitrocellulose filters and blots were processed (25). Regions of antibody binding were labeled with ^{125}I protein A (New England Nuclear) at 1 x 10^6 cpm per ml and the autoradiogram was developed after exposing the nitrocellulose to Kodak X-ray film.

Radioimmunoassay Procedures

Assay methods for both IL-1α and IL-1β utilize antisera that was developed in rabbits. Assay titers are established by reacting varying amounts of primary antisera with the corresponding ^{125}I IL-1 variant in the absence of unlabeled IL-1α or IL-1β. In general, dilutions of antisera that result in 50% binding are chosen for routine assays. Bound molecules are separated from the unbound molecules by precipitation with goat anti-rabbit gamma globulin (secondary antibody). The amount of radioactivity in the precipitate is quantitatively determined using a gamma scintillation spectrometer. IL-1 standards are included each time the assay is run and all assays are performed in duplicate. Detailed RIA procedure for IL-1α or IL-1β is described below.

All standard solutions, labelled reagents and dilution of secondary antisera utilized Tris-HCl buffer (0.05 M containing 1.0 g gelatin and 0.1 g thimersal per liter, pH 8.0). A 1:400 dilution of normal rabbit serum (NRS) was also prepared with this buffer for determining NSB. Primary antisera against IL-1α or IL-1β was diluted to the desired concentration using the 1:400 NRS buffer. Glass disposable culture tubes (12 x 75 mm) were marked for total counts, non-specific binding (NSB), standards and unknowns. Diluted primary antisera (200 ul) against IL-1α or IL-1β was added to all of the standard and unknown tubes while 1:400 diluted NRS was added to the tubes marked NSB. Fifty microliters of assay buffer was added to the tubes marked NSB and unknowns while varying amounts of IL-1α or IL-1β in

50 ul were added to standards. Unknowns samples (50 ul) to be assayed were added to the unknown tubes while an equal volume of the test vehicle, which contains no detectable IL-1 activity, was added to standards and NSB tubes. The mixture was allowed to incubate overnight at room temterature prior to adding the appropriate ^{125}I IL-1 (10000 cpm/0.1 ml). The reaction is allowed to continue for 20 hrs at room temperature. Goat anti rabbit gamma globulin titrated to give maximal precipitation is then added and the mixture is kept overnight at 4°C. Two ml of assay buffer (4°C) is then added to all tubes except for those marked total counts. Samples were then centrifuged for 30 minutes at 2000 rpm at 4°C. The amount of radioactivity in the precipitate is determined using a gamma scintillation spectrometer. A computerized data reduction system was used to calculate the IL-1 concentration.

RESULTS

Purified rhIL-1α (23) or rhIL-1β (15) for antisera production was homogeneous as judged by SDS polyacrylamide gel electrophoresis. The specificity of anti IL-1α or IL-1β was determined by Western blot analysis (Fig. 1). When anti IL-1α was used to probe Western transfers of rhIL-1α and rhIL-1β, only IL-1α cross-reacted while no reactivity towards rhIL-1β was observed. (Fig. 1, panel A). It is also shown that antibodies to the β form of IL-1 react well with IL-1β but do not cross react with IL-1α (panel B). These results indicate that both antisera are highly specific for each of these IL-1 variants.

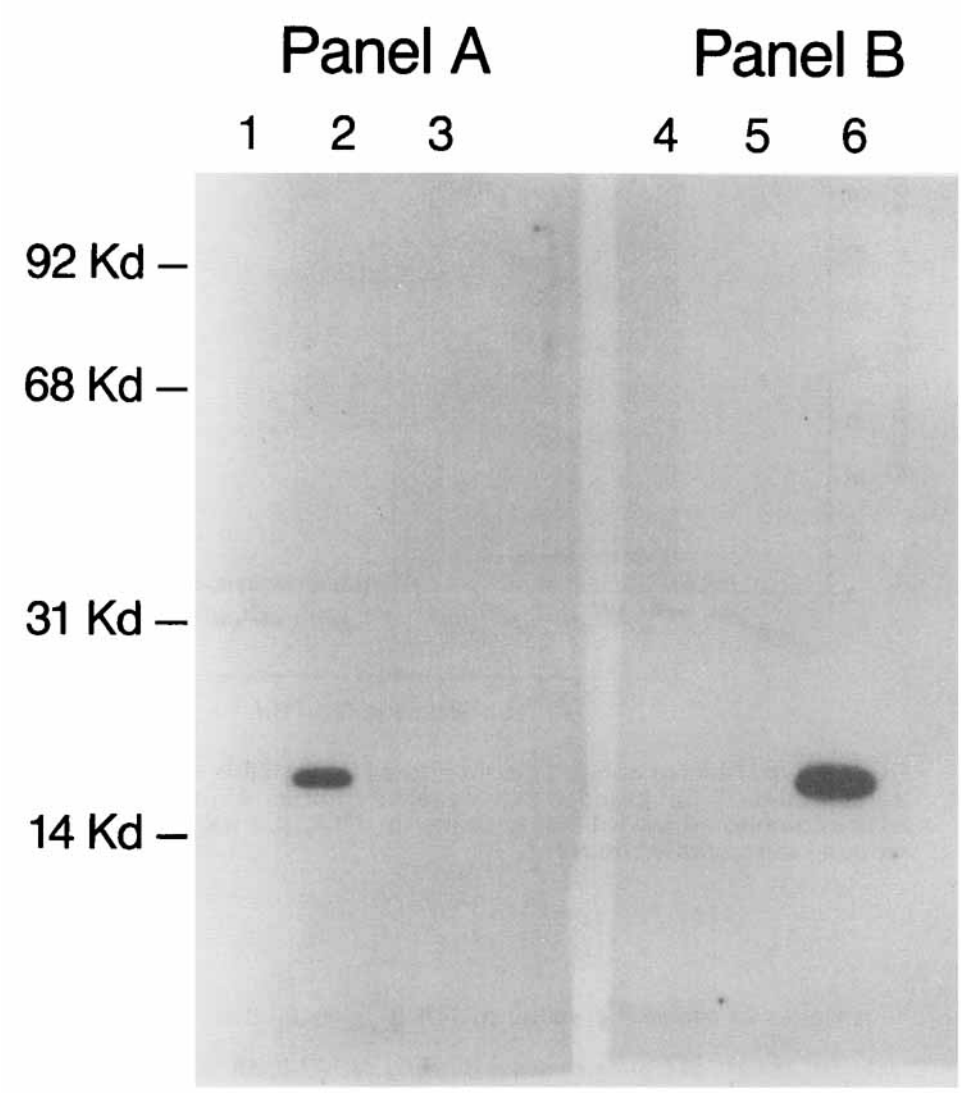

FIGURE 1. Specificity of antibodies to rhIL-1α and rhIL-1β using Western blotting. Panel A, after transfer the nitrocellulose was incubated with anti IL-1α sera. Lane 1, ^{14}C-labeled high molecular weight markers; Lane 2 rhIL-1α; Lane 3, rhIL-1β. Panel B, after transfer the nitrocellulose was incubated with anti IL-1β sera; Lane 4, ^{14}C-labeled high molecular weight markers; Lane 5, rhIL-1α; Lane 6, rhIL-1β.

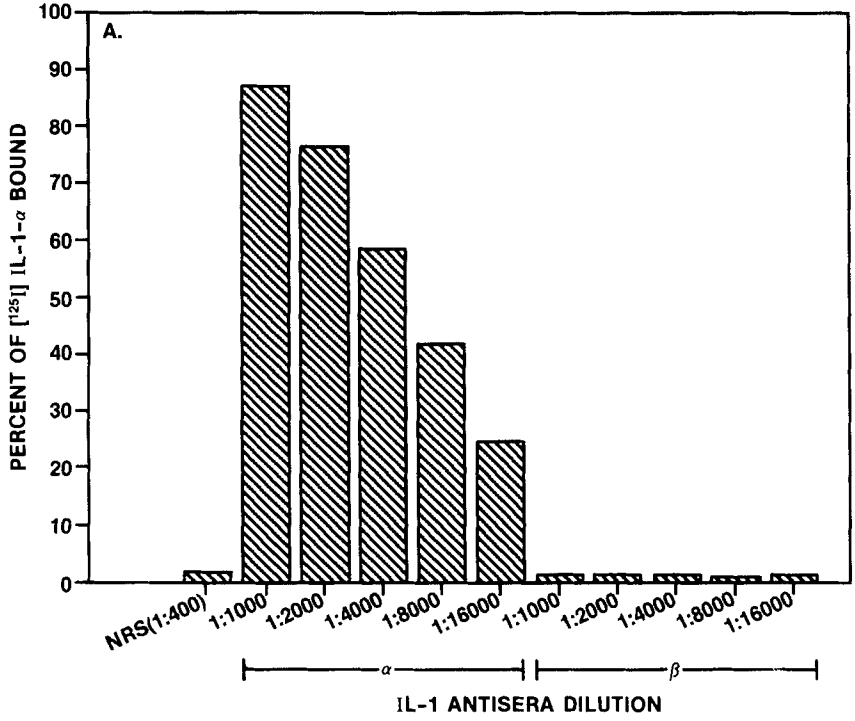

FIGURE 2. A, Titers of anti IL-1α as monitored by its ability to bind ^{125}I IL-1α. Anti-IL-1β was included as a negative control. B, Titers of anti IL-1β as monitored by its binding ability to ^{125}I IL1β. Anti IL-1α was included as a negative control.

Figure 2a shows the ability of ^{125}I IL-1α to bind to anti IL-1α. Likewise, results of Figure 2b show binding of ^{125}I IL-1β to anti IL-1β. As expected from results shown in Fig. 1, no significant binding was observed with anti IL-1α. When the studies of Fig. 2b were repeated using a commercial preparation (DuPont) of ^{125}I IL-1β, similar results were obtained (data not shown). It is concluded that our antisera to

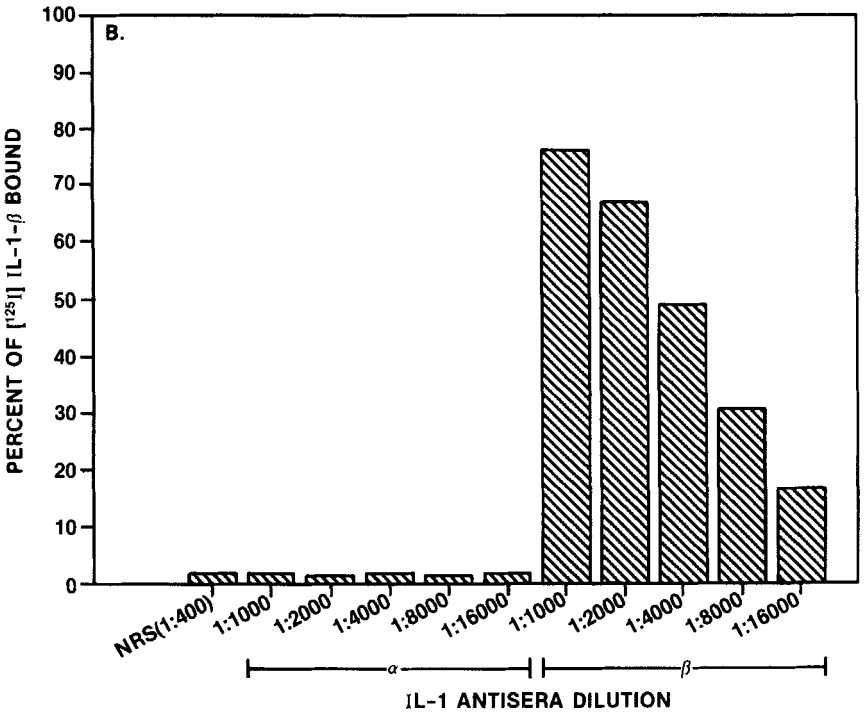

FIGURE 2b

IL-1α or IL-1β is very specific, and, thus, should be useful for the development of radioimmunoassays for each of these IL-1 variants.

Figure 3 shows dose response curves for rhIL-1α (Fig. 3a) and rhIL-1β (Fig. 3B). Under the experimental conditions used, a 50% displacement was observed with 0.5 ng of rhIL-1α and 50 pg of rhIL-1β at a concentration of 10 ng/ml and 1 ng/ml respectively.

As shown in Fig. 3A, IL-1α RIA was validated with two different sources of IL-1α as well as with two negative controls, rhIL-1β and tumor necrosis factor (TNF). Likewise, The international IL-1β standard

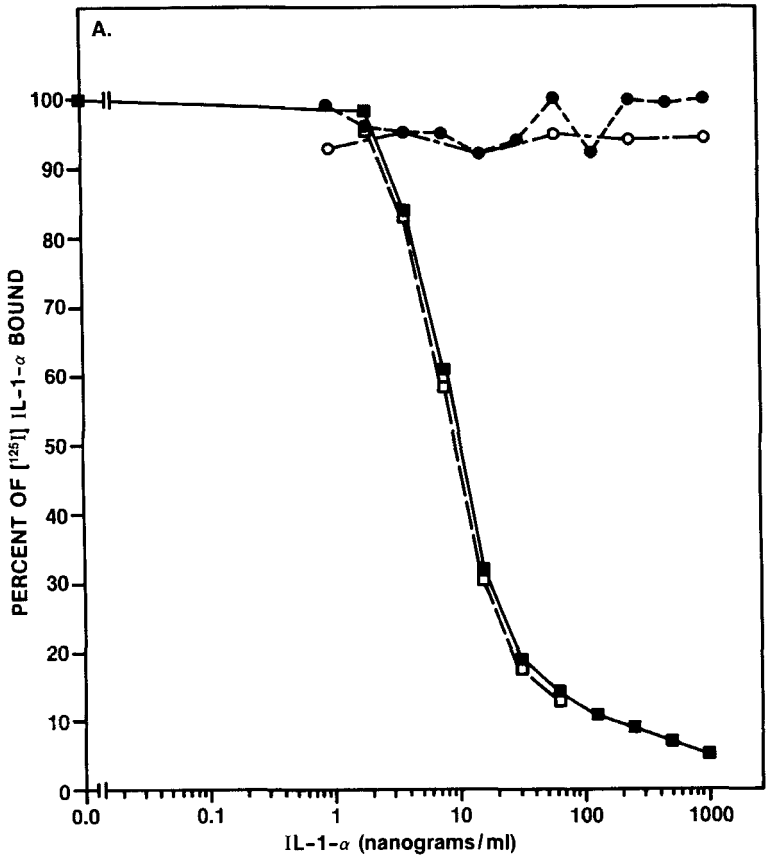

FIGURE 3. A, Effect of various concentrations of rhIL-1α (■—■), International IL-1α standard (□—□), rhIL-1β (●—●) and TNF (o—o) on the binding of ^{125}I IL-1α with rabbit anti IL-1α. B, Effect of various concentrations of rhIL-1β (■—■), International IL-1 standard (□—□), rhIL-1α (o—o) and TNF (●—●).

(86/552) compares favorably with our IL-1β standard. As expected, no cross reactivity (<0.1%) towards rhIL-1α or TNF was observed (Fig. 3B).

Assay precision and recovery at various IL-1α levels are shown in Table 1. Four spiked samples were analyzed and high recoveries (≧93%) were obtained with a coefficient of variation of less than 10%.

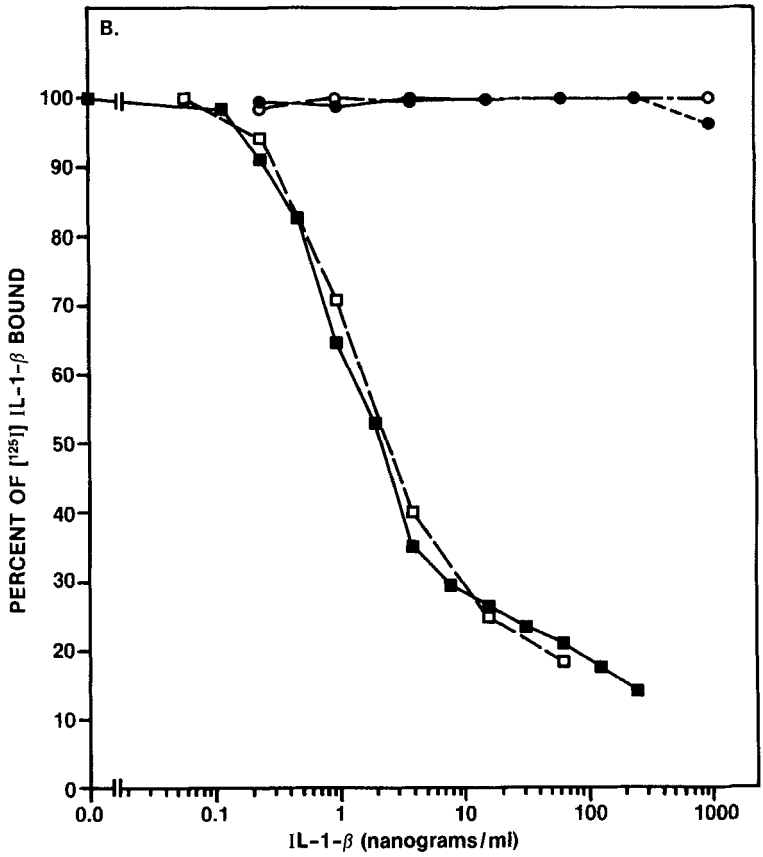

FIGURE 3B

Table 2 shows the assay variation and recovery for samples to which IL-1β was exogenously added. Again, good recoveries were achieved. These experimental results have indicated that these RIA procedures are expected to be of practical use in distinguishing IL-1α from IL-1β in biological fluids, as well as, in their quantitation from various other sources.

TABLE 1

Recovery of Recombinant Human IL-1α Added to Human Serum [a]

rhIL-1α Added (ng/ml)	rhIL-1α Measured (ng/ml)	Recovery of rhIL-1α (%)	CV[b] (%)
0	n.d.	–	–
8	7.8 ± 0.07	97.5	2.3
16	14.9 ± 0.14	93.1	2.3
32	34.0 ± 0.59	106.3	4.2
64	63.7 ± 2.56	99.5	9.8

a The standard RIA conditions for IL-1α are described under Materials and Methods. No IL-1 activity was detected in these samples when subjected to IL-1β RIA. Data represent mean ± S.D. (n = 6).

b Coefficient of variation

TABLE 2

Recovery of Recombinant Human IL-1β Added to Human Serum [a]

rhIL-1β Added (ng/ml)	rhIL-1β Measured (ng/ml)	Recovery of rhIL-1β (%)	CV[b] (%)
0	n.d.	–	–
0.5	0.44 ± 0.02	88	11.8
1.0	0.76 ± 0.02	76	5.9
2.0	1.63 ± 0.03	81	4.1
4.0	3.76 ± 0.05	94	3.5
8.0	6.69 ± 0.24	84	8.8
16	16.18 ± 0.85	101	12.9

a The standard RIA conditions for rhIL-1β are described under Materials and Methods. No IL-1 activity was detected when samples were subjected to IL-1α RIA. Data represent mean ± S.D. (n = 6).

b Coefficient of variation

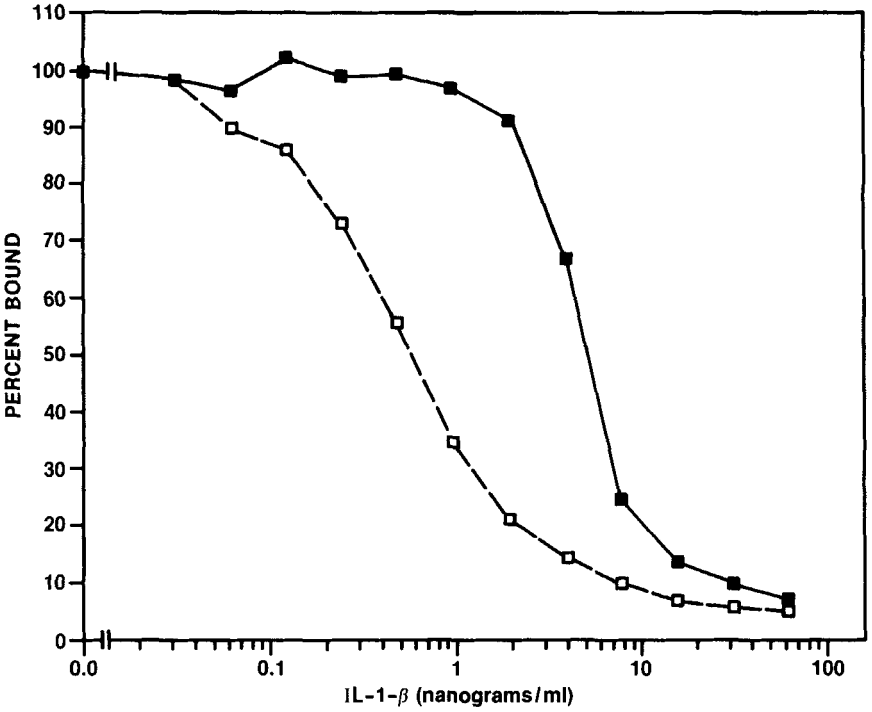

FIGURE 4. Comparison of dose response curves for rhIL-1β., 1 :1000 anti IL-1β dilution; ■—■ ,1:8000 anti IL-1β dilution; □—□.

The sensitivities of these assays can be increased by using higher dilutions of the antibodies. Fig. 4 shows a comparison of dose response curves at two different antibody dilutions for rhIL-1β. At the 1:8000 dilution the 0.12 ng/ml standard gives a response that is significantly different from zero, indicating that about 6 pg is the lower detection limit of the assay for IL-1β. In contrast, at a 1:1000 antibody dilution the lower detection limit was about 125 pg. Similar adjustments can be made to increase the sensitivity of IL-1α RIA by an order of magnitude or more.

The lower detection limit of IL-1α or IL-1β is likely to vary depending upon the specific activity and integrity of the label used. The lowest concentration of IL-1β that can be detected by the IL-1β RIA kit (Cistron) is 25 pg and it is recommended for the quantitative measurement of IL-1β levels in biological fluids. Data reported here thus would suggest that it should be possible to detect, quantitate, and distinguish IL-1α and IL-1β from various biological fluids.

DISCUSSION

The results presented here clearly demonstrate that there are antigenic determinants present on human IL-1α that are not carried on human IL-1β and vice versa. Tissue preparations and bodily fluids, as well as, many cell lines produce variable amounts of either IL-1α or IL-1β. In many instances, it is likely that the translation of a specific gene product may predominate. However, since this phenomena has to date been a problem due to the inability to test for each protein separately, the reported RIA's would definitely serve a useful purpose in future biological analyses. Knowledge of, or the ability to detect specific gene products may ultimately be a critical factor in the design of specific antagonists in inflamanatory diseases treatment regimens.

Practically all biological fluids and many cell lines, in addition to producing IL-1α or IL-1β, may also produce specific biological inhibitors. Examples include a urine derived human IL-1 inhibitor (26) and a monocyte derived IL-1 IL-1 inhibitor (27). It is likely that these

inhibitors are under strict physiological balance, although the production of such proteins is still poorly understood. For this reason, direct assays of IL-1 using traditional bioassays (17-20) may be misleading, and in many cases, unreliable. A major reason for the lack of information is due to lack of isolation to homogeneity of an IL-1 inhibitor. The RIA's reported herein provide a reliable method for screening for IL-1α or IL-1β directly based on the protein sequence of these agonists, but are not influenced by the biological antagonism routinely ignored to date.

Our antisera should also prove useful in immunoaffinity purification of rare sources of IL-1α or IL-1β for which current purification technologies are unsatisfactory. Preliminary results show that immunoaffinity columns prepared using either purified anti IL-1α or anti IL-1β IgG's successfully adsorb out, specifically, the antigen of choice (28).

During the course of this work, we have learned that anti-human IL-1 sera is available from a number of companies. However, at least two companies claim that their sera bind to both IL-1 forms. Likewise, a RIA kit for IL-1β is commercially available from Cistron Biotechnology (New Jersey). As far as we know this is the first time antisera specific for the IL-1α and its side by side comparison with anti IL-1β have been documented. We believe these reagents and RIA's will prove useful in a variety of experimemtal studies some of which are discussed above.

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