

Biological assays for interleukin 1 detection

Comparison of human T lymphocyte, murine thymocyte and NOB-1 assays

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Various biological assays are used for qualitative and quantitative measurements of interleukin 1 (IL-1) in supernatants from cell cultures. The purpose of the present study was to compare the specificity and variability of three cellular IL-1 bioassays: the PHA co-stimulatory human T lymphocyte proliferation assay, the PHA co-stimulatory murine thymocyte (THY) proliferation assay, and the 2-step NOB-1 conversion assay. Three different ways of IL-1 unit calculation, based on a semi-logarithmic plot, a double-logarithmic plot, or a probit-analysis plot were also compared. The T lymphocyte assay can be used only to demonstrate qualitative differences in IL-1-like activity, whereas the THY assay is excellent as a semi-quantitative assay, with a low intra-assay variability, but also with a low specificity. The NOB-1 assay is probably more specific with respect to IL-1 measurement, although, with a high intra-assay variance. The THY and the NOB-1 assays both have a high inter-assay variability, and measurement of samples from longitudinal clinical studies must be done in one and the same analysis if quantitative differences are to be illustrated. Probit analysis for unit calculation is recommended. To generate a consensus view as to assay performance, collaborative laboratory studies are needed.

Key words: interleukin 1; NOB-1; thymocytes; T lymphocytes; specificity; variability.

Accepted for publication 16 June 1990

CLINICAL ASPECTS

Cytokines, immune hormones, are presently attracting increasing attention as fundamental mediators of inflammation and immunopathogenesis, and methods for cytokine detection are now widely used in clinical studies. Bioassays appear to be, physiologically, the most relevant way to demonstrate cytokine activity. The actual production and release of the cytokine is measured through the amount available for interaction with the target cells. Moreover, inhibitors of the biological cytokine activity can be demonstrated. To interpret data from interleukin 1 (IL-1) analyses in clinical investigations, one should be aware of the low specificity of some IL-1 bioassays. Other cytokines and unknown biological factors, as well as IL-1-inducing factors and drugs can interfere with biological IL-1 detection. Due to a high inter-assay variability in cellular IL-1 bioassays, it is recommended in longitudinal clinical trials to perform the IL-1 measurements using one and the same test. Only then can reliable demonstration of quantitative differences be made.

In 1979, the term interleukin 1 (IL-1) was proposed as the common denominator for the T- and B-cell activating factors present in supernatants from monocyte (M ϕ) cultures (18). To-

day IL-1 activity can be demonstrated by use of bioassays (22), histological technics (20), solid-phase methods (10), and by expression of IL-1 mRNA in activated cells (9).

Bioassays appear to be, physiologically, the most relevant way to demonstrate IL-1 activity. The actual production and release of the cytokine is measured through the amount available for interaction with the target cells. Moreover, inhibitors of the biological cytokine activity can be demonstrated.

Various bioactivities have been attributed to IL-1 (2). One of these is the induction of lymphokine production by T lymphocytes, in particular the T-cell growth factor or interleukin 2 (IL-2). This lymphokine induces T-cell and thymocyte proliferation, thus being the basis of the most commonly used bioassays for IL-1. Assays based on this principle comprise techniques which use human thymocytes (17) or T lymphocytes (7, 18), murine thymocytes and T-cell lines (11, 22).

In this study three different IL-1 bioassays are compared with regard to specificity and inter- and intra-assay variability, namely a) the PHA co-stimulatory human T lymphocyte proliferation assay, b) the PHA co-stimulatory murine thymocyte proliferation assay, and c) the 2-step NOB-1 conversion assay. In addition three mathematical ways to calculate IL-1 content are compared.

MATERIAL AND METHODS

Mø and monocyte depleted cell (MDC) culture medium: RPMI 1640 with 25 mM HEPES, (Boehringer-Mannheim, Mannheim, Germany, batch No. 67971102) was supplied with 0.8 M L-glutamine (Gibco, Paisley, Scotland), 400 units/ml penicillin (Leo, Ballerup, Denmark) and 500 µg/ml streptomycin (Novo, Copenhagen, Denmark). Pooled normal human serum (NHS), was added at various concentrations. The endotoxin content of these culture media and reagents was less than 5 pg/ml (1). Using MDC, the RPMI 1640 with 25 mM HEPES was from Gibco.

Thymocyte (THY) and CTLL-2 culture medium: MDC culture medium composed as described above was supplied with 2.5×10^{-5} M 2-mercaptoethanol (2-ME) (Sigma, St. Louis, Mi, USA). In this case, however, the serum was heat-inactivated (HI) at 56°C for 30 min.

NOB-1 culture medium was composed as the THY culture medium but without 2-ME.

Mø- and MDC-suspensions were prepared as described previously (7). Briefly, mononuclear cells (MNC) were isolated from heparinized whole blood after density gradient centrifugation on Lymphoprep®. The MNC were washed thoroughly, resuspended in medium with 15% NHS and plated on plastic Petri dishes for 90 min at 37°C. After decanting the non-adherent cells and washing the dishes, the remaining adherent cells, termed Mø, were detached with chilled 10^{-2} M EDTA-phosphate buffered saline. The non-adherent cells were further purified on nylon-wool columns and finally resuspended in medium with 15% NHS at 2×10^5 cells/ml. These cells, mainly T cells, were termed MDC.

THY-suspension. Thymus was aseptically removed from C3H/SSI female mice, 6–8 weeks of age (Statens Serum Institute, Copenhagen, Denmark), teased in THY culture medium, and passed through a sterile No. 60 mesh stainless steel sieve. Cells were washed twice in medium with 5% HI-NHS and finally resuspended in medium with 10% HI-NHS at 1×10^7 cells/ml.

NOB-1 cell-line was purchased from Eur Collection of Animal Cell Cultures (Porton Down, UK). The cells were maintained in NOB-1 culture medium with 5% (v/v) heat-inactivated foetal calf serum (HI-FCS) (Gibco). The cultures were split and re-fed when cell densities reached $5\text{--}10 \times 10^5$ cells/ml.

CTLL-2 cells. This cell line was purchased from American Type Culture Collection, (Rockville, MD, USA). The cells were maintained in THY medium supplemented with 5% (v/v) HI-FCS and biological IL-2 (EL-4 cell line, own production), 40 U/30 ml. The cultures were split when cell density reached $5\text{--}10 \times 10^5$ cells/ml.

Production of Mø-supernatants. Mø cultured in medium with 1% NHS, at 3×10^5 cells/ml in 1.5 ml aliquots, were stimulated in round-bottomed polystyrene tubes (110 × 16 mm, Nunc, Roskilde, Denmark) with 2 µg/ml of lipopolysaccharide (LPS) (*E. coli* 055:B5, Westphal method, Difco, Detroit, Mich., USA), or cultured with pyrogen-free isotonic NaCl (Dispen-

sary, Rigshospitalet, Copenhagen, Denmark) for 18 h at 37°C. After centrifugation, cell-free LPS- and NaCl-induced supernatants were reconstituted with NaCl or LPS, respectively, and either stored in cryo-tubes (38 × 12.5 mm, Nunc) at -20°C or further purified.

IL-1 standard. A pool of eight crude supernatants of Mø-cultures stimulated with LPS as mentioned above.

Recombinant human IL-1α (rIL-1α), kindly donated by S. Gillis (Immunex, Seattle, WA, USA), was diluted in RPMI-1640 with 5% NHS to 100 U/ml.

Assays for IL-1 activity

a) *The MDC proliferation assay* was performed as previously described (7). Briefly, samples to be analysed and the standard were tested at 2% (v/v) or in serial 2-fold dilutions from 10% (v/v). MDC, 1×10^5 cells/500 µl MDC culture medium with 15% (v/v) NHS, were added followed by submaximal concentrations, 3 µg/ml, of phytohaemagglutinin (PHA) (PHA-P, Difco). The 72-h proliferation was measured by ^{14} C-thymidine incorporation for the last 24 h.

b) *The THY proliferation assay.* Samples and standard were tested in serial 2-fold dilutions from 10% (v/v) in 96-well round-bottomed microtitration plates (Nunc). THY, 1×10^6 cells 100 µl THY culture medium with 10% HI-NHS, and finally 20 µg/ml of PHA were added giving a total volume of 220 µl/well. The 72-h THY proliferation was measured after ^{3} H-thymidine addition, 0.5 µCi/well (New England Nuclear, Dreieich, Germany, specific activity 6-7 Ci/mmol) for the last 24 h. Preliminary experiments revealed that maximum proliferation was reached with this assay set-up.

c) *The NOB-1 assay* was performed by a slight modification of the two-stage IL-1 assay described by Gearing et al. (11): IL-1 standard diluted 1:40, fractions from chromatographies diluted 1:10 or undiluted rIL-1α were added in volumes of 100 µl to appropriate wells in a microtitration plate (Nunc). NOB-1 cells, 5×10^5 cells/ml, washed and resuspended in NOB-1 culture medium with 1% HI-NHS,

were finally added giving a total volume of 200 µl/well. After 24 h at 37°C and 5% CO₂, 50 µl supernatant from each well was transferred into a replicate microtitration plate. The plates were frozen at -80°C for 20 min in order to kill remaining NOB-1 cells, and thawed supernatants were tested for IL-2 content by the CTLL-2 assay (12).

Assay for IL-2. The CTLL-2 cells, 4×10^3 /100 µl THY culture medium, supplemented with 5% HI-NHS, were cultured with 50 µl samples in microtitration plates (Nunc) for 28 h. The cell proliferation was measured by ^{3} H-thymidine incorporation, 0.5 µCi/well (New England), for the last 4 h of the culture period.

Proliferative response. IL-1 activities were calculated on the basis of the medians of triplicate determinations in counts per minute (CPM). The results are given in CPM, Δ CPM or as a stimulation index (SI):

$$\Delta \text{ CPM} = \text{CPM}_{\text{PHA} + \text{supernatant}} - \text{CPM}_{\text{PHA} + \text{culture medium}}$$

$$\text{SI} = \frac{\text{CPM}_{\text{PHA} + \text{supernatant}}}{\text{CPM}_{\text{PHA} + \text{culture medium}}}$$

Unit calculation. Estimations of IL-1 activity in Mø-supernatants, expressed in units ml, were done by 3 methods:

1. *Semi-logarithmic linear regression analysis:* a linear regression curve was calculated from the ^{3} H-thymidine incorporation data, generated from a log₂ dilution series of the IL-1 standard. The X-axis dilution at which the curve crosses the two times background Y-axis ordinate was defined as 1 unit of IL-1 activity.
2. *Double logarithmic linear regression analysis:* The linear regression was calculated as above, but in a double logarithmic plot. The X-axis dilution at which the curve crosses the 1000 CPM Y-axis ordinate was defined as 1 unit of IL-1 activity.
3. *Probit analysis:* This method has been described previously for IL-2 (12). Briefly, the probit-transformed ^{3} H-thymidine incorporation data (CPM), generated from a log₂ dilution series of the IL-1 standard, was plotted by linear regression. The X-axis dilution corresponding to 50% of maximum activity was defined as 1 unit of IL-1 activity.

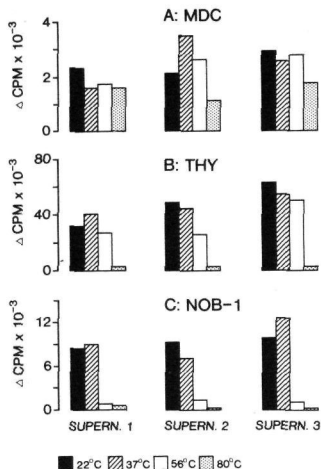


Fig. 1. Effect of heat-inactivation on the IL-1 like effect of 3 LPS-induced supernatants (supern. 1, 2 and 3) from Mø-cultures. Supernatants were tested A: At 2% (v/v) in the PHA, 3 µg/ml, co-stimulatory human MDC proliferation assay. B: At 5% (v/v) in the PHA, 20 µg/ml, co-stimulatory THY proliferation assay, and C: 10% (v/v) in the NOB-1 assay.

The three calculation methods were embodied in a computer programme which estimates the IL-1 activities in the standard and in samples with provision of the coefficients of correlation. Estimations of IL-1 units in samples were done by the parallel bioassay principle (3).

Ultra- and diafiltration: An Amicon ultrafiltration device (model 8050, Amicon Corp., Danvers, MA., USA) was used for ultra- and diafiltration of the Mø-supernatants through a 5 kilodalton (kD) cut-off filter (YM 5, Amicon) as previously described (21). In the diafiltration step, the supernatant was extracted 3 times with NaCl (0.15 M) - HEPES (0.05 M) buffer, pH 7.5, 10 times the volume.

Sephadex gel filtration chromatography: The IL-1 standard was concentrated 10-fold by ultrafiltra-

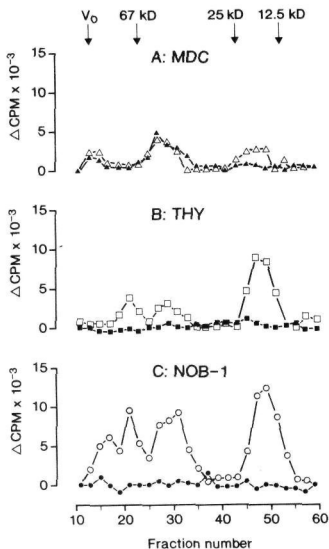


Fig. 2. IL-1 activities of fractions after Sephadex G-100 sieve chromatography of the IL-1 standard.

Fractions were tested before (open symbols) and after heat-inactivation at 80°C (closed symbols), at 10% (v/v) in: A: the MDC proliferation assay (▲/△), B: the THY proliferation assay, (□/■), and at 5% (v/v) in C: the NOB-1 assay (○/●).

tion through YM 5 and subsequently diafiltered through the same filter, as described above. Samples of 2 ml were applied to a 1.5 × 90 cm Sephadex G-100 column (Pharmacia, Uppsala, Sweden) equilibrated with NaCl (0.15 M) - HEPES (0.05 M) buffer, pH 7.5. Chromatography was performed at 4°C at a rate of 10 ml/h. Fractions of 2 ml were collected and stored at -20°C until tested for IL-1 activity. The column was calibrated with blue dextran, human serum albumin, chymotrypsinogen and horse cytochrome C (Sigma).

Supernatant heat inactivation: Sealed cryo-tubes (Nunc) containing Mø-supernatants were placed in a 37°C, 56°C or 80°C water-bath for 1/2 h.

Statistics

Differences in variability within assays were evaluated either by the Wilcoxon or the Friedman two-way analysis of variance. The variability between assays was evaluated by the Mann-Whitney or the Kruskal-Wallis one-way analysis of variance. Student's *t*-test was used for comparison of inter- and intra-assay variability.

RESULTS

Specificity of assays

IL-1 activity in supernatants from Mø-cultures was not completely destroyed by heat treatment (80°C for 30 min) when analysed by the MDC assay, whereas the activity was abolished when measured on THY or NOB-1 cells. (Fig. 1 A, B & C). The molecular weight(s) of the factor(s) was estimated by sieve-chromatography (Fig. 2). All the assays were sensitive to factors eluting

with the 12.5–25 kD and the 40–60 kD MW markers, whereas the THY and the NOB-1 assays also reacted to factor(s) in the 60–80 kD MW area. The heat-stable MDC co-stimulatory factor(s) had a molecular weight of 40–60 kD (Fig. 2). Heat-inactivated fractions from this MW area had no effect in the THY or in the NOB-1 assays.

The effect of the IL-1 standard on THY cells differed from the effect of rIL-1 α : The cell proliferation reached a higher maximum with the standard than with rIL-1 α , and the slope of the dilution curve was steeper (Fig. 3B). In the NOB-1 assay, there was no difference between the two preparations (Fig. 3C).

All supernatants and active chromatographic fractions were without measurable amounts of IL-2, judged by CTLL-2 assay.

Variability in unit estimation

With the MDC assay, in contrast to the THY and the NOB-1 assays, the proliferation resulted in a low SI and a shallow slope in the dilution curve (Fig. 4). Further analyses with the MDC assay were not performed. With the THY and the NOB-1 assays the 2-fold dilution curves resulted in significant decreases in SI ($P < 0.001$, Friedman).

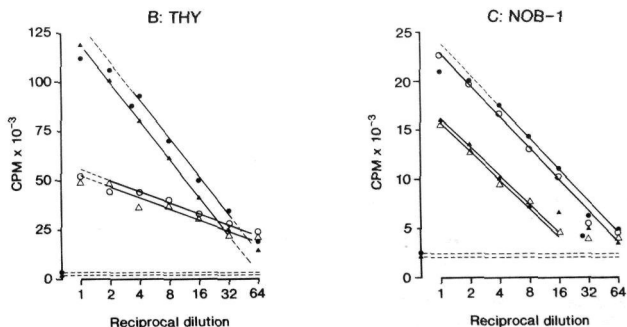


Fig. 3. Effect of the IL-1 standard (closed symbols) and of rIL-1 α (open symbols), both tested at 10% (v/v) after 2-fold dilution in B: the THY proliferation assay ($n = 2$) and C: the NOB-1 assay ($n = 2$).

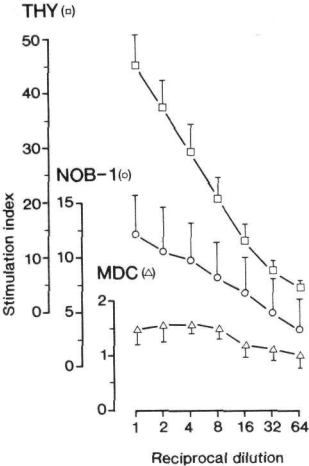


Fig. 4. Effect of an IL-1 standard tested after 2-fold dilution at 2 % (v/v) in: A: The MDC proliferation assay (Δ) and at 10 % (v/v) in B: The THY proliferation assay (□) and C: The NOB-1 assay (○). Proliferative response is given as mean stimulation index (SI) ($n = 12$), and bars represent 2 times mean SD from triplicate determinations of SI.

In contrast to the THY assay, the NOB-1 assay showed a significant difference ($P < 0.001$, Friedman) in variability of triplicate CPM determinations with increased dilution of the sample (Table 1).

The inter-assay variability in unit estimation was high (Table 2), especially when using double-logarithmic linear regression analysis. The semi-logarithmic analysis also gave a high inter-assay variation in the NOB-1 assay. Therefore, these two calculation methods were not employed in the following analyses of variability.

Using the probit-analysis for unit calculation, the mean THY and mean NOB-1 inter- and intra-plate variations in units (Table 3), were significantly below the inter-assay variability (Table 2) ($P < 0.01$, t-test). The intra-plate

Table 1.

Triplicate CPM variability in THY and NOB-1 assays, expressed as coefficients of variation in %. Variability was analysed at n repetitive days by 3-fold test of the sample within each dilution. Figures represent median with ranges in brackets

Dilution	Coefficient of variation in %		
	THY IL-1 standard ($n = 11$)	NOB-1 § IL-1 standard ($n = 11$)	NOB-1 § rIL-1 ($n = 13$)
1/1	6 (2-35)	8 (2-27)	15 (3-24)
1/2	6 (1-40)	10 (1-35)	14 (3-38)
1/4	10 (4-31)	7 (1-41)	12 (4-36)
1/8 #	8 (1-29)	15 (4-32)	16 (7-33)
1/16	10 (1-31)	9 (4-44)	18 (2-74)
1/32 #	7 (3-16)	19 (4-56)	23 (8-51)
1/64	14 (4-49)	20 (0-90)	36 (12-67)
BACKG #	13 (5-47)	17 (1-60)	35 (21-59)

Abbreviations: CPM: counts per minute, THY: thymocyte proliferation assay, NOB-1: two stage IL-1 assay, IL-1: interleukin 1, rIL-1a: recombinant IL-1a, BACKG: background.

Significant changes in coefficient of variation between assays within a dilution ($P < 0.04$, Kruskal-Wallis).

§ Significant changes in coefficient of variation between dilutions within an assay ($P < 0.001$, Friedman).

variability differed significantly ($P < 0.03$, Kruskal-Wallis) between assays (Table 3). No

Table 2.

Inter-assay variabilities in the THY and the NOB-1 assays, expressed as coefficient of variation in %, when a sample was measured in units/ml at repetitive days ($n = 10$). Unit calculation was performed by 3 different methods

	Coefficient of variation in %		
	THY IL-1 standard	NOB-1 IL-1 standard	NOB-1 rIL-1a
Semi-logarithmic linear regression analysis	24	152	242
Double-logarithmic linear regression analysis	159	296	116
Probit- analysis	25	33	60

Abbreviations: THY: thymocyte proliferation assay, IL-1: interleukin 1, NOB-1: two-stage IL-1 assay, rIL-1a: recombinant IL-1a

significant differences were found between inter- and intra-plate variation within assays, or in inter-plate variability between assays.

DISCUSSION

Biological assays for IL-1 activity are often sensitive not only to IL-1 precursors but even to IL-1 fragments (5). All three assays, in the modification used, are sensitive to IL-2 (11), and further, the THY assay is sensitive to IL-4 (4), to IL-6 (14), and to high concentrations of TNF- α (6). Apart from being sensitive to IL-1, the NOB-1 assay is responsive to IL-4 due to the CTLL-2 cells in the second step (13). Our investigation illustrates that the MDC assay is also sensitive to one or more heat-stable factors (Fig. 1) with apparent MW's in the range 40–60 kD (Fig. 2). These factors do not affect the THY or the NOB-1 assays.

Table 3.

Inter- and intra-plate variability, expressed as coefficient of variation in %, when a sample was measured in units/ml by use of the probit analysis quantitation programme. Inter-plate variability is based on unit-estimation from 4 different plates, column 1–3 in each plate and intra-plate variability on unit-estimation 4 times within the same plate, column 1–3, 4–6, 7–9 and 10–12, analysed at n repetitive days.

Figures represent median variability with (ranges) and [mean $\pm$ 2SD]

		<i>n</i>	Coefficient of variation in %	
			Inter-plate **	Intra-plate *
THY	(IL-1 standard)	5	10 (6-17) [11±11]	6 (3-13)*** [7±8]
NOB-1	(IL-1 standard)	6	15 (13-34) [18±16]	18 (9-23)*** [19±18]
NOB-1	rIL-1 α	7	not done	19 (8-39) [20±22]

Abbreviations: IL-1: interleukin 1, rIL-1 α : recombinant IL-1 α , THY: thymocyte proliferation assay, NOB-1: two-stage IL-1 assay.

* Significant changes in coefficient of variation between assays ($P < 0.03$, Kruskal-Wallis).

** No significant difference between assays ($P > 0.05$, Mann-Whitney).

***No significant differences within assays ($P > 0.05$, Wilcoxon).

The steep titration curve obtained in the THY assay with the IL-1 standard compared with the curve obtained with rIL-1 α (Fig. 3) indicates that M ϕ -supernatants contain other co-stimulatory factor(s) than IL-1 α . Thus, a shift in the slope of the titration curve during the course of a purification procedure may indicate the removal from a sample of other co-stimulatory or inhibitory factors. This illustrates the problem of using a homogeneous preparation as standard, when samples containing complex mixtures of active molecules are analysed in non-specific assays. With such assays, the standard should ideally be of a similar grade of purity as the test sample. The parallel and almost identical titration curves of IL-1 α and standard with the NOB-1 assay, indicate that this assay has a higher specificity for IL-1 than the THY assay (Fig. 3).

As cytokines seldom show linear dose-response curves and because of a considerable variance in the level of these curves, the proliferative response in CPM values can not be used for quantitative comparison of samples. Thus, although often neglected, the biological effect of a cytokine should be quantified through titration curves (15).

Due to the steep slopes of the titration curves, the THY and the NOB-1 assays are usable as semi-quantitative assays in contrast to the MDC assay (Fig. 4), which can only be used to analyse differences in IL-1 activities between samples (8).

The lowest inter-assay variability in unit estimation was obtained with the THY assay, both when using semi-logarithmic linear regression analysis and probit analysis (Table 2). With the NOB-1 assay and the semi-logarithmic analysis, the variability increases. This is explained by the data at the tail of the titration curve, which have the highest variability in triplicate CPM determinations (Table 1). Thus, the semi-logarithmic analysis is useless for unit estimation of IL-1 with the NOB-1 assay. Likewise, the double-logarithmic plot is useless no matter the assay, contrary to the results of others (19). This difference is probably due to the PHA concentration used during THY proliferation and the way to define a unit.

In conclusion: The MDC assay can be used only to demonstrate qualitative differences in IL-1 like activity, whereas the THY assay is excellent as a semi-quantitative assay, with a low intra-assay variability and a low specificity. The NOB-1 assay is probably more specific with respect to IL-1 measurement, although, with a high intra-assay variance. Due to the high inter-assay variability, the testing of samples from longitudinal clinical studies must be done in one and the same analysis if quantitative differences are to be illustrated. Probit analysis for unit calculation is recommended.

Despite the emergence of apparently specific immuno-chemical assays for cytokines, biological assays are still necessary in order to ascertain bioactivity of a given cytokine and to register the presence of substances that interfere with their bioactivity. However, collaborative laboratory studies testing several IL-1 preparations by a variety of techniques are needed in order to generate a consensus view as to assay performance.

ACKNOWLEDGEMENTS

We are grateful to Ms Bente Andersen, Ms Lone Bruhn and Ms Kirsten Junker for excellent technical assistance. We also thank Dr. L. Baek for the endotoxin measurements, Dr. S. Gillis for his kind gift of rIL-1 α and Dr A. R. Thomsen for delivering C3H/SSI mice. The critical revision of the manuscript by consultant V. Andersen, and the help with the statistical analysis by Dr. A. Dirksen, is much appreciated.

Financial support was obtained from the Danish Rheumatism Association, the Danish Medical Research Council and the Nordic Assurance Company Jubilee Foundation.

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