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Development and validation of a bioassay for Interleukin-2

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Abstract: A reliable and precise method for the determination of IL-2 activity, based on stimulation of CTLL cell proliferation, was developed. Cells were incubated with different concentrations of IL-2 for 24 h in microtiter plates. The stimulatory effect was measured on a plate-reading spectrophotometer by reading the optical density of formazan, which is produced by viable cells from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The bioassay was designed as a four-dose parallel line test, fulfilling pharmacopoeial requirements for assay validity, and the inter-assay relative standard deviation (RSD) for a group of four experiments was 2.6%. The International Standard for human IL-2 and the Reference Reagent for Recombinant DNA-derived IL-2 were employed for potency determinations. The method was found suitable for potency assessments of pharmaceutical formulations of IL-2.

Keywords: *Interleukin-2; bioassay; CTLL-2 cells; formazan; WHO International Standard; pharmacopoeial standards.*

Introduction

Interleukin-2 (IL-2) is a potent lymphokine that induces mitogenic stimulation and changes in surface receptor expression on activated T-lymphocyte subsets [1–3].

Pharmaceutical preparations of human IL-2 prepared by recombinant DNA technology (rhIL-2) have recently been approved for marketing in several countries. Preparations based on expression of rhIL-2 in *Escherichia coli* contain a modified molecule, 125-serine hIL-2, to enable the biosynthesis in prokaryote cells of a polypeptide with a tertiary structure that conserves bioactivity [4–6].

A number of methods have been developed for determination of IL-2 activity [7–14]. Some of these use the proliferation of murine (C57BL/6) cytotoxic T-lymphocytes (CTLL) as an index of IL-2 activity [7, 9, 10, 13]. However, none of these assay methods has been designed to fulfil pharmacopoeial requirements for the quantitation of pharmaceutical preparations of biologicals. The authors have developed and validated a four-dose parallel line assay for IL-2 based on its stimulatory effect on the proliferation of CTLL-2 cells, using the MTT colorimetric detection system [8], and designed to fulfil established pharmacopoeial statistical validity requirements for bioassays. The method determines the potencies of IL-2 preparations as a percentage of the recently established International Standard for Human IL-2 [15] or the Reference Reagent for Recombinant DNA-derived IL-2 [15]. The assay is easy to perform and gives reliable results with high sensitivity and precision.

Materials and Methods

Cells and culture conditions

The murine cytotoxic T-cell line CTLL-2 [16] was obtained from the American Type Culture Collection (Rockville, MD, USA). CTLL-2 is dependent on IL-2 for its survival in culture. The cells were grown in RPMI 1640 medium (The National Veterinary Institute, Uppsala, Sweden), supplemented with 5% foetal calf serum, 2 mM L-glutamine, 100 IU ml^{-1} benzylpenicillin, 100 μg ml^{-1} streptomycin, 2 mM sodium pyruvate, and 40 mM HEPES, pH 7.4 (Flow Laboratories, Irvine, UK) and 50 μM β-mercaptoethanol (Sigma Chemical Co., St Louis, MO, USA). IL-2 was added to a final concentration of 65 IU ml^{-1} (complete medium). Cells were maintained in a humid atmosphere, with a composition of 5% CO_2/95% air in 50 ml Nunclon Delta plastic flasks (A/S Nunc, Roskilde, Denmark). Before

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use in an experiment the cells were washed three times in ice-cold phosphate-buffered saline (PBS) (The National Veterinary Institute, Uppsala, Sweden) and resuspended in complete culture medium, with IL-2 excluded (assay medium), at a density of 2×10^5 cells ml^{-1}.

Reagents

The International Standard for Human IL-2 (WHO, 86/504; prepared from lectin-stimulated cells of the human T-cell line Jurkat) and the Reference Reagent for Recombinant DNA-derived IL-2 (WHO, 86/564; derived from rDNA-expression in *E. coli*) were provided by the National Institute for Biological Standards and Control (Potters Bar, UK). These preparations were stored in sealed ampoules at −20°C until use. They were dissolved in assay medium or in PBS. Recombinant human IL-2 (rhIL-2) was provided by two manufacturers. The commercial rhIL-2 preparation Ia was dissolved just before analysis in assay medium to the manufacturer's recommended concentration (tested against WHO, 86/504) or to 3.2×10^4 IU ml^{-1} (stored at −70°C for up to several months before testing for potency against WHO, 86/564). Preparations Ib and II were stored as lyophilized powder at +4°C and reconstituted in distilled water according to the manufacturer's instructions.

Human insulin (Actrapid^R Human, Novo Nordisk A/S, Bagsvaerd, Denmark) and human growth hormone (Genotropin^R, Kabi Pharmacia AB, Stockholm, Sweden) were obtained from a local pharmacy. Substance P was from Peninsula Laboratories Europe Ltd (St Helens, Merseyside, UK). Interleukin-3 was a gift from Dr Jim Ihle, St Jude Children's Research Hospital (Memphis, TN, USA). Interleukin-4 was provided by Kabi Pharmacia AB (Uppsala, Sweden).

A 6.5 mg ml^{-1} sterile stock solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was prepared by dissolving MTT in PBS and passing it through a 0.2-μm membrane. MTT and sodium dodecyl sulphate (SDS; dissolved to 25%, w/v, in 0.033 M HCl) were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Assay conditions

Potency determinations. The cytokine preparations were diluted in assay medium in two separate series, each comprising four distinct concentrations of IL-2. These concentrations were selected from the approximately linear portion of the log dose–response curve. Samples were transferred in pentaplicate to 96-well flat bottom microtiter plates and mixed with an equal volume of CTLL-2 cell suspension that had been prewashed three times in ice-cold PBS and resuspended in assay medium. Microwells, which contained only indicator cells and assay medium as well as those with an excess of IL-2 (65 IU ml^{-1}), served as an estimate of the minimum and maximum proliferative stimulation, respectively. The cell suspensions were then incubated at 37°C (5% CO_2/95% air) for 24 h before determination of the proliferative stimulation. Protocols, specific for two types of assays, are detailed below.

Cell density. A 125 μl volume of the dilute rhIL-2 samples was mixed with an equal volume of CTLL-2 cell suspension (final cell density: 1×10^5 cells ml^{-1}). Following incubation and careful mixing, 200 μl from each culture well was diluted with 19.8 ml Isoton II solution (Coulter Electronics Ltd, Luton, UK) and analysed for cell numbers using an automatic cell counter (Coulter Counter, D, Industrial from Coulter Electronics Ltd, Luton, UK). The arithmetic mean of these records was filed as the final cell density.

MTT reduction. Samples, containing IL-2 or assay medium only, of 90 μl were mixed with 90 μl of a CTLL-2 cell suspension of 2×10^5 cells ml^{-1}. Following the 24-h incubation, 20 μl of a 6.5 mg ml^{-1} solution of MTT, was added to each well. The plates were thereafter allowed to incubate for another 4.5 h, in the same environment, before termination with 50 μl $well^{-1}$ of the acidified SDS solution. Formazan crystals were dissolved by an overnight incubation of the plates at room temperature in a dark chamber. Following brief agitation, the absorbance values were determined on a multi-channel spectrophotometer (SLT 340 ATTC, SLT-Labinstruments, Salzburg, Austria). The plates were read three times using 570 and 620 nm as test and reference wavelengths, respectively. The arithmetic mean values were used for processing.

Dose–response analysis. Two-fold dilutions

of IL-2, encompassing the range of proliferative response of CTLL-2 cells, were analysed in microtiter plates according to the protocols described above for cell density determination and MTT colorimetric assay, respectively.

Data processing

The potency of each test preparation was calculated by analysis of variance for a three- or four-dose assay according to principles described in the European Pharmacopoeia [17]. The statistical weight is defined as the reciprocal value of the variance of the $\log_{10}$ potency estimate [17]. The index of precision was calculated by dividing the standard deviation of the responses by the negative slope of the log dose–response relationship [18]. After testing for homogeneity, the weighted log potency estimates were used for combination of results from separate assays [17]. The RSD (or coefficient of variation) is defined as the ratio between the standard deviation of responses and the arithmetic mean value of these responses [17].

Results

Dose–response relationship

Growth of CTLL-2 cells was dependent on the presence of IL-2. Using different sources of hIL-2, the log dose–response curve was steep at IL-2 concentrations of 0.2 to about 3 IU per ml (Fig. 1). Proliferation was not affected when cells were exposed to any one of the following substances: human growth hormone, insulin and substance P (not shown). Interleukin-3 and interleukin-4 were employed in analogous experiments; these cytokines were found to be devoid of growth-inducing activity on CTLL-2 cells over a broad dose range, with 50 and 100 IU ml^{-1}, respectively, as maximal concentrations (not shown).

Potency determinations

In order to acquire an estimate of the precision and inter-assay variation of the method, the potencies of an rhIL-2 preparation, diluted to various known concentrations, were determined. In four sets of experiments, samples (pentaplicates) were determined by the MTT colorimetric method, single examples from each group being depicted in Fig. 2. The results are summarized in Table 1. The estimated and the nominal potencies were very close and the inter-assay

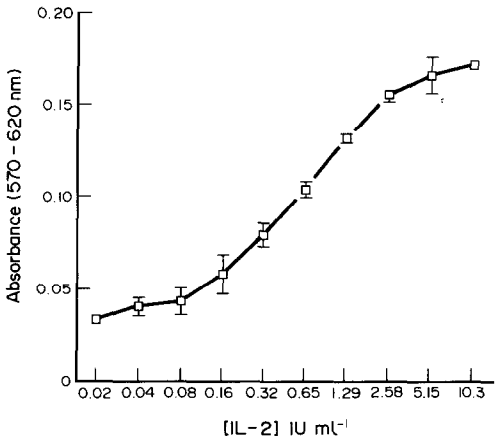

Figure 1
CTLL-2 cellular dose–response curve for dilutions of IL-2 following 24-h stimulation. Preparation Ia served as a source of IL-2. The proliferative stimulation was measured by dual-wavelength plate reading of the formazan concentration. Bars indicate the range (two variates).

RSD was about 3% in a series of four separate experiments. In some potency determinations, especially when analysed by a four-dose assay, variation between the identically prepared incubation series was so limited that minute deviations from linearity or parallelism became statistically significant. In some cases such determinations have been accepted, in other cases the significance of the deviations disappeared when data were analysed as a three-dose assay excluding the results of the lowest or highest IL-2 concentration. The fiducial limits were often more narrow, and the index of precision lower, for determinations with the colorimetric assay, suggesting an even higher precision compared with the results obtained with the cell density method (Tables 1 and 2).

In order to evaluate whether an extended incubation time of CTLL-2 cells, in the presence of IL-2, could further improve the bioassay, the stimulatory response was measured at 48 h following addition of the cytokine. As summarized in Table 3, the statistical weight tended to be lower and the index of precision higher, indicating a somewhat greater variability and lower precision with the 48-h assay (cf. Tables 1 and 3). Thus, the more rapid procedure was preferred.

Two commercial preparations of rhIL-2 were assayed against the International Standard (WHO, 86/504) and/or the Reference Reagent (WHO, 86/564) (Table 4). Two batches of a single preparation, (Ia and Ib) were then found to possess 33 and 40%,

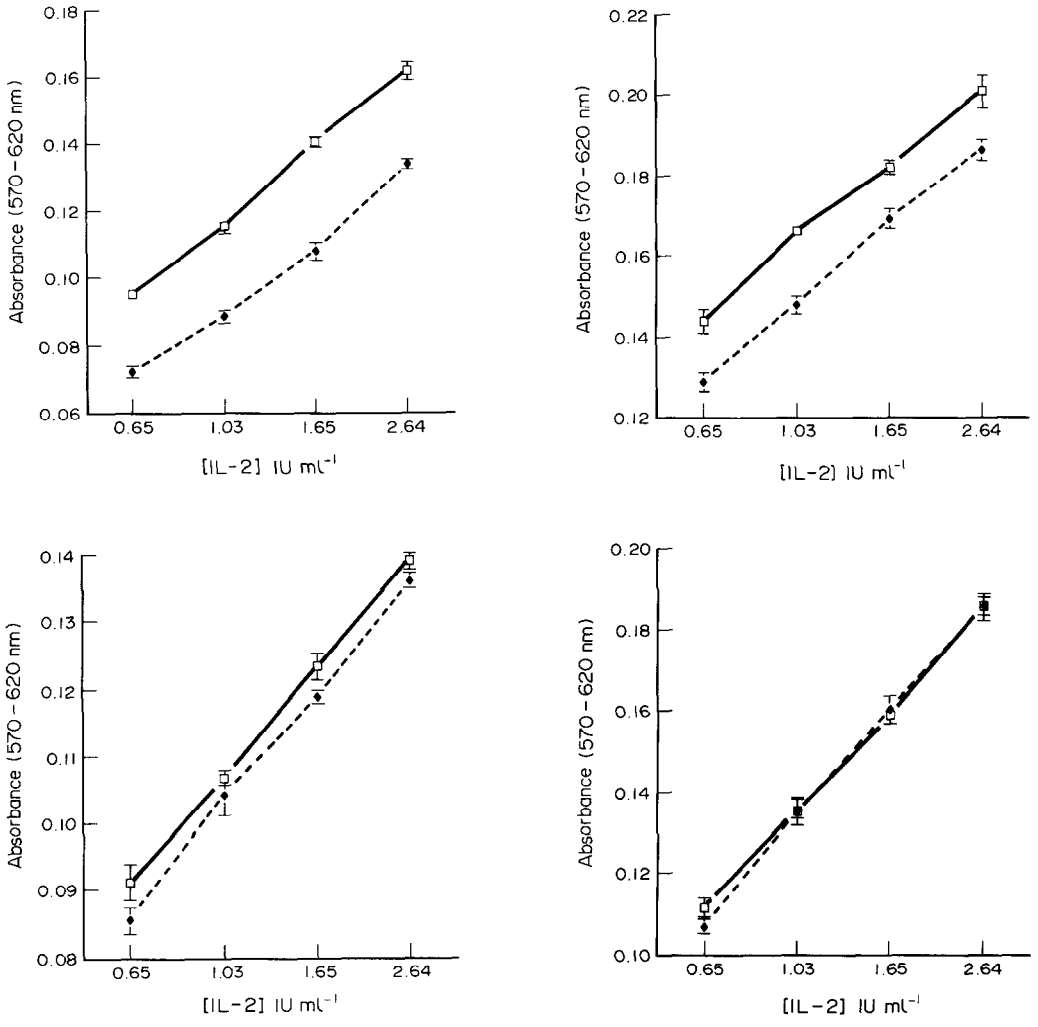

Figure 2
Quantitative titration of rhIL-2 (preparation Ia) using the MTT colorimetric assay (dual wavelength: 570 vs 620 nm). Arithmetic mean values ±S.E.M. of five variates in a single experiment are indicated. Hatched lines represent various test concentrations that are diluted to certain fractions of the reference titer (filled lines). (A) The test solution was diluted to a nominal concentration of 50% of the reference ($T = 0.5R$); (B) $T = 0.7R$; (C) $T = 0.9R$; (D) $T = R$.

respectively, of the potency of the International Standard. A figure, in the lower range of these values, was obtained when batch Ia was tested against the Reference Reagent. However, the second IL-2 preparation was estimated as having *ca* two-fold higher potency, compared with preparation I, and thus aligned more closely to the nominal value.

In a final experiment, a dissolved sample of IL-2 stored at 56°C for 6 h was assayed against the corresponding preparation stored frozen. The sample kept at 56°C retained 49% of the control activity. Limits of error ($P = 0.95$) were 93–108% (combination of two titrations).

Discussion

Due to recent advances in molecular biology and biotechnology, lymphokines of various types are now readily available and pharmaceutical preparations of IL-2 have already been registered for marketing in various countries. Therefore, it is essential that an assay for IL-2 is designed that fulfils pharmacopoeial requirements for bioassays of biologicals [17]. The potent stimulatory effect of IL-2 on CTLL cell proliferation was easily determined both by cell density measurements and recordings of MTT formazan formation. The precision

Table 1
Estimated potencies of four different dilutions of a rhIL-2 preparation (Ia) as determined by MTT reduction assay

Potency (%)		Limits of error (%)			
Nominal	Estimated	$P = 0.95$	Statistical weight	Index of precision	RSD
Single experiments					
50	55.4	89.5–111.7	1877	0.04996	
50	50.9	93.0–107.6	4331	0.03119	
50	55.4	92.2–108.5	3482	0.03672	
70	68.4	92.4–108.2	3692	0.04036	
70	68.6	91.5–109.4	2787	0.05639	
70	80.7	85.5–117.6	845	0.10662	
90	89.2	92.9–107.6	4137	0.04888	
90	102.9	87.7–114.0	1350	0.07447	
90	90.4	94.2–106.2	6403	0.03393	
100	98.2	94.0–106.4	5817	0.04146	
100	98.1	93.4–107.1	4743	0.04591	
100	102.0	95.3–105.0	9874	0.02755	
100	103.1	89.6–111.6	1928	0.06231	0.0257
Combined results					
50	53.3	95.4–104.8	9709	3 experiments	
70	69.8	94.7–105.6	7299	3 experiments	
90	91.3	95.8–104.3	11891	3 experiments	
100	100.3	97.0–103.1	22989	4 experiments	

Table 2
Estimated potencies of a single dilution of a rhIL-2 preparation (Ia) as determined by cell counting

Potency (%)		Limits of error (%)		
Nominal	Estimated	$P = 0.95$	Statistical weight	Index of precision
Single experiments				
100	112.9	84.8–117.9	822	0.10990
100	100.3	88.7–112.2	1607	0.06831
Combined results				
100	104.2	91.0–109.9	2433	2 experiments

Table 3
Determination of potency of a single dilution of a recombinant IL-2 preparation (Ia). The growth stimulation was measured by the MTT reduction assay following 48 h of incubation

Potency (%)		Limits of error (%)			
Nominal	Estimated	$P = 0.95$	Statistical weight	Index of precision	RSD
Single experiments					
90	91.6	86.0–116.4	970	0.10118	
90	92.5	89.7–111.5	1960	0.06153	
90	87.0	85.0–117.6	844	0.10794	
90	81.6	84.9–117.8	860	0.09018	0.0567
Combined results					
90	89.2	93.4–107.0	4630	4 experiments	

appeared to be on comparative levels but the MTT method was considerably less time-consuming and therefore preferred. The present results showed minimal variability. With the precision of the present assay it appears possible to adapt fairly strict potency requirements for IL-2 preparations with limits for the estimated potency of 90–111% of the stated potency and with fiducial limits of error ($P = 0.95$) of 80–125% of the stated potency.

The specificity of the assay was demonstrated by the lack of proliferative effects on

Table 4
Potency determination of two separate commercial rhIL-2 preparations using the MTT colorimetric assay. Ia and Ib represent two separate batches of the same preparation

Preparation	Potency (% of WHO 86/504)	Potency (% of WHO 86/564)	Limits of error (%) $P = 0.95$	Statistical weight	Index of precision
Single experiments					
Ia	35.5		87.9–113.8	1332	0.07730
Ia	30.9		88.0–113.6	1412	0.06474
Ia		34.3	86.1–116.2	1029	0.07873
Ia		29.8	87.5–114.3	1238	0.08863
Ib	41.5		87.5–114.3	1242	0.08834
Ib	34.8		77.0–129.9	325	0.16585
II		84.5	84.5–118.4	784	0.11289
II		74.7	88.6–112.9	1567	0.06869
Combined results					
Ia	33.0		91.5–109.2	2740	2 experiments
Ia		31.8	90.7–110.2	2268	2 experiments
Ib	40.0		89.0–112.4	1567	2 experiments
II		77.8	90.9–110.0	2347	2 experiments

CTLL-2 cells by human growth hormone, insulin and substance P, which are peptides known to bind to receptors on a human lymphoblastoid cell-line, IM-9 [19–21]. Moreover, neither interleukin-3 nor interleukin-4 induced CTLL cellular growth. In previous reports, the murine T-helper cell line HT-2, has been described as an indicator line in a similar bioassay system. By including monoclonal antibodies, specific for either IL-2 or IL-4, the assay could be designed as specific for one of these factors [14]. However, a CTLL-based quantification of IL-2 presently offers the least complex titration method of the bioactivity of this particular lymphokine.

A limited but reproducible regressive effect on different tumours has been observed with a combination of adaptive immunotherapy and IL-2 infusions [22–25]. However, a high incidence of serious toxic effects of this treatment has been observed and would appear to be dose-related within a narrow range [25–28]. By employing moderate doses of IL-2 the frequency of adverse effects can be substantially reduced [3, 25; for a review on IL-2 efficacy and toxicity, see 29]; this strategy requires assay methods of high reliability and throughput. The assay described gives precise and reliable results and is fairly simple to perform and should therefore be a candidate as a pharmacopoeial method for potency assessments of preparations of IL-2, but should also be useful for quantitation of IL-2 in other circumstances.

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