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Analysis of the heterogeneity of the biological responses to native and mutant human interleukin-6

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Abstract: The structure-function relationships of the biological activities of mutant varieties of the pleiotropic cytokine interleukin-6 (human) were measured by three assays: induction of immunoglobulin M (IgM) secretion from an Epstein-Barr virus-transformed human B cell line and induction of fibrinogen secretion from either a human hepatoma cell line or a rat hepatoma cell line. The biological effects of the cytokine were characterized by three parameters as determined by a novel analysis: effectiveness (the maximal response attainable), efficiency (the concentration yielding a half-maximal response), and complexity (a measure of heterogeneity and feedback control). Substitution of serine for cysteine was associated with a reduction in the effectiveness of interleukin-6 in both fibrinogen secretion assays. In the assay with human hepatoma cells, there was also a profound reduction in efficiency. Serine substitution in the human IgM synthesis assay appears mainly to reduce the efficiency. Deletion of amino acids 4 to 23 increased the efficiency in the rat hepatoma assay. The complexity parameter suggests the presence of multiple receptor classes or negative feedback in all three assays. Use of the proposed sequential approach to the analysis of dose-response relations in bioassays provides a more useful quantitative assessment of activities as well as more insight into the complexity of the reactions. *J. Leukoc. Biol.* 52: 415-420; 1992.

Key Words: bioactivity • bioassay • interleukin-6

INTRODUCTION

Interleukin 6 (IL-6) is a member of a class of pleiotropic cytokines. Among its activities are stimulation of growth and differentiation of T lymphocytes, stimulation of maturation of B lymphocytes and B cell-derived hybridoma cell lines to produce immunoglobulin, stimulation of hematopoietic cells, and induction of synthesis of various acute-phase proteins, including fibrinogen, by hepatocytes [1, 2]. IL-6 was initially found to be secreted by fibroblasts but is now known also to be produced by T lymphocytes, monocytes, keratinocytes, and endothelial cells [3-6].

The sequence of IL-6 is fully known [7-9]. Its gene has been successfully expressed in transfected eukaryotic cells, in bacteria, and in cell-free lysates [7, 10, 11]. This gene and variants containing replacements by serine of the naturally occurring cysteines and/or deletions all along its length have been constructed in vitro [12-15]. The cysteine-free variant and several of the deletion mutants retained biological activity to varying degrees in hybridoma growth and differentiation assays and in hepatocyte stimulation assays [15].

However, quantitation and standardization of the activities of the IL-6 variants proved difficult because of the complexity of the binding to cell surface receptors, the complexity of the biological responses, and the fact that, in some instances, a saturation response could not be attained.

We have performed an analysis of the dose-response relationship of the IL-6 variants that retained significant biological activity in three assays, hepatocyte stimulation in human and rat hepatoma cell lines and stimulation of synthesis of immunoglobulin M (IgM) from Epstein-Barr virus (EBV)-transformed human B cells, by a technique that was found to be useful in estimating the activity of interleukin-1 (IL-1) [16]. We characterized the action of IL-6 in terms of three parameters: (1) its effectiveness, i.e., the maximal response attainable under the experimental conditions; (2) its efficiency, i.e., the increment in dose necessary to achieve a specified increment in response, conveniently taken as the concentration of the preparation that yields a half-maximal response; (3) its complexity, i.e., the degree to which the responsiveness of the cells is attenuated or enhanced as the dose is increased.

MATERIALS AND METHODS

Construction of genes encoding recombinant IL-6 mutants

The construction of the gene encoding IL-6S, in which the four naturally occurring cysteines of human IL-6 are replaced by serines, has been described previously [12]. Briefly, this gene was assembled from 22 synthetic oligonucleotides and initially cloned into a modified pBS M13+ cloning vector (Stratagene). The natural stop codon of this gene was converted to a serine codon by cassette mutagenesis to allow expression as a fusion protein. The gene encoding an IL-6S analogue with the natural pattern of cysteines (IL-6) was constructed by cassette mutagenesis of the IL-6S gene as described previously [15]. We also constructed a deletion mutant (from amino acids 4 through 23 near the NH₂ terminal) of IL-6 (IL6 δ 4-23) and a deletion mutant of IL-6S (IL-6S δ 4-23) using a previously published method for oligonucleotide mutagenesis [17], modified as described previously [15].

Abbreviations: EBV, Epstein-Barr virus; ELISA, enzyme-linked immunosorbent assay; IgM, immunoglobulin M; IL-6, interleukin-6.

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Following sequence verification by dideoxy sequencing, genes encoding each of the IL-6 variants were subcloned into the p340 expression vector. This method was described in detail previously [12, 15]. This vector allows high-level expression of IL-6 variants as β -galactosidase fusion proteins.

Expression, purification, and quantitation of IL-6 variants

Expression, purification, and quantitation of IL-6 variants for use in bioassays were carried out as described previously [15]. Briefly, expression vectors containing genes for IL-6 variants were transformed into *Escherichia coli* JM101. Single ampicillin-resistant colonies were used to inoculate 10-ml broth cultures, and expression of the IL-6/ β -galactosidase fusion protein was induced by adding isopropyl β -D-thiogalactopyranoside. When β -galactosidase activity reached a maximum, bacteria were pelleted by centrifugation and stored at -20°C . Bacteria were resuspended and lysed by freezing and thawing after lysozyme treatment. The lysate was sonicated to reduce its viscosity, and the fusion protein, along with other insoluble material, was pelleted by centrifugation. The pellet was washed to remove soluble contaminants, and the fusion protein was solubilized in 2% sodium lauroyl sarcosine. Insoluble contaminants were removed by centrifugation, and the fusion protein was further purified by two rounds of selective ammonium sulfate precipitation. Before bioassay or quantitation, the IL-6 variants were cleaved from β -galactosidase with collagenase. Proteins were quantitated by denaturing polyacrylamide gel electrophoresis under reducing conditions and were stained by Coomassie Blue and scanned by laser densitometry.

Assay of the activity of IL-6 variants

Bioassays were carried out as described previously [15]. Briefly, cells were treated with varying concentrations of IL-6 in 96-well microtiter plates. For each mutant, two or three independent protein preparations were tested in duplicate in each bioassay. Hepatocyte stimulation activity was determined by measuring IL-6-stimulated secretion of fibrinogen from human (HEP 3B2) and rat (FAZA 967) hepatoma cells. Fibrinogen was quantitated using a sandwich enzyme-linked immunosorbent assay (ELISA) specific for human or rat fibrinogen. B cell differentiation activity was determined by measuring IL-6-stimulated secretion of IgM from a human EBV-transformed B cell line (SKW6.4). IgM was quantitated by a sandwich ELISA.

Analysis of the activity of IL-6 variants

If the response in a biological assay is directly proportional to the amount of the cytokine bound, and if the receptors are independent and equivalent, then the dose-response relation can be represented by

$$N/L = s \times (M - N) \quad (1)$$

where N = response

M = maximal response attainable in the assay

L = concentration of free cytokine (not bound)

s = proportionality constant

M , the effectiveness parameter, and s are estimated by linear regression.

If the assumptions specified above do not hold, e.g., if the receptors are not independent and equivalent or if the response is not simply proportional to the amount of cytokine bound, then the linear relation between N/L and N will not

hold. However, the quantity M may still be approximately estimable if a sufficient portion of the plot of N/L versus N , in the high-response range, is linear.

M having been estimated, the fractional response $T = N/M$ may be calculated and the dose-response relation recast into the form

$$\ln(T/(1 - T)) = a \times \ln(L) + \ln(k) \quad (2)$$

where L = concentration of free cytokine

a = coefficient estimated by regression, the complexity parameter

k = coefficient estimated by regression

The efficiency parameter is the value of L when $T = 1/2$, i.e., the concentration of the cytokine giving a half-maximal response.

This formulation is consistent with a variety of complex ligand-binding processes and responses to hormones and other biologically active processes [18, 19].

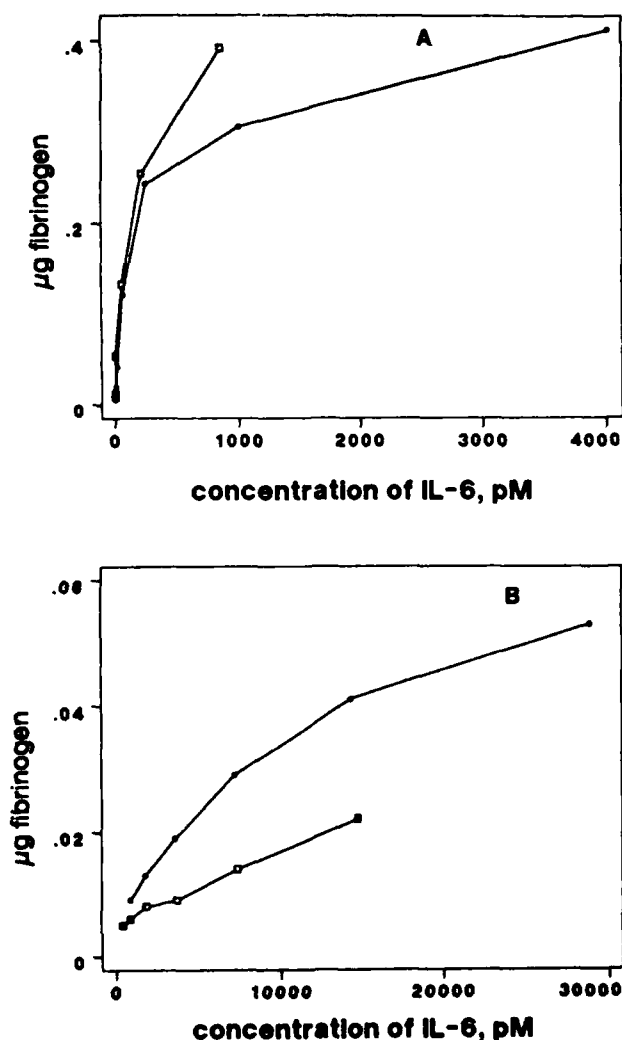


Fig. 1. Dose-response curves of human IL-6 variants in the hepatocyte stimulation assay using human hepatoma cell line. (A) (●) IL-6, native IL-6; (□) IL-6S4-23, deletion mutant of IL-6. (B) (●) IL-6S, serine-substituted IL-6; (□) IL-6S4-23, deletion mutant of IL-6S.

RESULTS

Figure 1 presents the dose-response relations observed for the IL-6 variants in the hepatocyte stimulation assay using the human hepatoma cell line. These conventional plots gave no indication of the maximal response. Saturation of the response was not achieved experimentally (**Fig. 1B**) because the concentrations that would have been required were not attainable. The data suggest that the serine-substituted IL-6 (IL-6S) and its deletion mutant (IL-6S δ 4-23) were less active than native IL-6. The same data are shown in **Figure 2** in the form of plots of the ratio of the response to the concentration of free cytokine on the vertical axis against the response on the horizontal axis [see equation (1) of Materials and Methods]. In no instance is the simple linear relationship observed with pure preparations of IL-1 encountered with IL-6. The plots are consistent with proportionately decreasing responses as the dose of the cytokine is increased and suggest heterogeneity or negative feedback control in the biological response to IL-6 and its variants. Extrapolation of the data at the high doses permits approximate estimation of the maximal response.

Figures 3-5, show the data for the three assays (hepatocyte stimulation using human and rat hepatoma cell lines, stimulation of synthesis of IgM from EBV-transformed human B cells) in the form suggested by equation (2). These plots permit the estimation of the two additional parameters

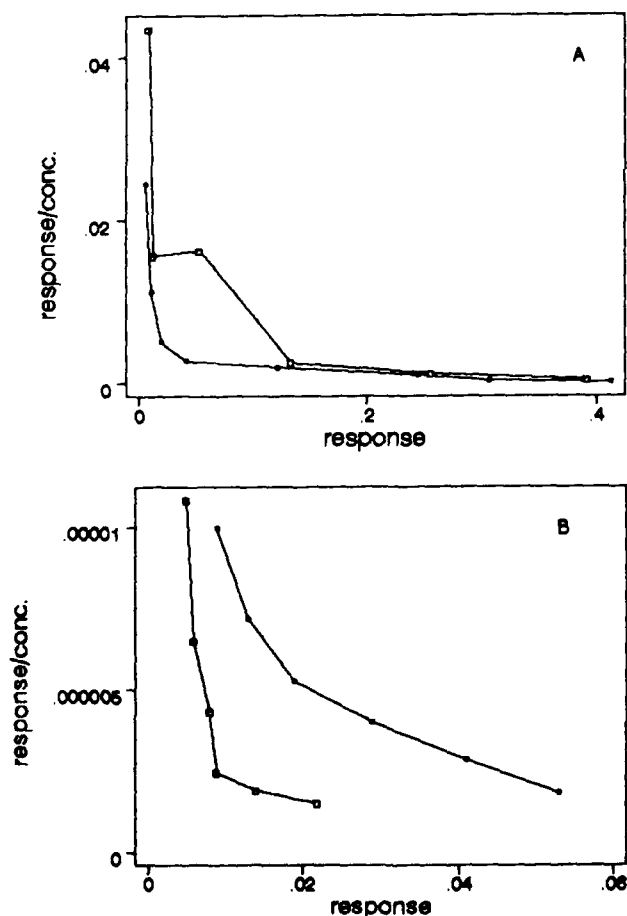


Fig. 2. Replot of the dose-response curves in **Figure 1** according to equation (1).

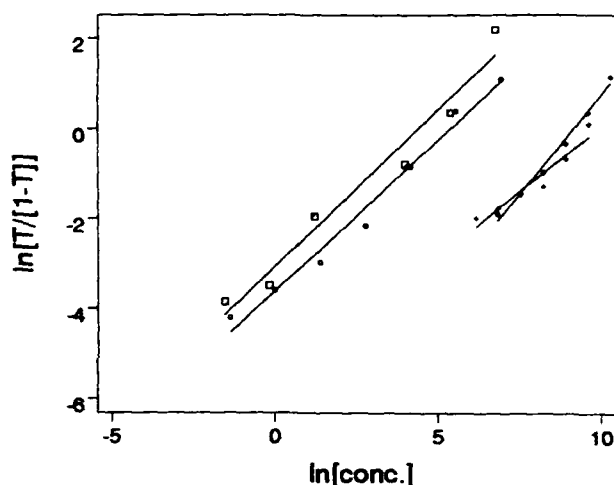


Fig. 3. Summary of the dose-response relationships of human IL-6 variants in the hepatocyte stimulation assay using human hepatoma cell line. (○) IL-6, native IL-6; (□) IL-6 δ 4-23, deletion mutant of IL-6; (◇) IL-6S, serine-substituted IL-6; (+) IL-6S δ 4-23, deletion mutant of IL-6S. Concentrations are in picomolar (pM) units.

of the dose-response relation. The first, the slope of the line, is an indication of the attenuation (or increase) of the responsiveness of the cells as the dose of the cytokine is increased. In the case of the IL-6 variants in this report, the slopes were all less than 1, indicating attenuation. The second parameter, the concentration of the cytokine that induced half of the maximum response, is a measure of the efficiency of the cytokine in the assay.

Figures 3-5 clearly demonstrate the differences among the cytokine variants in both the efficiency and the complexity of the stimulatory effects. This is probably attributable to multiple heterogeneous receptors for IL-6 on the cells used in the bioassays (see the Discussion).

Table 1, showing averages of two to three replicates of each of the three assays, summarizes the results obtained in all three assays. From this table, it is clear that substantial differences exist among the IL-6 variants in the maximal

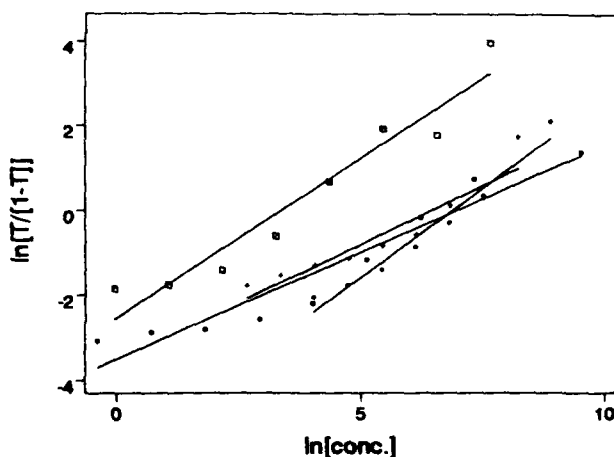


Fig. 4. Summary of the dose-response relationships of human IL-6 variants in the hepatocyte stimulation assay using rat hepatoma cell line. (○) IL-6, native IL-6; (□) IL-6 δ 4-23, deletion mutant of IL-6; (◇) IL-6S, serine-substituted IL-6; (+) IL-6S δ 4-23, deletion mutant of IL-6S. Concentrations are in picomolar (pM) units.

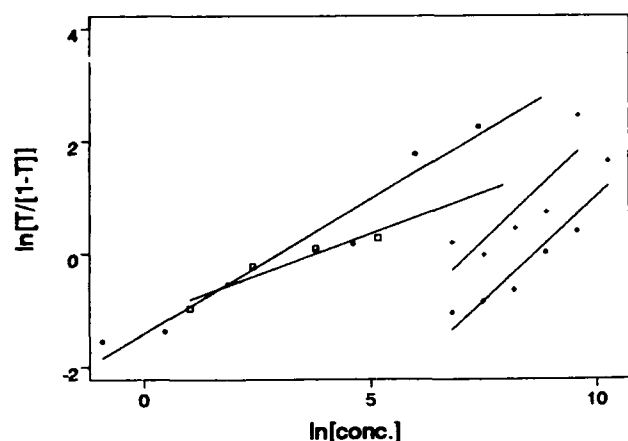


Fig. 5. Summary of the dose-response relationships of human IL-6 variants in the B cell differentiation assay using a human EBV-transformed B cell line. (○) IL-6, native IL-6; (□) IL-6δ4-23, deletion mutant of IL-6; (◇) IL-6S, serine-substituted IL-6; (+) IL-6Sδ4-23, deletion mutant of IL-6S. Concentrations are in picomolar (pM) units.

responses they can elicit: the serine-substituted mutants were less capable of stimulating hepatocytes to produce fibrinogen than the natural, cysteine-containing forms. The reduction in the maximal response was more pronounced in the bioassay using human hepatoma cells than in the rat hepatoma bioassay. In all three assays, the deletion mutation rendered the cytokine's effectiveness more vulnerable to serine substitution. Cysteine-containing deletion mutants were not significantly less effective than their intact analogues.

The mutations under evaluation had quite distinct effects on the efficiency parameter in the assays using human and rat cells. Replacement of the cysteines with serines (IL-6S, IL-6Sδ4-23) reduced the efficiency of the cytokine by over two orders of magnitude in the case of the human hepatoma cells (Table 1), increasing the concentration needed to achieve a half-maximal response from 217–113 pM to 18,500–13,500 pM, but hardly at all in the case of the rat cells (Table 1). Deletion of amino acids 4 to 23 in the cysteine-containing IL-6 (IL-6δ4-23) substantially increased its efficiency in the rat cell assay (from 835 to 70 pM), but not in the assay using human cells. The deletion in the serine-containing mutant (IL-6Sδ4-23) appears to cause a minor increase in efficiency in the rat hepatoma and B cell differentiation assays, while also reducing effectiveness or maximal response (see below).

DISCUSSION

The response observed in biological assays of cytokines results from an extended and complex series of steps. The initial step is the binding of the cytokine to a cell surface receptor. If all subsequent steps in the process are fully and unalterably set in motion at this point, the observed response will be linearly related to the quantity of the cytokine bound. Even then, however, the dose-response relation may not be a simple one. Because of potential interactions among the receptors, their affinity for the cytokine may increase or decrease with the amount bound. Under certain circumstances—for example, in the case of pure IL-1—a single class of independent and equivalent receptors appears to be present and the response appears to be governed by a simple equilibrium binding of the cytokine to the cell surface receptor [16]. Plots of the type displayed in Figure 2, in that case,

yield straight lines. Such simple behavior is not displayed by IL-6 and a more complex characterization of the effect of the cytokine in the assay is needed. This can be usefully accomplished in terms of the three parameters described above. For example, in the case of IL-6, it has been shown that at least two classes of receptors exist on murine B cells (but only one on T cells) [20, 21]. Figure 2, in which an attenuation of the response with increasing amount of IL-6 bound is evident, is consistent with the presence of multiple receptors with different affinities for the cytokine, although other explanations of the concavity of the plots, such as negative feedback in the binding step or rate limitations in subsequent steps, are also possible. However, although the presence of receptor heterogeneity detected in binding studies is consistent with the subunit structure of the IL-6 receptor, with the binding to the receptor site on the gp80 component stabilized by its association with the gp130 signaling component, if there is an excess of gp80 over gp130 molecules, it is not consistent with the dose-biological response relationship that we observe. That is because the only binding visible in our assay is that which leads to association with the gp130 component and, therefore, to initiation of the biological effects. Replacement of the cysteines with serines and/or introduction of deletions may alter the affinity of the cell surface receptors for the cytokine, resulting in a change in the efficiency parameter (the concentration giving a half-maximal response). However, the affinity for the various classes of heterogeneous receptors may be differentially affected, or the interaction among the receptors may be altered. This will result in a changed complexity parameter. The maximal response (effectiveness) may also be altered if the various components of the response involve different receptors. For example, if both cellular proliferation and synthesis of the protein assayed are stimulated by the cytokine, but through different receptors, a reduction in the affinity for the receptors involved in proliferation but not for those that control protein synthe-

TABLE 1. Dose-Response Parameters of IL-6 Mutants

Sample	Effectiveness ^a		Efficiency ^b (pM)	Complexity ^c
	Experimental	Calculated		
<i>Hepatocyte stimulation assay using human hepatoma cell line</i>				
	μg/ml fibrinogen			
IL-6	0.41	0.42 ± 0.02 ^d	217 ± 29	0.79 ± 0.15
IL-6δ4-23	0.40	0.43 ± 0	113 ± 42	0.66 ± 0.03
IL-6S	0.06	0.08 ± 0.02	13,500 ± 4,900	0.88 ± 0
IL-6Sδ4-23	0.03	0.04 ± 0	18,500 ± 4,900	0.69 ± 0.17
<i>Hepatocyte stimulation assay using rat hepatoma cell line</i>				
IL-6	1.90	1.88 ± 0.50	835 ± 457	0.69 ± 0.04
IL-6δ4-23	1.23	1.20 ± 0.27	70 ± 58	0.58 ± 0.23
IL-6S	0.83	1.06 ± 0.16	1,143 ± 248	0.76 ± 0.08
IL-6Sδ4-23	0.43	0.49 ± 0.04	497 ± 180	0.57 ± 0.03
<i>B cell differentiation assay using human EBV-trans-formed B cell line</i>				
	ng/ml IgM			
IL-6	254	247 ± 39	12 ± 7	0.54 ± 0.12
IL-6δ4-23	323	316 ± 42	19 ± 13	0.46 ± 0.12
IL-6S	207	240 ± 45	4,300 ± 2,200	0.61 ± 0.03
IL-6Sδ4-23	127	132 ± 11	1,000 ± 500	0.71 ± 0.06

^aMaximal response.

^bConcentration of IL-6 that produced a half-maximal response.

^cCoefficient *a* in equation (2).

^dThe measures of variability are standard deviations based on two or three replicate measurements.

sis may reduce the maximal response attainable over the fixed duration of the assay. Thus, all three aspects of the dose-response relationship must be examined. A further potential complication results from the possible presence of other biologically active substances in the complex mixtures used in the assays, including residual contaminants in the partially purified preparations of the cytokines. We have not, however, detected any systematic pattern in the parameter estimates that might be attributable to the presence of biologically active contaminants.

The efficiency parameter, the concentration of the cytokine giving a half-maximal response, may be thought of as a measure of the affinity of the receptor(s) for the cytokine. Indeed, if the response is entirely governed by the equilibrium binding of the cytokine to a single class of independent receptors, it is the reciprocal of the binding constant. In more complex cases in which the response is still fully determined by the quantity of the cytokine bound, it is an indication of a weighted mean affinity. This is the parameter that lends itself best to comparisons among assays. Thus, the effectiveness parameter, the maximal response, is clearly a function of the product being assayed, but the efficiency parameter is a measure of the sensitivity of the cells to the cytokine, irrespective of the nature and quantity of the product. Thus, the efficiency of IL-6 is about one order of magnitude greater in the human IgM production assay than in the human fibrinogen synthesis assay and is decreased by two orders of magnitude by substitution of serines for cysteines. It is of interest that binding studies with ^{125}I -labeled IL-6 revealed a parallel increase of about two orders of magnitude in the dissociation constant (K_d) of IL-6 from receptors on U937 cells resulting from the substitution of serines for the cysteines ($K_d = 1.2 \text{ nM}$ and 760 nM for IL-6 and IL-6S respectively) [22].

The complexity parameter is best taken as, principally, a qualitative guide to the control mechanisms that guide the binding of/or response to the cytokine. Thus, values less than 1 indicate the presence of receptor heterogeneity or of negative feedback mechanisms and values greater than 1 the presence of threshold effects or positive feedback.

In our previous report, we described the activities of the serine-substituted with-or-without-deletion mutants relative to recombinant IL-6 by comparing concentrations required to double the response in the assays [15]. We can now state more specifically that the reported increase in the activity of the $\delta 4$ -23 mutant in the hepatocyte stimulation assay using rat hepatoma cells appears to be due to an increase in the efficiency of the mutant. On the other hand, the reported reduction in the activity of the serine-substituted mutants in this assay is associated with a reduction in their effectiveness. In the case of the assay using human hepatoma cells, the profound reduction in activity of the serine-substituted mutants appears to be due to a reduction of both the effectiveness and the efficiency. The principal effect of serine substitution in the human IgM synthesis assay appears to be the reduction of efficiency.

We conclude that the proper characterization of the dose-response relation of biologically active agents in complex biological assays should include quantitative estimation of their properties of effectiveness, efficiency, and complexity. As we have demonstrated, this characterization can be carried out with minimal effort. The availability of the three quantities, obtained by standard procedures, will greatly facilitate the comparison of results among laboratories.

TECHNICAL NOTE

Alternative approaches to the estimation of the parameters are available. For example, although we estimate the effectiveness before calculating the efficiency and complexity parameters, using linear regression in both instances, all three parameters may be computed simultaneously by nonlinear regression techniques by a formulation analogous to equation (2). Such an approach is embodied in the computer program ALLFIT [23], developed at the National Institutes of Health. Although it offers the convenience of one-step estimation and, in principle, an added statistical rigor, we believe that there are clear advantages of the stepwise approach described here when applied to the highly complex biological assays of the interleukins. It provides more information and clearer insights into the pattern of the response of the cell systems to stimulation by the cytokines.

We also employ a least-squares nonlinear regression analysis program based on the efficient Marquardt algorithm (A. Goolsby, Emory University, personal communication) as a final step. The function used is analogous to that in ALLFIT, without the parameter giving the background response because all our results are corrected for background. To succeed at all, this approach requires the provision of reasonable estimates of the parameters as "starting values." In addition, because of the very high correlation among the parameters to be estimated and the quite limited precision of the experimental data, convergence is often not achieved or convergence to a "local" rather than true minimum may occur. When all goes well, the results are very similar to those obtained by the method we describe. For example, nonlinear regression yields estimates of the effectiveness, efficiency, and complexity parameters of 0.45, 370, and 0.67 in the human hepatocyte stimulation assay with native IL-6 (cf. 0.42, 217, and 0.79 in Table 1) and of 1.93, 937 and 0.68 in the rat hepatocyte stimulation assay with native IL-6 (cf. 1.88, 833, and 0.68 in Table 1). We, therefore, strongly recommend that at least a preliminary analysis of the data by the method described herein be carried out before the more complex nonlinear regression approach is attempted.

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