Quantification of the Neutralization of Cytokine Biological Activity by Antibody: The Ten-Fold Reduction Bioassay of Interleukin-6 as Growth Factor

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The measurement of neutralizing antibodies (NABs) to biological therapeutic agents is important clinically as well as for the preclinical evaluation of product immunogenicity. To determine whether the theoretical concepts and experimental data from studies of the nature of antibody neutralization of interferons (IFNs) can apply to unrelated protein effector molecules, neutralization experiments were undertaken with interleukin-6 (IL-6), a proinflammatory, highly pleiotropic cytokine. By following IL-6 induction of hybridoma cell growth, we demonstrated that anti-IL-6 monoclonal and polyclonal NABs can be measured with a bioassay design structured to reduce 10 Laboratory Units (LU)/mL to 1 LU/mL. Results are reported in Ten-fold Reduction Units (TRU)/mL, as recommended for the standardization of IFN NAB unitage. The bioassay was shown to be sensitive, reproducible, and robust in measuring IL-6 potency and NAB titer, as well as for evaluating dose-response curve slope differences. This bioassay design should be applicable to any cytokine, growth factor, protein hormone, or similar effector molecules for which an adequately sensitive cellular response can be quantified.

Introduction

THE DEVELOPMENT OF ANTIBODIES to biological therapeutic agents following administration to patients is a matter of increasing clinical importance. Antibody inducibility is also a major consideration in the preclinical evaluation of the immunogenicity of products being developed for therapeutic use. In view of the multiplicity of very different biotechnology products inducing neutralizing antibodies (NABs) (Porter 2001; Schellekens 2003) and of factors affecting the design and optimization of cell-based assays to evaluate immunogenicity (Gupta and others 2007), there is a need for a more unified approach to such measurements that can be related, at least in part, to theoretical analyses of the nature of the interactions between antigens and NABs.

By applying the law of mass action to an analysis of the reaction between NABs and the interferons (IFNs) as the biologically active, soluble protein antigens, Kawade and colleagues developed a mathematical model to quantify neutralization (Kawade 1980; Kawade and Watanabe 1984, 1985; Kawade 1986; Grossberg and others 2001a; Kawade and others 2003). Neutralization is thereby calculated in relation to antigen potency, measured in internally determined units of biological activity, and to the mode of the

neutralization reaction. The neutralization reaction mode can be characterized by the dose-dependent rate of neutralization gauged by slope. Biological activity is operationally defined as 1 Laboratory Unit (LU) at the titration end point, and since dose-response is drug concentration-dependent, is expressed per unit volume. For antigens having World Health Organization (WHO) International Standard Preparations, such as several IFNs and interleukins, the unit of biologically active antigen actually measured in any given type of bioassay, that is, 1 LU/mL, may be greater or smaller than 1 International Unit (IU)/mL of the specific antigen concerned, depending on the assay sensitivity measured in relation to the assigned potency of the particular WHO International Standard Preparation (Grossberg and others 2001b). The analysis of data from several laboratories engaged in a WHO international collaborative study of WHO human serum anti-interferon- α and - β antibody preparations assessed interlaboratory comparability. This study resulted in a recommendation to calculate the titers from appropriately designed neutralization bioassays to be expressed as $t = f(n - 1)/9$, where t is the titer, f is the dilution of antibody at the end point, and n is the amount of antigen measured in LU/mL mixed with antibody, with

1 LU as the end point (Grossberg and others 2001a). This formula defines a unit of antibody neutralization as the Ten-fold Reduction Unit, expressed as TRU/mL, which it was postulated might be used with any quantitative, similarly designed neutralization bioassay of sufficient relative sensitivity to the antigen (Grossberg and others 2001b; Grossberg and Kawade 2006).

Although it was considered likely that the principles derived from the studies of the neutralization of IFNs with antiviral bioassays might be applicable to different assays of other soluble protein effectors (Grossberg and Kawade 1997; Grossberg and others 2001a, 2001b; Kawade and others 2003), the current study was undertaken as experimental proof of principle with a completely unrelated cytokine as antigen, interleukin-6 (IL-6). IL-6 is a 21–26 kDa inflammatory, multi-functional glycoprotein secreted by lymphoid and nonlymphoid cells, including mononuclear phagocytes, fibroblasts, keratinocytes, and endothelial cells. IL-6 is able to (1) induce the growth of B lymphocytes and their differentiation into antibody-producing plasma cells, (2) promote the activation and differentiation of T lymphocytes, and (3) stimulate hepatocytes to produce acute-phase reactants, including C-reactive protein and fibrinogen (Van Snick 1990; Janeway and others 2005). In addition to its hematologic, immunologic, and hepatic effects, IL-6 has multiple endocrine and metabolic actions, specifically as a potent stimulator of the hypothalamic–pituitary–adrenal axis (Papanicolaou and others 1998). IL-6 is elevated in several inflammatory diseases, such as rheumatoid arthritis, and has been associated with the appearance of autoantibodies (Morales-Montor 2005; Nakahara and Nishimoto 2006). Recently IL-6 was shown to promote the development of Th17 cells, the differentiation of which was inhibited almost completely by anti-IL-6 antibody (Veldhoen and others 2006; Ohsugi 2007). Antibodies to IL-6 or to IL-6 receptor have been used therapeutically, for example, in rheumatoid arthritis (Wendling and others 1993; Veldhoen and others 2006). The experimental design presented herein represents an extension of elements employed in the bioassay of IFNs and their NABs (Grossberg and others 2001a, 2001b; Grossberg and Kawade 2006) to the measurement of a very different protein effector, the cytokine IL-6.

Materials and Methods

IL-6

Highly purified human recombinant IL-6 having a specific activity of 2×10^8 units/mg was purchased from Becton-Dickinson Collaborative Biomedical Products (lot 905734) (Bedford, MA). Sigosix, human recombinant IL-6 produced for clinical use, and batch L093-PD01-A were gifts from Serono Labs (Randolph, MA). WHO IL-6 International Reference Reagent (recombinant DNA human type) 88/514, having an assigned potency of 130,000 IU/mL (Gaines Das and Poole 1993), was produced at the National Institute for Biological Standards and Control (Potters Bar, England) and obtained from the Biological Resources Branch, Developmental Therapeutics Program, National Cancer Institute, Bethesda, MD. IL-6 preparations were diluted in 4% human serum albumin to a final concentration of 100 µg/mL, and stored at -80°C .

Antibodies

Murine monoclonal antibody (mAb) (S062/L1) to human IL-6 was provided by the Cesare Serono Institute of Research, Rome, Italy. Murine anti-IL-6 mAb clone 34-1 (Novick and others 1989) was obtained from InterPharm (Ness Ziona, Israel). Purified polyclonal anti-human-IL-6 rabbit serum antibody was purchased from Endogen (Cambridge, MA).

Cells

An IL-6-dependent murine hybridoma cell line B9 (Helle and others 1988) was obtained from Cesare Serono Institute of Research, Rome, Italy. B9 cells were propagated in RPMI-1640 containing 10% fetal bovine serum (FBS), glutamine, ciprofloxacin, β -mercaptoethanol 50 µM, plus 50 units/mL of IL-6 (B-D Collaborative Biomedical, Bedford, MA).

IL-6 bioassay

To determine the potency of IL-6 preparations, a 2-fold duplicate dilutional series of IL-6 was mixed with B9 cells, which were suspended in medium without supplemental IL-6 to a density of 10^4 cells/0.1 mL/well. The cells were then incubated at 36°C in 5% CO_2 for 3 days, at which time MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide, Sigma M2128) 0.5% in freshly prepared phosphate-buffered saline, 0.2 mL/well was added and incubated for 2 h at 36°C . To 0.15 mL of supernatant medium from each well was added 0.2 mL of acidic isopropanol, and absorbance at 540 nm, the experimentally observable measure of biological response, was determined in a 96-well plate reader (Lab Systems MultiScan MS). Controls included multiple wells of B9 cells without IL-6 (negative control) and B9 cells with IL-6 200 units/mL (positive control). The midpoint between the mean value of these controls defines the 50% end point as well as 1 Laboratory Unit of potency (1 LU/mL). Spectrophotometric data were directly processed to construct the dose-response curves, perform statistical analyses, and determine biological potency by a custom-built computer application (see below). The relative sensitivity of this IL-6 assay is 0.95 IU/LU, that is, it measures 1.05 times the 130,000 IU assigned to the IL-6 International Reference Reagent 88/514.

IL-6 neutralization bioassay

The constant antigen, varying Ab method was used. Serial duplicate 2-fold dilutions of antibody were incubated with an equal volume of 20 Laboratory Units (LU)/mL of IL-6 for 2 h at 36°C , to which 10^4 B9 cells/50 µL/well were distributed into 96-well plates for a 3-day incubation at 36°C . A simultaneous IL-6 control titration was included on each plate, using serial duplicate 2-fold dilutions of the targeted 10 LU/mL, to measure the actual potency of IL-6 (since it can vary from the targeted value). Determination of the absorbance of MTT-colored product and of the 50% end point was as described above.

Computational operations

To measure objectively and efficiently the results of cytokine assays having sigmoidal dose-response curves, a

suite of computer applications were written in Mathematica (Wolfram Research Inc., Champaign, IL). The applications developed read as input operator-keyed values and spectrophotometric data files. A Marquardt-Levenberg nonlinear regression, informed by programmatically selected starting values, data-point weights, and a four-parameter logistic model, is used to fit the curves. This algorithm uses a repetitive process to find values for the parameters of the sigmoidal model for which the sum of the squares of the vertical distances of data points from the determined curve is a minimum. The model's equation is

$$y = p_1 + (p_2 - p_1) / \left\{ 1 + e^{[(x - p_3) / p_4]} \right\}$$

where p_1 to p_4 are treated as parameters, x is in log units of concentration, and y is in log units of biological activity. Assay results in quantitative characteristics, such as titer and slope, were calculated from the equation, with slope determined over a range of one dilutional interval centered on the dose-response curve's inflection point. NAb titers were calculated by the formula $t = f(n - 1)/9$ to provide results in TRU/mL (Grossberg and others 2001a, 2001b). Quality control and statistical evaluations were also performed.

Results

Figure 1 portrays a characteristic sigmoidal dose-response curve of IL-6 induction of the growth of B-lymphoblastoid cells. Such curves are generated as the simultaneous control on each plate in every neutralization antibody titration to verify the actual number of LU/mL of

FIG. 1. A typical interleukin-6 (IL-6) simultaneous control curve, in this case of IL-6 antigen 88/514, obtained by the B9 hybridoma, MTT, cell-proliferation method. The horizontal broken line defines 1 LU/mL at the 50% end point, the median of the dynamic range between maximum cell growth obtainable by high-dose supplemental IL-6 and the minimum control level as control without IL-6; its intersection with the dose-response curve establishes the potency (ie, titer) of the antigen read off the lower abscissa 5.1812 log₁₀, or 152,050. The vertical dashed lines within the rectilinear portion of the dose-response curve relate the IL-6 dose as LU/mL to the IL-6 dilution of antigen, for which the upper and lower abscissa scales are drawn in opposite directions.

antigen used, a dose previously estimated to be 10 LU/mL as the final target dose in each well with serum. The 50% end point in the simultaneous antigen control titration determines the end point of antibody titrations run on the same plate, providing the necessary, internally coherent measure of antigen. To illustrate the nature and quality of the kinds of data generated by this IL-6 bioassay, Table 1 summarizes the multiple measurements of potency and dose-response slopes of the different antigen sources utilized, including the determination of the assay's relative sensitivity (0.95 IU/LU) against the IL-6 International Reference Reagent 88/514.

Figure 2 shows representative dose-response curves of IL-6 neutralization by a hyperimmune anti-IL-6 rabbit serum polyclonal antibody (pAb) and of a mAb, illustrating the difference in curve slopes. Table 2 summarizes the multiple determinations of NAb titers and curve slopes of the antibodies tested.

The mean standard deviation of the maximum controls was found to be 0.0482 and the mean dynamic range of the assay (the difference between the maximum and minimum controls) was 1.06, as further measures of assay quality. The results indicate that the measurement of IL-6 NAb by this bioassay method is highly reproducible and robust.

It was found that the slopes of the mAbs are about the same in magnitude as those of the simultaneous IL-6 control curves, slightly less so, but given the degree of overlap, not importantly. The difference in slope of mAb 34-1 from that of the IL-6 control is 0.198 (SD 0.193) and that of mAb S062/L1 is 0.179 (SD 0.297), with mean slope ratios of -0.82 (SD 0.17) and -0.87 (SD 0.34), respectively. In contrast, the mean slope of the pAb (2.204) is strikingly greater than the mean slope of the mAbs [0.71 (SD 0.17)] by a factor of 3.1.

Discussion

The NAb bioassay design described herein illustrates as proof of principle its applicability to a protein effector, in this case, the cytokine IL-6, which is very different in many ways from the IFNs. Elements of the design thus apply whether the biological effector is inhibitory (like the IFNs) or stimulatory (like IL-6 and other growth factors). The approach taken to setting the limits of the control dose-response curve of the IL-6 NAb assay is rather analogous to the IFN-inducible luciferase gene reporter or the MxA protein induction assays but unlike that of the IFN antiviral assay. In the antiviral assay, maximum response is taken to be the absorbance of untreated cell control, with the expectation that that represents the healthiest, virus-resistant cells, considered equivalent to the maximally tolerable dose of IFN protecting the cells against virus challenge. For the IL-6 assay, a maximum tolerated dose (50–200 LU/mL) was utilized to ascertain the maximum response level. The upper asymptotes of the dose-response curves are not taken as maximum since these depend on too few data points, often only two, and may vary greatly; instead, the mean is used of several (six or eight) replicate maximum dosage controls. Likewise, the minimum is established as the mean of several negative controls.

The end point in LU/mL of the IL-6 assay, like that for IFN assays, be they antiviral, MxA induction, or luciferase gene reporter, is based on a measurable biological effect that, when coupled in a neutralization assay to a simultaneous antigen

TABLE 1. POTENCY VALUES AND CURVE SLOPES OF IL-6 PREPARATIONS FROM DIFFERENT SOURCES

IL-6 Preparation	<i>n</i>	Potency (LU/mL)		Potency (LU/mL) Mean antilog	Potency (IU/mL)	Curve slope	
		Mean log ₁₀	(SD)			Mean	(SD)
88/514 ^a	16	5.1348	(0.105)	136,390	130,000 ^a	-0.9663	(0.244)
905734	13	5.4215	(0.086)	263,940	251,371	-0.9671	(0.221)
Sigosix	10	7.4877	(0.074)	3.07×10^7	2.92×10^7	-0.9971	(0.305)
L093-PD01	5	8.8919	(0.095)	7.79×10^8	7.42×10^8	-1.0252	(0.374)

^aWorld Health Organization IL-6 International Reference Reagent with an assigned value of 130,000 IU, such that the sensitivity (IU/LU) of the bioassay is 0.95.

Abbreviations: IL-6, interleukin-6; *n*, number of titrations; SD, standard deviation.

dose-response curve, allows the total and the residual antigen potency values to be measured and thereby neutralizing capacity. This methodology is distinct from attempting to assess neutralization by directly relating arbitrarily selected weights of antigen instead of biological effect, especially for antigens having high specific activities.

Based on theoretical and experimental studies of the neutralization of IFNs, hypothetical modes of neutralization reactions have been posited and designated as Constant Proportion and Fixed Amount. Each mode represents different conditions of antibody affinity and concentrations of total to residual antigen (Grossberg and others 2001a, 2001b;

Kawade and others 2003). In the Constant Proportion mode, antibody acts to reduce the effect of 10 LU/mL to 1 LU/mL, or 30 LU to 3 LU, or 50 LU to 5 LU, and so on, a ratio representing a 90% reduction, for which neutralization is reported in TRU/mL. This neutralization model best fit the data on NABs in patients' sera with different IFN bioassays performed in an international collaborative assay study (Grossberg and others 2001a, 2001b). The neutralization reaction in the Constant Proportion mode characteristically exhibits a dose-response curve with a slope of magnitude similar to that of the antigen. Like IFN NAb dose-response curves previously observed (Grossberg and others 2001a), as well as in a study of over 700 anti-IFN- β NAb-positive human sera (Grossberg, unpublished studies), the slopes of the IL-6 mAb curves were generally similar in magnitude to those of the antigen control curves and thus consistent with the Constant Proportion mode such that titers were calculated as TRU/mL.

The neutralization curve slopes of the anti-IL-6 hyperimmune (pAb) rabbit serum used are much more acute than either the IL-6 or the mAb curves and represent a special case hardly ever observed with anti-IFN NAb curve slopes. The unusually steep curves of this pAb more nearly meet the criteria for the Fixed Amount hypothesis. In this neutralization mode, antibody is expected theoretically to neutralize a certain definite amount of biologically active antigen to reduce the effect of 10 LU/mL to 1 LU/mL, that is by 9 LU/mL, and thereby 20 LU to 11 LU, or 50 LU to 41 LU, and so on. Inasmuch as the bioassay end point is 1 LU/mL and the amount of target antigen is 10 LU/mL, it is possible to calculate the titer of this pAb by the $t = f(n - 1)/9$ formula as 12,160 TRU/mL. Given this bioassay's relative sensitivity (0.95 IU/LU) and as Fixed Amount neutralization reaction, the titer could be reported as 12,010 IU/mL (see equation (2) in Grossberg and others 2001a, and equation (5) in Kawade and others 2003). The biological factors that may likely contribute to the acute slope are a preponderance of high affinity antibodies in this hyperimmune antiserum as well as the possibly greater antibody heterogeneity in recognizing additional epitopes. Kawade and Watanabe (1985) had previously observed that the extent of neutralization by anti-IFN mAbs increased with antibody concentration, but with a rate considerably lower than in the case of pAbs; there often appeared to be a limit in the maximum amount of IFN that can be neutralized by mAbs, suggesting that the two parameters, antibody affinity and efficacy of neutralization, may be independent. Observations on the characteristics of other IL-6 pAbs, especially of patients' NABs, are warranted.

FIG. 2. Neutralization curves illustrative of the slopes for the hyperimmune rabbit polyclonal antibody (pAb) P-620 and for the monoclonal antibody (mAb) 34-1. Note that the direction of these curves is opposite to that of the interleukin-6 (IL-6) antigen curve in Figure 1. The slanted dashed lines indicate the graduated theoretical curve slope indices provided for comparison. In this titration, the slope of the P-620 pAb curve is 3.250, and the slope for the 34-1 mAb curve is 0.750, a difference of 4.3-fold. [The neutralization titers, *t*, (13,782 TRU/mL for the pAb, and 51,435 TRU/mL for the mAb) are calculated by the formula $t = f(n - 1)/9$, where *n* is the simultaneously measured antigen potency, and *f* is the antilog of the log₁₀ dilution at the 50% endpoint, with log₁₀ dilutions 3.886 for the pAb and 4.458 for the mAb.]

TABLE 2. INTERLEUKIN-6-NEUTRALIZING ANTIBODY (NAb) TITERS AND CURVE SLOPES OF POLYCLONAL (pAb) AND MONOCLONAL (mAb) ANTIBODIES

Antibody	n	NAb Titer (TRU/mL)		NAb Titer (TRU/mL) Mean antilog	Curve slope	
		Mean log ₁₀	(SD)		Mean	(SD)
mAb 34-1	12	4.6248	(0.076)	42,150	0.7725	(0.153)
mAb S062/L1	17	4.7567	(0.175)	57,108	0.6653	(0.173)
pAb P-620	17	4.1007	(0.091)	12,610	2.204	(0.673)

Abbreviations: n, number of titrations; SD, standard deviation.

The use of this bioassay design and the approach to calculation of NAb potency as well as the reporting of NAb results in TRU/mL may have application to the neutralization of any cytokine, growth factor, protein hormone, or similar effector molecule for which a cellular effect can be quantified with adequate sensitivity. The ten-fold reduction approach has been successfully operative with very different methodologies to measure NAb to the IFNs, involving different biological effects, whether they be, for example, antiviral (Grossberg and others 1986), MxA protein induction (Pungor and others 1998; Files 2007), cell-growth inhibition (Redlich and Grossberg 1989), or luciferase reporter gene induction (Lallemand and others 2008; Lam and others 2008). Further, NAb titers calculated as TRU/mL determined on the same serum samples by the luciferase gene induction and by antiviral assays were comparable (Lallemand and others 2008). In addition, NAb titers as TRU/mL can be used not only with the commonly employed constant antigen method, like those mentioned above, but also with the constant antibody method, which is 10- to 20-fold more sensitive than constant antigen assays (Grossberg and others 2009). The ten-fold reduction approach for IFN NAb has been repeatedly endorsed by the World Health Organization (Berg and others 1983; Billiau and others 1985; Andzhaparidze and others 1988; Calam and others 1995), and should be considered for use with other protein effector molecules, for which it appears quite applicable with bioassays of appropriate design.

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