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A sensitive immunoradiometric assay for the quantification of murine monoclonal antibodies in human serum

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Abstract: The clinical investigation of murine monoclonal antibodies (MoAbs) as potential immunotherapeutic agents necessitates their quantification in human serum. The present paper reports the development of a sensitive, non-competitive "sandwich" immunoradiometric assay specifically optimized for measurement of murine IgG in human serum. Affinity-purified goat anti-mouse IgG antibody covalently bound to acrylic microspheres serves as the solid-phase antibody and ^{125}I-labelled goat anti-mouse IgG antibody functions as tracer. All assay reagents were obtained from commercial sources. The assay is sensitive (capable of detecting 20 ng ml^{-1} of murine IgG), specific (<0.0001% cross-reactivity with human IgG), reproducible (intra-assay %RSD <6.3%), and rapid (a 100-tube assay can routinely be processed in 3 h). The assay has a working concentration range of 20–2000 ng ml^{-1} and is suitable for measuring MoAbs of any antigenic specificity. The assay was validated for use with human serum and cell culture media by comparison studies with flow cytometric and immunonephelometric methods and by high-performance size-exclusion chromatographic studies. Application of this assay should facilitate further investigation of murine MoAbs as potential immunotherapeutic agents.

Keywords: *Monoclonal antibody; monoclonal antibody–drug conjugate; immunoradiometric assay; immunotherapy.*

Introduction

A number of diverse analytical methods have been developed for measuring murine immunoglobulins in biological matrices [1, 2]. However, the quantification of murine monoclonal antibodies (MoAbs) in human serum during clinical investigation presents a number of unique analytical problems. First, the assay must be highly sensitive in order to quantify the low serum concentrations of MoAb achieved during therapy. Secondly, the assay must be compatible with human serum, a matrix which routinely interferes with immunologic-based assays. Thirdly, the assay must be specific for murine IgG due to the high concentration of endogenous human immunoglobulins present in serum. Finally,

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reagent availability is an important issue with respect to the widespread usage of the assay.

The present paper reports the development of a non-competitive immunoradiometric assay (IRMA) specifically optimised for measuring murine IgG in human serum. In current work, this IRMA was used for quantifying KS1/4, a murine MoAb that is reactive with a surface antigen expressed by the P3/UCLA human adenocarcinoma cell line. This IRMA was also used to quantify KS1/4 and KS1/4-DAVLB in a number of biological matrices in addition to human serum, including human pleural fluid, cell culture fluid, and chimpanzee monkey serum. In conclusion, this assay should facilitate further clinical studies of MoAbs as potential immunotherapeutic agents.

Experimental

Assay reagents

Affinity-purified goat antibody specific for mouse IgG (heavy and light chains) covalently attached to uniform, spherical 3-μm acrylic particles was obtained from Kirkegaard and Perry Laboratories (Gaithersburg, MD). ^{125}I-labelled affinity-purified goat anti-mouse IgG antibody (sp. act., 9.0 μCi mg^{-1}) was purchased from DuPont/NEN Research Products (Boston, MA). Affinity-purified mouse IgG was obtained from ICN Immunobiologicals (Lisle, IL). Non-fat powdered milk was from Carnation Co. (Los Angeles, CA). Human IgG and all other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

KS1/4, a murine MoAb of the IgG2a subclass, was raised against an antigen expressed by the P3/UCLA human adenocarcinoma cell line [3]. Preparation of KS1/4-DAVLB, the desacetyl vinblastine succinate conjugate of KS1/4, has been reported previously [4]. Highly purified KS1/4 and KS1/4-DAVLB (lot Nos RS0023 and RS0024, Eli Lilly and Co.) were divided into 1-ml fractions (1.0 mg ml^{-1}) and stored at −20°C.

Methods

Serum concentrations of human IgG were determined using a radial immunodiffusion kit purchased from Kallestad Laboratories, Inc. (Austin, TX). Cell culture KS1/4 concentrations were estimated by a standard immunonephelometric assay (Behring, La Jolla, CA). Serum KS1/4-DAVLB concentrations were estimated by a flow cytometric assay, described previously [4], using an EPICS-C flow cytometer (Coulter Corp., Hialeah, FL).

High-performance liquid chromatographic analyses of murine IgG were performed with the Pharmacia FPLC system using a Superose 6 HR 10/30 size-exclusion column. The mobile phase (pH 7.5) consisted of 50 mM sodium phosphate with 0.1% (m/v) sodium chloride, 0.1% (m/v) sodium azide and 0.01% (v/v) Tween-20. The column was eluted at a flow rate of 0.5 ml min^{-1}, and 0.25-ml fractions were collected and assayed for murine IgG concentration using the IRMA procedure.

IRMA procedure. The assay buffer (pH 7.5, adjusted with 10 M NaOH) consisted of 50 mM sodium phosphate with 0.1% (m/v) NaCl, 0.1% (m/v) EDTA, 0.1% (m/v) sodium azide and 1.0% (m/v) non-fat powdered milk. Lyophilized goat anti-mouse IgG antibody-coupled microspheres (200 mg) were reconstituted with 2 ml of sterile water and diluted to 40 ml with assay buffer yielding a 5 mg ml^{-1} suspension. The binding reaction was carried out in 12 × 75 mm borosilicate glass culture tubes and consisted of a

100-μl addition of sample or standard and a 200-μl addition of microspheres (well mixed). Each assay tube was vortexed and incubated for 1 h at room temperature. After the addition of 2 ml of assay buffer, each tube was centrifuged for 10 min at 3500*g* and the supernate was removed by aspiration. ^{125}I-labelled goat anti-mouse IgG antibody (100 μl) was then added (125 nCi; 200,000 cpm) and each tube was incubated, washed, centrifuged, and the supernate aspirated as described above. The radioactivity remaining in the pellet was quantified for 1 min in an Apex 600 automatic gamma counter (ICN Micromedic, Horsham, PA).

Assay data were analysed with the IN system (Iso-data Inc., Rolling Meadows, IL), an IBM-PC based immunoassay data reduction system, employing a French curve (smoothing factor = 0) or cubic spline algorithm. The murine IgG concentration of serum test samples was estimated from a standard curve of either KS1/4 or affinity purified mouse IgG reference material, prepared in assay buffer using the following concentrations: 0, 20, 50, 100, 200, 500, 1000, 1500 and 2000 ng ml^{-1}.

Results

Typical IRMA standard curves of reference KS1/4 diluted in assay buffer and human serum are shown in Fig. 1. Standards prepared in these matrices yielded comparable curves. In addition, when KS1/4 was analysed in assay buffer with an increasing percentage of human serum, no deviation in murine IgG recovery was observed (data not shown). These results demonstrated that human serum does not affect murine IgG measurement and therefore indicated that this assay is compatible for use with human serum.

Assay specificity was evaluated using KS1/4, KS1/4-DAVLB and commercial preparations of affinity-purified polyclonal murine IgG and human IgG (Fig. 2). Human IgG produced negligible cross-reactivity at concentrations up to 10 mg ml^{-1}. Fresh human serum samples (n = 8) produced a mean response in the assay equivalent to 12.5 ng ml^{-1} of murine IgG. The mean concentration of human IgG in these serum samples was 14.0 mg ml^{-1}, indicating that human IgG yielded a cross-reactivity of <0.0001%.

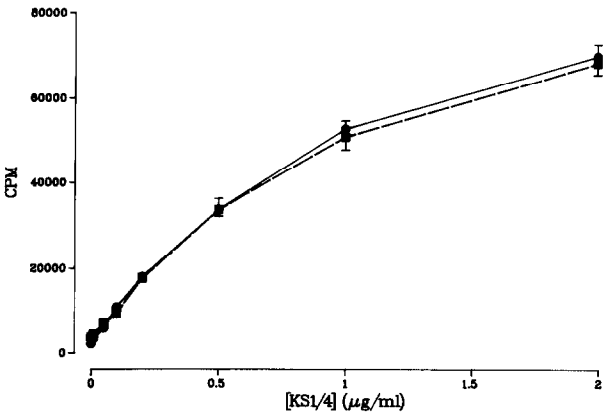

Figure 1
IRMA standard curves of KS1/4 prepared in assay buffer (●) and serum (■).

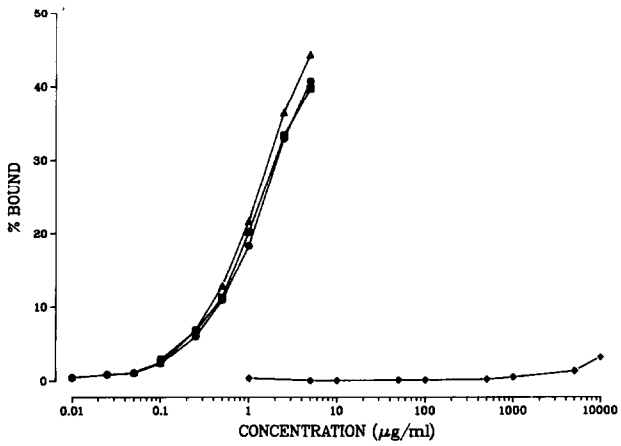

Figure 2
Specificity of the IRMA. Standards were prepared in assay buffer and analysed as described in the Experimental section. ●, KS1/4; ■, KS1/4-DAVLB; ▲, affinity-purified polyclonal mouse IgG; and ◆, human IgG.

The observed level of non-specific binding (zero dose response) was routinely 1% of the total cpm added to each assay tube. The minimal detectable concentration of murine IgG based on the apparent concentration 2 standard deviations (SD) above the zero dose response was <10 ng ml^{-1}; however, 20 ng ml^{-1}, the lowest standard concentration, was routinely used as the limit of assay sensitivity.

Precision and accuracy data obtained with spiked human serum samples are summarized in Table 1. Assay accuracy was estimated by calculating the mean percent recovery after addition of KS1/4 to fresh human serum. Mean recoveries were 90.9–117% for three different levels of KS1/4. Accuracy was also evaluated by serially diluting a spiked human serum sample with assay buffer and then measuring the murine IgG concentration. After correction for dilution, the mean murine IgG recovery was 102%. Assay precision was evaluated by calculating the relative standard deviation (RSD) of results obtained for spiked serum samples within an assay and between several assays. Intra-assay RSD (n = 5) was 4.7–6.3%; the inter-assay RSD (n = 10) was 6.1–12.2%.

Table 1
Precision and accuracy data for human serum samples

	μg ml^{-1}	Observed	% Recovery†	% RSD
Intra-assay (n = 5)*				
	1.0	0.99	99.0	6.3
	5.0	5.83	116.6	5.5
	10.0	9.09	90.9	4.7
Inter-assay (n = 10)				
	1.0	1.09	108.6	11.5
	5.0	5.41	108.2	12.2
	10.0	10.19	101.9	6.1

*KS1/4 was added to fresh human serum and assayed as described in the Experimental section after a 1:10 dilution in assay buffer.
†% Recovery = (observed value/expressed value) × 100.

Buffer composition and incubation parameters such as volume, time and temperature were systematically evaluated in order to maximize assay performance. Several proteins were examined for their ability to reduce the overall level of non-specific binding in the assay. As shown in Table 2, a buffer solution containing 1% non-fat powdered milk yielded the lowest non-specific binding (1.1%) without affecting the binding of a 5 μg ml^{-1} murine IgG sample. A time course for each binding reaction demonstrated that both reactions reached equilibrium within 1 h and were temperature insensitive, whereas the level of non-specific binding increased slightly with temperature (data not shown). A 200-μl addition containing 1 mg of microspheres was found to be optimal, based on a routine 100-μl addition of the sample. This volume of microspheres minimized any matrix effect of serum on the assay and permitted attainment of equilibrium in <1 h. A 100-μl addition of tracer containing 125 nCi (approximately 200,000 cpm) of labelled goat anti-mouse IgG antibody yielded a standard curve with a 100-fold working standard concentration range.

The IRMA was validated by direct comparison with a laser nephelometric assay for murine IgG (Fig. 3) and with a flow cytometer assay for functional antibody (Fig. 4). Comparison of the IRMA with the nephelometric assay was performed using cell culture samples of KS1/4 (n = 20); comparison with the flow cytometer assay was performed using serum samples (n = 122) from subjects who received KS1/4-DAVLB. Regression analysis of the comparison data demonstrated a high level of correlation for the IRMA with the immunonephelometric (r = 0.8) and the flow cytometric (r = 0.94) assay methods.

The application of this IRMA for measuring murine MoAb concentrations in human serum was also validated by high-performance size-exclusion chromatographic analyses. As shown in Fig. 5, immunoreactive murine IgG present in the serum of a patient who had received KS1/4-DAVLB co-migrated with reference KS1/4 spiked in assay buffer.

Table 2
Impact of various proteins on the performance of the IRMA

Protein*	% (w/v)	% NSB†	% Binding‡
BSA	1.0	5.9	36.4
	2.5	2.8	38.4
GSA	1.0	3.4	43.5
	2.5	2.5	42.3
	5.0	2.2	39.1
NGS	1.0	3.7	39.0
	2.5	2.9	38.1
	5.0	1.9	32.9
	10.0	1.5	36.1
NFPM	1.0	1.1	38.3
	2.5	1.4	33.1
	5.0	1.8	34.2

*The buffer was prepared with the stated percentage of the following proteins and the assay was performed as described in the Experimental section: BSA, bovine serum albumin; GSA, goat serum albumin; NGS, normal goat serum; NFPM, non-fat powdered milk.

†% NSB = (cpm of blank/cpm total) × 100.

‡% Binding = (cpm bound/cpm total) × 100, for a 5 μg ml^{-1} sample.

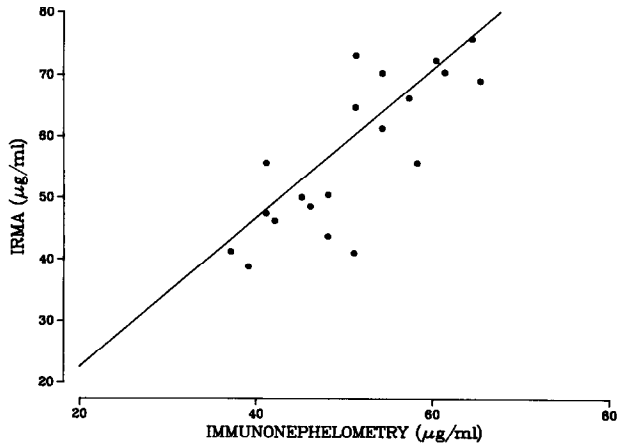

Figure 3
Comparative study of cell culture KS1/4 concentrations determined by the IRMA and immunonephelometric assays. The best fitted line was $y = 1.164x + 1.84$ ($r = 0.8$, $n = 20$), as determined by least-squares linear regression analysis.

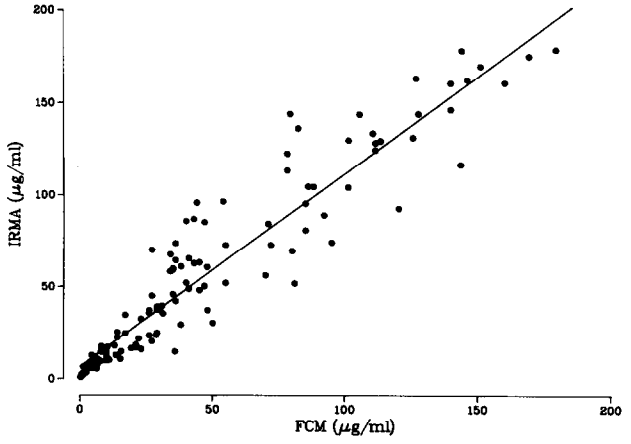

Figure 4
Comparative study of serum KS1/4-DAVLB concentrations determined by the IRMA and flow cytometer assays. The best fitted line was $y = 1.053x + 6.24$ ($r = 0.94$, $n = 122$), as determined by least-squares linear regression analysis.

Discussion

A number of diverse analytical methods have been developed for the quantification of IgG in biological matrices. For example, radial immunodiffusion [5], immunonephelometry [6, 7], RIA [8] ELISA [1], and IRMA [2] methods have been reported for the measurement of IgG with varying degrees of success in respect of assay sensitivity, specificity and precision. Despite the existence of a large number of methods, none has been reported in detail specifically optimized for the quantification of murine IgG in human serum. Several recent studies have utilized cell-based immunoassays to measure MoAbs during clinical studies [9–14]. However, these procedures required unique

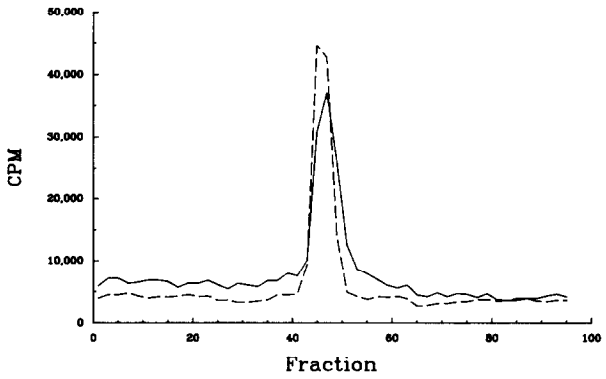

Figure 5
High-performance size-exclusion chromatography. Elution profiles of KS1/4 in assay buffer (---) and immunoreactive murine IgG present in human serum after i.v. administration of KS1/4-DAVLB (———). As shown above, immunoreactive murine IgG present in the serum sample co-migrated with reference murine IgG spiked in assay buffer.

immunological reagents (i.e. cells expressing the surface antigen reactive with the MoAb of interest) and most were not described in detail [11, 15]. These factors prompted the authors to develop a non-competitive IRMA specifically optimized for the quantification of murine IgG in human serum. The resulting assay is sensitive (20 ng ml^{-1}), specific (<0.0001% cross-reactivity with human IgG), reproducible (intra-assay RSD of 4.7–6.3%), and rapid (a 100-tube assay can routinely be processed in 3 h). Another major advantage is that the IRMA is composed entirely of commercially available reagents, a beneficial feature when widespread usage of the assay is considered.

Non-competitive IRMA methodology, originally reported by Miles and Hales [16], has been shown to exhibit potentially greater sensitivity, broader working ranges, and to be less susceptible to interference from matrix constituents than are other immunoassay procedures [17, 18]. The IRMA described here is characterized by a working concentration range of 20–2000 ng ml^{-1}. At murine IgG concentrations >2000 ng ml^{-1}, the authors routinely observed a high-dose hook effect. This phenomenon is common to most non-competitive binding assays and may result from steric hindrance at high analyte concentration [18]. Accordingly, test samples containing high concentrations of murine IgG were analysed after an appropriate dilution with assay buffer.

The monosized acrylic-based polymer particles employed in this study have been used previously as a solid-phase antibody support in several immunoradiometric assays [19–21]. The desirable characteristics of these beads include: a low level of non-specific binding; an excellent and reproducible binding capacity; long-term antibody stability; and relative ease of dispersing and sedimenting.

Because the IRMA does not discriminate between functional and non-functional murine IgG, this assay measures both biologically active and inactive forms of murine IgG. The murine IgG concentration has been measured in serum from patients to whom KS1/4-DAVLB had been administered; both a flow cytometer assay and the IRMA were used. The flow cytometer assay for KS1/4 [4] is similar to other reported cell-based immunoassays [9–14] and requires the presence of functional murine antibody for murine IgG measurement. Comparison of serum KS1/4-DAVLB concentrations obtained using both assay methods demonstrated a high degree of correlation (Fig. 4)

suggesting that the two assays yielded similar results and that the IRMA is well-suited for use during clinical studies involving administration of murine IgG. Correlational analysis with immunonephelometric data also demonstrated that the IRMA can be used for routine measurement of murine IgG in cell culture fluid (Fig. 3).

This assay can potentially be adapted to measure any immunoglobulin class or subclass by simply substituting the affinity-purified reagent antibodies and reference standard. This feature could represent a significant advantage over cell-based assays when several different MoAbs are undergoing simultaneous clinical investigation. During the course of this study, it was observed that unconjugated KS1/4 and DAVLB conjugated KS1/4 produced equivalent responses in the assay; this observation indicated that the IRMA may be useful for quantifying murine MoAb concentrations during *in vivo* studies involving MoAbs conjugated with oncolytic drugs or toxins.

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