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Short communication

Effect of double antigen bridging immunoassay format on antigen coating concentration dependence and implications for designing immunogenicity assays for monoclonal antibodies

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Abstract

The double antigen bridging immunoassay has been used extensively for detection of immunogenicity responses to therapeutic monoclonal antibodies. We have analyzed parameters affecting performance of this type of immunoassay including microtiter plate antigen coating concentration, enzyme-labeled antigen conjugate dilution and assay format (one-step versus two-step). We present results demonstrating that the format of the assay has a significant impact on the optimal parameters to maximize assay performance. A one-step assay format achieves maximal sensitivity across a broad range of coating concentrations and at a lower concentration of conjugate than that in a two-step format. In contrast, a two-step format requires very low coating concentrations and higher conjugate concentrations to achieve maximal sensitivity and suffers from significantly reduced sensitivity at higher coating concentrations. Together, these findings indicate that a one-step assay format can greatly reduce the effect of coating concentration variation on assay performance.

Keywords: Enzyme immunoassay; Double antigen; Bridging; Antigen coating

1. Introduction

There are currently 18 monoclonal antibodies approved in the United States for human therapeutic use (Table 1). These antibodies include fully murine, murine–human chimeras, humanized, and fully human monoclonal proteins. The first antibodies used for clinical trials were murine monoclonals. Results from clinical studies indicated that these proteins elicited human anti-murine antibodies (HAMA) in most patients, result in limited effectiveness due to neutralization and altered pharmacokinetics [1]. The introduction of recombinant DNA technology resulted in generation of mouse–human chimeras where murine monoclonal variable genes were fused with human heavy and light chain constant genes.

As molecular biology technology improved, efforts to increase the humanization of antibodies resulted in the transfer of murine complementarity determining regions (CDRs); encoding for the antibody binding sites into human frameworks resulting in "humanized" antibodies. The CAMPATH (anti-CD52) antibody was one of the first therapeutic antibodies in this category [2]. Fully human monoclonals have recently become available through phage display technology with adalimumab (Humira) being approved in 2002 [3]. As monoclonals have become progressively more humanized, the reported incidence of antibodies has generally decreased (Table 1). However, even the fully human monoclonal antibody adalimumab has been shown to elicit antibodies in 5% of patients [4].

Immunogenicity testing results for therapeutic monoclonals are dependent on the sensitivity and specificity of the assay methods utilized and may be influenced by a variety of factors including sample handling, timing of sample

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Table 1
FDA-approved monoclonal antibodies

Product	Name	Antigen	Indication	Form	Approval	Immunogenicity	Company
Orthoclone OKT3	Muromonab-CD3	CD3	Graft reject	Murine IgG2a k	1986	86% IgG	Ortho Biotech
ReoPro	Abciximab	GP IIb/IIIa-R	Angioplasty	Chimeric Fab	1994	6–44%	Centocor
Rituxan	Rituximab	CD20	NHL	Chimeric IgG1 k	1997	1% (3/356)	Genentech
Zenapax	Daclizumab	CD25	Graft reject	Humanized IgG1	1997	14–34%	Roche
Remicade	Infliximab	TNF	RA, CD	Chimeric IgG1 k	1998	10%	Centocor
Simulect	Basiliximab	CD25	Graft reject	Chimeric IgG1 k	1998	4/339, 2/138	Novartis
Herceptin	Trastuzumab	HER-2/neu	Breast cancer	Humanized IgG1 k	1998	<1% (1/903)	Genentech
Synagis	Palivizumab	RSV	RSV	Humanized IgG1 k	1998	1–2%	MedImmune
Mylotarg	Gemtuzumab ozogamicin	CD33	AML	Humanized IgG4 k	2000	0% (0/277)	Wyeth
Campath	Alemtuzumab	CD52	CLL	Humanized IgG1 k	2001	2% (4/211)	ILEX
Zevalin	Ibritumomab tiuxetan	CD20	NHL	Murine IgG1 k	2002	4% (8/211)	IDEC
Humira	Adalimumab	TNF	RA	HumanIgG1 k	2002	5% (58/1062)	Abbott
Xolair	Omalizumab	IgE	Asthma	Humanized IgG1 k	2003	<0.1% (1/1723)	Genentech
Bexxar	Tositumomab	CD20	NHL	Murine IgG2a l	2003	99% (219/220)	Corixa
Raptiva	Efalizumab	CD11a	Psoriasis	Humanized IgG1 k	2003	6.3% (67/1063)	Genentech
Erbitux	Cetuximab	EGF-R	Colorectal CA	Chimeric IgG1 k	2004	5% (28/530)	Imclone
Avastin	Bevacizumab	VEGF	Colorectal CA	Humanized IgG1	2004	0/500	Genentech
Tysabri	Natalizumab	α4-Integrin	MS	Humanized IgG4k	2004	10%	Biogen Idec

Data from relevant package inserts and www.fda.gov.

collection, concomitant medications, and underlying disease. A variety of different methods have been used to evaluate the immune response to therapeutic monoclonal antibodies including double antigen (bridging) ELISA [5,6], sandwich ELISA [7], radio-immune assay (RIA) [8], surface-enhanced laser desorption/ionization mass spectrometry (SELDI-MS) [9] and surface plasmon resonance (Biacore) [10].

We have developed one-step double antigen bridging enzyme immunoassays for assessment of patient response to humanized and fully human monoclonal antibodies to be tested in human clinical trials. These assays have demonstrated high levels of sensitivity, the ability to detect both idiotypic and non-idiotypic antibodies and avoid issues of cross reactivity between the monoclonal antibodies and anti-human IgG detection reagents found in traditional sandwich type assays. During optimization of these assays we found that both assay format and antigen coating concentration can have significant effects on the ability of the assay to detect human anti-human antibody (HAHA) responses. We describe here the interactions between these parameters and their effects on assay performance.

2. Materials and methods

2.1. Reagents

Therapeutic antibody candidate (LY) was supplied by Eli Lilly and Co. (Indianapolis, IN). Affinity-purified rabbit anti-human IgG (AffiniPure rabbit anti-human IgG (H + L), was obtained from Jackson ImmunoResearch Laboratories Inc., West Grove, PA. Wash buffer for all assays was Tris-buffered saline with Tween (TBST) consisting of 25 mM Tris–buffered saline (TBS) (Fisher Scientific, Fair Lawn, NJ) with 0.05% Tween 20 (Sigma–Aldrich, St. Louis, MO).

2.2. Horseradish peroxidase-LY conjugate (LY-HRP)

Therapeutic antibody (LY) was labeled with horseradish peroxidase (HRP) using the EZ-Link Plus Activated Peroxidase Kit (Pierce Biotechnology, Rockford, IL, #31489). The LY antibody was diluted in Buph carbonate–bicarbonate conjugation buffer (0.1 M carbonate–bicarbonate buffer, pH 9.4, Pierce, #28382) to a concentration of 1.0 mg/ml. One milliliter of this antibody solution was added directly into a 1 mg vial of EZ-Link Plus activated peroxidase (Pierce, #31487) and incubated for 1 h at room temperature. Ten microliters of 5 M sodium cyanoborohydride was added and allowed to incubate for 15 min at room temperature. Labeled protein was transferred to a 10,000 MWCO Pierce Slide-A-Lyzer (Pierce, #66380) and dialyzed overnight at 4 °C against 2 l of TBS with constant stirring. The resulting solution was mixed with an equal volume of glycerol (EMScience, Gibbstown, NJ #GX01E5-6) and stored at −20 °C until use.

2.3. ELISA procedure

Assays were performed in both one-step and two-step formats (Table 2). The one-step format refers to addition of LY-HRP conjugate followed by test sample for simultaneous incubation of these reagents and subsequent bridge

Table 2
Assay format comparison

One-step assay	Two-step assay
Coat plates overnight with antigen at 4C and block	
Add LY-HRP conjugate	Add test samples and incubate for 1 h
Add test samples and incubate 2 h	Wash
	Add LY-HRP conjugate, incubate for 1 h
Wash, add TMB substrate and develop for 30 min	
Stop with 2M phosphoric acid and read at 450 nm	

formation. The two-step format refers to addition of test sample followed by incubation and washing prior to addition of the LY-HRP conjugate. ELISA plates (Nunc Maxisorp 96 Microplates, Nunc, Rochester, NY) were coated with therapeutic antibody at various concentrations in BupH carbonate–bicarbonate buffer by adding 100 μl per well and incubating overnight at 4 °C. Plates were washed three times with TBST. After washing, for the one-step assay 50 μl of a suitable dilution of LY-HRP conjugate was added followed by 50 μl of appropriate dilution of test sample, and the mixture was incubated for 2 h at room temperature. The two-step assay was performed by adding 50 μl of similarly diluted test sample, incubating for 1 h at room temperature, washing the wells three times with TBST, adding 50 μl of LY-HRP conjugate, and incubating for 1 h at room temperature. Both one-step and two-step assays were then washed three times with TBST and 100 μl of tetramethyl benzidine (TMB) substrate (BioFX, Ewings Mills, MD) was added and incubated at room temperature for 30 min. The reaction was stopped by addition of 100 μl of 2 M phosphoric acid, and plates were read at 450 nm in a SpectraMax Plus plate reader.

2.4. Data analysis

Data was transferred to Excel 2000 where averages and standard deviations were calculated. The calculated numbers were transferred to Sigma Plot 8.0 for graphical presentation.

3. Results

Initial experiments were performed to determine a suitable dilution of LY-HRP conjugate to be used in the assay. One-step and two-step format assays were performed using serial two-fold LY-HRP conjugate dilutions ranging from 1:250 to 1:32,000 on ELISA plates coated overnight with antigen (homologous LY molecule) at concentrations of 10, 50, 100 and 500 ng/well. Since no human positive samples were available, an affinity-purified rabbit anti-human IgG (250 ng/ml) was used to bridge the plate-bound monoclonal antibody and LY-HRP conjugate using the basic one-step and two-step formats outlined in Table 2. Results shown in Fig. 1 demonstrate significantly different profiles for the two assay formats at different LY antigen coating concentrations. In the case of the one-step assay, there is a relatively broad maximum signal strength observed with 1:4000–1:8000 LY-HRP conjugate dilutions giving maximal signal (Fig. 1A). In addition, there are relatively minor signal variations over the 10–500 ng/well range of LY antigen coating concentrations used at each of the LY-HRP conjugate dilutions tested.

In contrast, the two-step format shows significantly different signal strengths for the four LY coating concentrations tested, with a strong inverse correlation noted between signal detection and coating concentration. Signals for the 10 ng/well LY antigen coat are maximal. However, signals for higher LY antigen coating concentrations show progressively

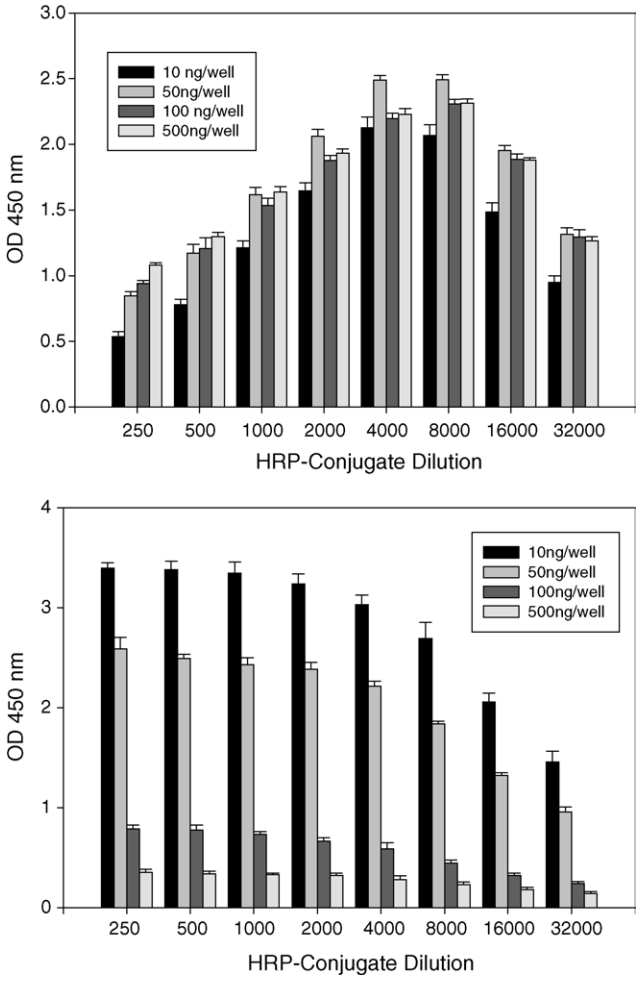

Fig. 1. Optimization of LY-HRP conjugate concentration. (Top) A one-step assay was performed with microtiter plates coated with 10, 50, 100, or 500 ng/well of homologous monoclonal antibody. LY-HRP conjugate was used at dilutions from 1:1000 to 1:32,000. In each experiment, 250 ng/ml of rabbit anti-human IgG antibody was used to form the bridge. Results are representative of eight sets of observations from two independent experiments. (Bottom) A two-step assay was performed with the same parameters described above. Results are representative of eight sets of observations from two independent experiments.

lower signals with the 500 ng/well LY antigen coating giving the lowest signals at all LY-HRP conjugate dilutions tested (Fig. 1B). Two-step assay signals reach maximum levels at LY-HRP conjugate dilutions of 1:250–1:2000 and drop-off gradually and continuously at progressively higher dilutions.

A comparison of the one-step versus two-step assay formats was next performed utilizing various anti-human IgG levels across a broad range of antigen coating concentrations. ELISA plates were coated overnight with serially two-fold diluted LY monoclonal antibody antigens ranging from 1000 ng/well down to 1 ng/well. One-step and two-step assays were performed using the protocols outlined in Table 1 with varying concentrations of anti-human IgG at 31, 63, 125 and 250 ng/ml tested with a monoclonal LY-HRP conjugate dilution of 1:2000. The concentrations of anti-IgG used

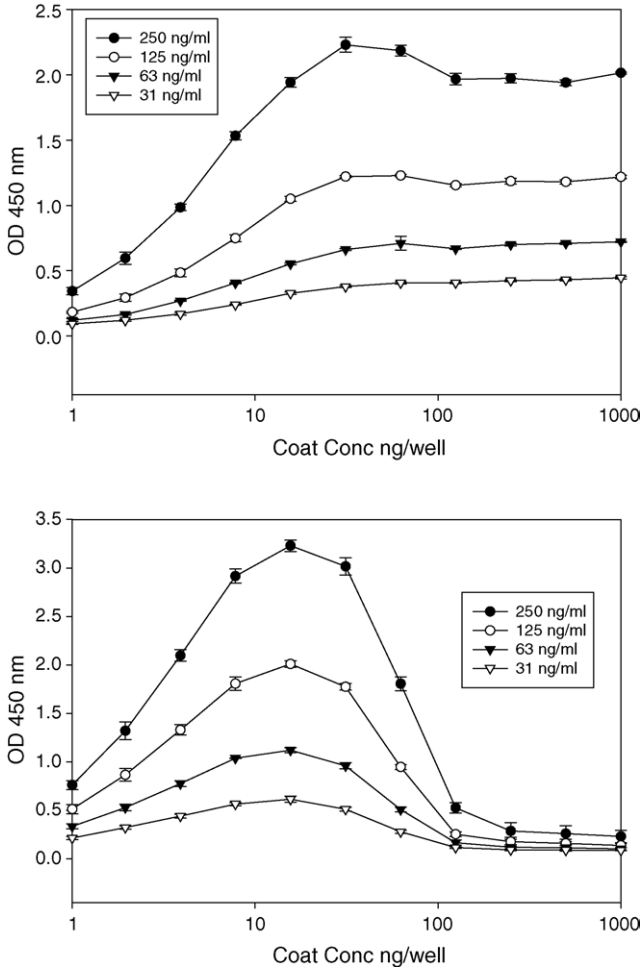

Fig. 2. Optimization of microtiter plate antigen coating concentration. (Top) In a one-step assay, optimization of microtiter plate antigen coating concentrations were optimized utilizing serial two-fold dilutions of monoclonal antibody starting at 1000 ng/ml. Rabbit anti-human IgG antibody was tested at 31, 63, 125 or 250 ng/ml for its ability to bridge the assay. The Ly-HRP conjugate was used at a 1/4000 dilution. Results are representative of eight sets of observations from two independent experiments. (Bottom) The same optimization was performed for a two-step assay. Results are representative of eight sets of observations from two independent experiments.

overlap the recommended sensitivity of 250–500 ng/ml suggested by the recent AAPS/FDA working group [11]. Results shown in Fig. 2 again demonstrate significant differences between the one- and two-step bridging assay formats. The one-step format resulted in relatively constant OD values for coating concentrations above 30 ng/well for each of the anti-human IgG antibody concentrations tested (Fig. 2A). In contrast, the two-step assay format achieved maximum signal detection at antigen coating concentrations of approximately 15 ng/well for all antibody concentrations tested but dropped off significantly at higher coating concentrations (Fig. 2B).

Finally, direct sensitivity comparisons of the one-step and two-step bridging assay formats were performed at different coating concentrations and LY-HRP conjugate dilutions. ELISA plates were coated overnight with LY monoclonal antibody antigen at concentrations of 10 or 500 ng/well, and a

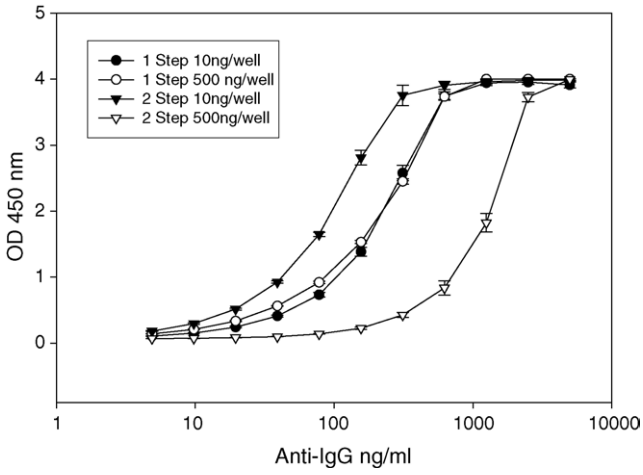

Fig. 3. Sensitivity levels of one-step vs. two-step formats. The sensitivity of one-step vs. two-step formats was determined by testing the ability of concentrations of anti-human IgG from 5 to 5000 ng/ml to bridge the respective assay. Microtiter plates were coated at concentrations of 10 or 500 ng/well, and an LY-HRP conjugate dilution of 1/4000 was used. Results are representative of eight sets of observations from two independent experiments.

dilution of LY-HRP conjugate of 1:2000 was employed. Rabbit anti-human IgG was tested at serial two-fold dilutions with concentrations ranging from 5 to 5000 ng/ml that encompass the recommended sensitivity of 250–500 ng/ml noted above. In these experiments, the one-step format showed virtually identical binding curves for the two coating concentrations employed with signals two- to three-fold above background obtained at rabbit anti-human IgG concentrations of 10–20 ng/ml (Fig. 3). Not surprisingly, the two-step format demonstrated marked dependence on coating concentration.

The concentration–response data for the two formats were fit using a Four Parameter Logistic Model with weighting, and a 90% confidence band of the fitted curve was determined using the Windows version of the S-Plus programming language, version 6.2. When the sensitivity of the assay is then defined as the lowest concentration where the 90% confidence band of the expected concentration does not overlap with the 90% confidence band of the expected response at zero concentration, the one-step 10 and 500 ng/ml and two-step 10 ng/ml curves give very similar sensitivities of 1.6, 0.8 and 1.4 ng/ml, respectively while the two-step 500 ng/ml curves give a sensitivity of 6.6 ng/ml.

While data presented here was obtained using affinity-purified rabbit anti-human IgG, similar results have been observed using cynomolgous anti-LY antisera (data not shown). This indicates that these results should be generally applicable to analysis of human samples.

4. Discussion

Many clinical protocols for detecting antibodies to monoclonal antibody human therapeutics have utilized antibody bridging (double antigen) assays for assessment of

immunogenicity [3,5,12]. The bridging assay allows for detection of all immunoglobulin isotypes and classes without the concern of secondary anti-human IgG detection antibody binding to the plate coating antigen or missing specific classes or isotypes of the sample anti-therapeutic antibody. Bridging assays utilizing both one-step [13] and two-step formats [14] have been reported in the literature. Results presented here indicate that the format of the bridging assay can have significant impact on the assay performance, particularly in regard to antigen coating density.

It is generally accepted for ELISA format assays that antigen should be coated to excess on the microtiter plate [15]. Results presented here indicate that different optimal coating concentrations were obtained for the one-step and two-step bridging assay formats. The two-step bridging format gives peak responses in the 15 ng/well antibody coating range that fall-off drastically at both higher and lower coating concentrations. The one-step format, on the other hand gives relatively constant responses at coating levels above 30 ng/well and thus lends itself well to coating to excess.

Similar problems with two-step assays were observed by Aggerbeck et al. in a two-step format double antigen, time-resolved fluorescence assay, where the signal strength dropped off rapidly as the coating antigen concentration increased [16]. Mire-Sluis et al. also reported lower signals in a bridging format assay at higher coating levels with some antisera [17]. These results suggest that at higher coating concentrations, in the two-step format, the coating density on the microtiter plate reaches a point where both binding arms of the anti-IgG antibody can bind to adjacent plate-bound antigens, preventing bridge formation with the enzyme-labeled antibody conjugate. In contrast, a one-step format, where the various components of the assay are all present simultaneously, allows for bridge formation to occur even at high (excess) coating densities.

The enzyme-labeled antibody conjugate concentration also has different effects in the two assay formats. The one-step assay shows a broad maximum at intermediate dilutions with decreased signal strengths at both higher and lower conjugate dilutions and little dependence on coating concentration. The two-step assay exhibits uniformly high signals at lower conjugate dilutions and a drop-off of signal at higher conjugate dilutions and an inverse correlation with coating concentration. Both assay formats appear to be losing signal at high conjugate dilutions due to limiting amounts of available HRP-LY conjugate. The one-step assay also shows decreased signal strengths at lower conjugate dilutions (higher concentrations). This is most likely due to high levels of HRP-LY saturating both binding arms of the anti-IgG antibody preventing formation of a bridge with the plate-bound antigen. The two-step format however, shows no evidence of decreased signals at high conjugate concentrations (but does show inverse dependence on coating concentration). In this case, since the anti-IgG is reacted separately from the HRP-LY conjugate, there is no opportunity for soluble complexes to form and the HRP-LY can bind to any free binding arms of the plate-bound anti-IgG antibody molecules in the second step of the assay.

Sensitivity comparisons for the two formats show that both methods can detect antibodies to levels below 10 ng/ml given appropriate assay parameters. This indicates that the bridging format ELISA exceeds the sensitivity of 250–500 ng/ml suggested by the recent AAPS/FDA working group [11]. The one-step format gives virtually identical results at widely different coating levels and appropriate conjugate concentrations. The two-step format shows sensitivities essentially equal to the one-step format at very low coat levels (10 ng/well) and similar conjugate concentrations but a lower sensitivity at high coat levels (500 ng/well). The higher assay signal observed for the two-step, 10 ng/well coat compared with the one-step format results at 10 or 500 ng/well coat (Fig. 3) at anti-IgG concentrations in the 50–300 ng/ml range could suggest that there is some bivalent binding in the one-step format. However, this does not seem likely in view of the virtually identical results for the 10 and 500 ng/well coating in the one-step format.

5. Conclusion

Results presented here indicate that the double antigen bridging assay represents an excellent method for sensitive detection of antibodies to therapeutic monoclonal antibodies. The format of the assay has a significant impact on optimal parameters required to achieve the desired results. Individual circumstances may affect the choice of format and subsequent assay parameters. The one-step assay achieves maximal sensitivity across a broad range of coating concentrations and at lower conjugate concentrations than the two-step format. The two-step format achieves maximal sensitivity at low plate coating concentrations and higher conjugate concentrations. The one-step format provides an assay in which plates can be coated to excess, removing coating variation as a variable in the assay.

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