The Journal of Immunology

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J Immunol 2007; 178:7467-7472; ;
doi: 10.4049/jimmunol.178.11.7467
http://www.jimmunol.org/content/178/11/7467

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Comparing ELISA and Surface Plasmon Resonance for Assessing Clinical Immunogenicity of Panitumumab

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Evaluation of the immunogenicity of panitumumab, a fully human anti-epidermal growth factor receptor mAb approved for use in colorectal cancer patients, led to the development of two separate immunoassays for the detection of anti-panitumumab Abs. The first immunoassay used a bridging ELISA capable of detecting 10 ng/ml positive control anti-panitumumab Ab. The ELISA incorporated an acid dissociation step to reduce drug interference and tolerated the presence of ~100-fold molar excess of drug. During eight clinical trials, the ELISA detected developing Ab responses in 2 of 612 (0.3%) subjects. In one of the ELISA positive subjects, neutralizing Abs were detected using an epidermal growth factor receptor phosphorylation bioassay. The second immunoassay used a Biacore biosensor immunoassay format capable of detecting 1 μg/ml positive control Ab while tolerating the presence of equal molar amounts of drug. Although less sensitive and less tolerant to competing drug in the assay, the Biacore assay detected developing Ab responses in 25 of the 604 (4.1%) subjects. Additionally, the Biacore assay identified eight subjects who developed neutralizing Abs. Mouse mAbs with affinities ranging from 1.1×10^{-6} to 8.4×10^{-10} M were used to characterize both assay types. The ELISA was more sensitive for the detection of higher affinity mAbs and detected high-affinity mAbs in the presence of higher molar ratio of drug to mAb. The Biacore assay was more sensitive for detection of lower affinity mAbs and detected low affinity Abs in the presence of higher molar ratios of drug to mAb. ***The Journal of Immunology,*** **2007, 178: 7467–7472.**

Epidermal growth factor receptor (EGFR)[2] is an established clinical target for the treatment of a variety of tumor types (1–3). Monoclonal Abs that bind to EGFR and block ligand-induced signal transduction have been applied in the treatment of a variety of solid tumor types (4). Anti-EGFR mAb therapeutics have evolved from the initial use of murine monoclonals to development of chimeric and fully human anti-EGFR Abs (5). The goal of reducing the extent of murine sequence in the therapeutic Ab is to reduce the possibility of developing human anti-mouse Abs. The development of human anti-human Abs can be associated with adverse events such as infusion reactions and reduced efficacy (6).

Panitumumab is a high-affinity fully human IgG2 mAb directed against the human EGFR. Panitumumab blocks epidermal growth factor (EGF) and TGF-α from binding to EGFR, inhibits tumor growth, and elicits both tumor regression and eradication of established tumors in some murine xenograft tumor models (7, 8). Panitumumab has efficacy as monotherapy and with chemotherapeutic agents in different cancer types including nonsmall cell lung cancer, renal and colorectal cancer (9). The fully human nature of panitumumab is expected to reduce the incidence of anti-panitumumab Ab development; however, anti-allotypic or anti-idiotypic Ab responses targeting the unique sequences in the variable regions are possible.

To evaluate the immunogenicity of panitumumab in clinical trials, two different immunoassays were developed to screen for anti-panitumumab Abs. The first assay was a bridging ELISA incorporating an acid dissociation step to reduce drug interference. The second assay was developed on a Biacore 3000 (Biacore AB) instrument using surface plasmon resonance to detect Ab binding to immobilized panitumumab. Samples testing positive in either screening assay were further tested for the presence of neutralizing Abs using a bioassay incorporating detection of EGF-induced EGFR phosphorylation.

Despite superior sensitivity and better drug tolerance, the ELISA detected fewer anti-panitumumab Ab positive subjects than the Biacore assay. Additionally, both assays detected subjects testing positive in only one assay type. The ability of the Biacore platform to detect low-affinity binding events is well established (10). The ELISA incorporates a number of extended incubations and wash steps during which low-affinity Abs may dissociate and therefore fail to be detected. To test the hypothesis that the ability of the Biacore platform to detect low-affinity binding events explains the observed results, anti-panitumumab mAbs were developed and screened using the Biacore to identify Abs with a range of affinities. Five mAbs were selected covering a range of affinities from 1.1×10^{-6} to 8.4×10^{-10} M. The monoclonals were used to further evaluate the performance of the ELISA and Biacore assay.

Materials and Methods

ELISA

ELISAs were performed as described previously (11). In brief, panitumumab was covalently attached to the wells of a 96-well immobilizer microtiter plate (Nalge Nunc International) via amine coupling. The wells were blocked with a nonfat dry milk diluent/blocking solution (Kirkegaard

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Received for publication November 8, 2006. Accepted for publication March 16, 2007.

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[2] Abbreviations used in this paper: EGFR, epidermal growth factor receptor; DPBS, Dulbecco's PBS; RU, response unit.

& Perry Laboratories). Duplicate serum samples were diluted 1/10 in 300 mM acetic acid and incubated at room temperature for 1 h. Biotinylated panitumumab and 1 M Tris (pH 9.5) were added to each well of the plates followed by the addition and mixing of the acid-treated serum samples. After a 3.5 h incubation at room temperature, the plates were washed, 100 μl of streptavidin peroxidase (Jackson ImmunoResearch Laboratories) was added, and the plates were incubated for 20 min at room temperature. The plates were washed, and 100 μl of *o*-phenylenediamine substrate (Sigma-Aldrich) was added to each well and incubated for 30 min at room temperature followed by the addition of 50 μl per well of 4 N sulfuric acid. The plates were read at 490 nm on a microtiter plate reader (Molecular Devices) to determine the OD of each well. The average signal-to-noise ratio for each sample was determined by dividing the average OD of each sample by the average OD of the negative control consisting of pooled normal human serum. The threshold of the assay was validated at a signal-to-noise ratio of 1.19 representing the 95% percentile above background. The assay sensitivity (at threshold) is ~10 ng/ml in neat serum. Any sample testing above threshold was considered anti-panitumumab Ab positive pending additional confirmatory testing. Drug-specificity confirmatory assays were conducted using samples spiked with 100 μg/ml panitumumab and analyzed for signal reduction indicating drug. Only samples that tested above the assay threshold and demonstrated drug specificity were considered positive for anti-panitumumab Abs. The appropriate Institutional Review Boards approved the studies and patients provided written consent for the reported results.

Biacore assay

In the Biacore immunoassay, panitumumab at 10 μg/ml was covalently immobilized through free amines to a carboxy-methylated sensor chip targeting a surface density of 2000 to 3000 response units (RU) on a Biacore 3000 instrument. Serum samples were diluted 1/4 in 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.05% Tween 20. Ten microliters of diluted serum samples and assay controls were individually injected sequentially across the panitumumab surface at 5 μl/min. The surface was regenerated using 10 μl of 0.39 M guanidine-HCl in 25 mM NaOH at 30 μl/min. The threshold of the panitumumab surface assay was validated at 168 RU for the panitumumab surface. The threshold value represents the 95th percentile above background provided by analysis of 60 serum samples including samples from 20 normal men volunteers, 20 normal women volunteers, and 20 subjects with colorectal cancer (gender unknown). Any sample testing at or above threshold was considered positive, pending additional confirmatory testing. The assay sensitivity at the threshold was ~1 μg/ml in neat serum based on titration of the anti-panitumumab positive control Ab. The specificity of binding to panitumumab observed in serum samples was confirmed by competition through the addition of 1 mg/ml panitumumab to the serum sample before testing. A 50% or greater reduction in net RU signal was required for a positive result for drug specificity. Only samples that tested above the assay threshold and demonstrated drug specificity were considered positive for anti-panitumumab Abs.

To confirm that the binding was attributed to anti-panitumumab Abs, limited isotyping experiments were conducted by sequentially injecting a goat anti-human IgG1, IgG3 and IgG4 (as a mixture) (The Binding Site GmbH) followed by a second injection of anti-IgM Abs (The Binding Site GmbH) after initial Ab binding. Of note, panitumumab is an IgG2 class Ab and thus IgG2 class Abs cannot be detected in this assay format because the surface is coated with panitumumab.

Antibody subclass determination

The Biacore 3000 instrument was used to determine Ab subclasses. Panitumuamb was diluted to 100 μg/ml in 10 mM sodium acetate (pH 5.0) and covalently immobilized at 2000 to 3000 RU onto a carboxymethyl-dextran-coated sensor chip by amine coupling chemistry. Previously confirmed IgG positive patient samples were diluted 1/4 in sample diluent containing HEPES-buffered saline (pH 7.4) containing 5 mM EDTA, and 0.005% Tween 20. Diluted samples were injected for 2 min across the ligand-immobilized surface at 5 μl/min and allowed to bind to the surface. The subclass of the captured anti-panitumumab IgG Abs was determined by sequentially injecting 10 μl of anti-IgG1, anti-IgG3, and anti-IgG4 at 300 μg/ml in sample diluent at 5 μl/min to allow binding and isotype determination. After determining the subclass for each sample, the surface was regenerated with a 10-μl injection of 25 mM NaOH/0.4 M guanidine-HCl at 30 μl/min.

Bioassay for neutralizing antibodies

A431 human epidermoid carcinoma cells were obtained from the American Type Culture Collection (CRL-1555) and maintained in high glucose DMEM (Invitrogen Life Technologies) with 10% FBS (Invitrogen Life Technologies) with 1× penicillin, streptamycin glutamine solution (Invitrogen Life Technologies) in a 37 ± 1°C incubator with 5 ± 1% CO_2 and 90 ± 10% humidity. Cells were passaged every 3 to 4 days at 1×10^5, 2×10^5, or 4×10^5 cells/ml in a 225 cm^2 flask. Cell monolayers were washed twice with Dulbecco's PBS (DPBS) without magnesium or calcium chloride (Invitrogen Life Technologies), followed by the addition of 5–15 ml of trypsin-EDTA (Invitrogen Life Technologies). Cells were incubated with trypsin-EDTA for up to 10 min at 37°C until detached. Growth medium was added at twice the trypsin volume and cells were counted for an assay or passaged at 1×10^5, 2×10^5, or 4×10^5 cells/ml in a 225 cm^2 flask.

A431 cells were plated in growth medium overnight at 1000 cells/well in 96-well, clear-bottom microtiter plates. Serum samples were subsequently diluted with DMEM low glucose (Invitrogen Life Technologies) to a final dilution of 1/10. Each serum dilution was preincubated with panitumumab (100 ng/ml final) for at least 30 min at room temperature before the addition to cells. The medium was removed from the plated cells and 50 μl of the serum mixture was added and allowed to incubate for another 30 min at 37 ± 1°C, 90 ± 10% humidity, and 5 ± 1% CO_2. Recombinant human EGF (R&D Systems) was added to the wells at a final concentration of 4 ng/ml for 15 to 25 min at 37°C. The medium was aspirated from the wells, and the cells were washed twice with 200 μl of ice cold DPBS. After washing, 50 μl of 0.15 M NaCl, 0.05 M Tris-HCl (pH 7.4), 6 mM sodium deoxycholate, 0.5% Nonidet P-40, and 30 μM orthovanadate (0.18% H_2O_2 activated), with protease inhibitor mixture (Sigma-Aldrich) was obtained internally and added to the wells and allowed to incubate on ice for at least 20 min. Subsequently, 50 μl of 2 μg/ml biotinylated goat anti-EGFR Ab (R&D Systems) and 50 μl of 2 μg/ml anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology) were added to the lysates and allowed to incubate for at least 30 min with shaking at room temperature. Following the incubation, 100 μl of this mixture was transferred to a streptavidin-coated MSD PR100 plate (Meso Scale Discovery) that had been blocked for 1 h with 1% BSA/DPBS. The plates were incubated for another 30 min with shaking at room temperature. Following this incubation, 50 μl of 1 μg/ml ruthenium-labeled goat anti-mouse IgG was added to each well for anther 30 min. The wells were washed three times with DPBS and 150 μl of 1× T buffer (Meso Scale Discovery) was added to each well. The plates were read on the MSD PR100 plate reader. The threshold of the assay was validated at a signal-to-noise ratio 1.32 representing the 95% percentile above background. The assay sensitivity (at threshold) was ~62.5 ng/ml in neat serum.

Generation of anti-panitumumab antibodies

Panitumamab-specific mAbs were generated using both rapid and standard immunization methods. For the rapid method, two 8- to 10-wk-old female NOD mice (Charles River Laboratory) and two 8- to 10-wk-old female BDF1 mice (Charles River Laboratory) each received immunizations with purified soluble panitumumab, every 2 to 3 days, five times over 13 days. The NOD mice have several defects in their thymic selection processes and were included to increase the likelihood of selecting epitopes leading to lower affinity Abs. For each immunization, 10 μg of Ag emulsified in Freund's complete adjuvant (Pierce) or RIBI adjuvant (Sigma-Aldrich) in a volume of 600 μl was administered subcutaneously to 12 sites proximal to draining lymph nodes, at 50 μl/site. For the standard method, five 8- to 10-wk-old female NOD mice (Charles River Laboratory) each received three rounds of immunizations with purified soluble panitumamab over 3 mo. For the initial immunization, 50 μg of Ag emulsified in Freund's complete adjuvant (Pierce) was administered subcutaneously at the nape of the neck. Twenty-one days later, the second boost of 25 μg of Ag emulsified in RIBI adjuvant was administered to the same site. Five days before fusion, the animals were boosted i.p. with 50 μg of Ag in PBS. The appropriate Institutional Review Boards approved the care and use of all animals.

B cell hybridomas were obtained by fusing immune lymphocytes or splenocytes with nonsecreting murine myeloma cells, Sp2/0-Ag14 (American Type Culture Collection), at a ratio of 2.5:1 by electrofusion. Cells were grown in 96-well plates in HAT (0.1 mM hypoxanthine, 4 mM aminopterin, 0.16 mM thymidine; Sigma-Aldrich) medium. Conditioned medium was screened by indirect ELISA using biotin-labeled panitumumab. Positive hybridomas were expanded and single-cell cloned.

Monoclonal Abs screening using ELISA

To detect anti-panitumamab Abs, 96-well enzyme immunoassay/RIA flat-bottom plates (Costar) were coated with goat anti-mouse IgG Fc (Pierce) at 1 μg/ml in PBS at 4°C overnight. The plates were blocked with PBS containing 1% BSA solution (Kirkegaard & Perry Laboratories) and 1% goat serum (Chemicon) at room temperature for 2 h. After washing the plates, 50 μl of hybridoma conditioned medium was added to each well and incubated on a shaker at room temperature for 1 h. The plates were

Table I. *Incidence of anti-panitumumab Abs and panitumumab-neutralizing Abs in clinical studies*

	Subjects Developing Non-Transient Anti-Panitumumab Abs[a]	Subjects Testing Neutralizing Ab Positive Postdose
ELISA		
Positive subjects	2	1
Total subjects	612	612
% Positive	0.3	0.2
Biacore		
Positive subjects	25	8
Total subjects	604	604
% Positive	4.1	1.3

[a] Subject tests positive postdose and remains positive at the final time point tested. Postdose positive subjects testing positive predose are excluded.

washed two times with washing solution (0.05% Tween 20 in PBS) and incubated with 50 μl/well of 100 ng/ml biotin-conjugated panitumumab premixed with 1/5000 dilution of NeutrAvidin HRP (Pierce) in blocking buffer containing 25% human serum (Chemicon) at room temperature for 1 h. After washing the plates four times, 50 μl/well of tetramethylbenzidine substrate (Sigma-Aldrich) was added and allowed to incubate at room temperature for 3 to 5 min. ELISA plates were read at 650 nm in a microplate reader. All positive clones with results greater than 8-fold above background were selected.

Kinetic analysis (affinity constant determination) of the mAbs

A Biacore assay using the Biacore 3000 instrument was developed for kinetic analysis of anti-panitumumab mAbs that bind to immobilized panitumumab. Panitumumab was diluted to 1 mg/ml in 10 mM sodium acetate (pH 5.0) and was covalently immobilized at a low density (i.e., 250 RU) onto a carboxymethyl-dextran-coated sensor chip using amine coupling chemistry. The mAbs were serially diluted from 6.25 nM to 500 nM in sample diluent containing HEPES-buffered saline (pH 7.4) containing 5 mM EDTA, and 0.005% Tween 20. Diluted mAbs were injected for 1 min across the panitumumab surface at 30 μl/min and allowed to dissociate for 10 min. After each sample, the surface was regenerated with a 10-μl injection of 50 mM NaOH at 30 ml/min. The dilution series sensograms generated for each mAb are evaluated using the BIAevaluation software (version 4.0.1) to determine the affinity constants using a 2:1 binding model.

Epitope mapping

The Biacore 3000 instrument mapped the epitopes bound by the anti-panitumumab mAbs using competitive inhibition of binding to a panitumumab-coated surface. Panitumumab diluted to 1 μg/ml in 10 mM sodium acetate (pH 5.0) was covalently immobilized at a low density (578 RU) onto a carboxymethyl-dextran-coated sensor chip by amine coupling chemistry. The mAbs were diluted in sample diluent containing HEPES-buffered saline (pH 7.4) containing 5 mM EDTA, and 0.005% Tween 20. Each mAb was separately evaluated by injection at a high concentration (100 to 800 μg/ml) for 2 min across the panitumumab surface at 5 μl/min followed by subsequent injections until the panitumumab surface was saturated as indicated by no additional binding. After panitumumab surface saturation, subsequent mAbs were sequentially injected for 1 min at 5 μl/min across the surface and any additional binding to available epitopes was determined for each mAb. After the final mAb was injected, the surface was regenerated with a 10-μl injection of 50 mM NaOH at 30 μl/min. The process was repeated after saturating the panitumumab surface individually with each of the five mAbs, and the order of addition of subsequent mAbs was changed as necessary to establish competitive binding relationships between the mAbs. Abs were considered to have mapped to the same epitope when surface saturation with one mAb completely inhibited binding of a second mAb.

Table II. *Affinity constants of selected anti-panitumumab monoclonal Abs*

Hybridoma Clone Designation	K_D (M)	K_a (M^{-1} min^{-1})	K_d (min^{-1})
2.2.1	8.4×10^{-10}	1.5×10^{4}	1.3×10^{-5}
3.7.1	3.2×10^{-9}	1.1×10^{5}	3.5×10^{-4}
18.2	3.8×10^{-8}	3.2×10^{5}	1.2×10^{-2}
19.1	6.5×10^{-7}	7.4×10^{4}	4.8×10^{-2}
12.2	1.1×10^{-6}	5.0×10^{4}	5.6×10^{-2}

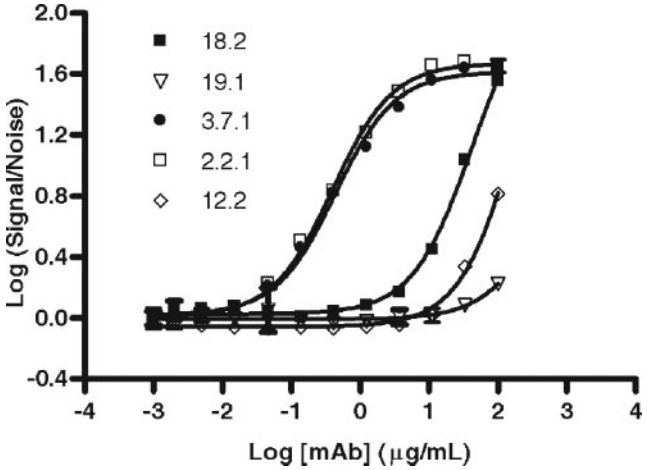

FIGURE 1. Titration of anti-panitumumab mAbs in the ELISA screening assay. Data represent the mean of three replicates of Log_{10} of signal-to-noise ratio ± 1 SD.

Results

Immunogenicity of panitumumab in clinical studies

The acid dissociation ELISA and Biacore assay were used to screen for Abs in eight panitumumab clinical studies. Subjects testing positive in either screening assay where further tested for the presence of neutralizing Abs in the EGFR phosphorylation bioassay. In the bioassay neutralizing Abs are detected by their ability to enable EGFR phosphorylation in the presence of a normally inhibitory concentration of panitumumab. Although immunogenicity assays are considered generally qualitative, the precision of the assays applied in the described studies enable positive postdose samples from predose negative subjects to be interpreted as developing Ab responses. It should be noted that postdose increases in signal intensity in the screening assays represent either an increase in Ab level or an increase in Ab affinity.

A transient anti-panitumumab Ab response is indicated when subjects who initially test positive at a postdose time point test negative at the last time point tested in a given study. The ELISA

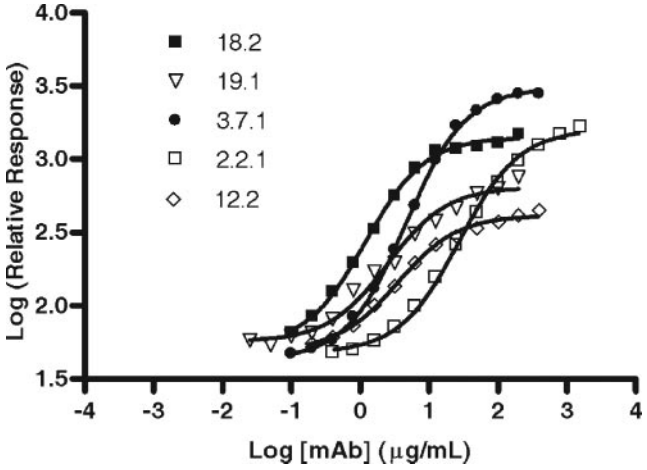

FIGURE 2. Titration of mAbs in the Biacore screening assay. Data represent the Log_{10} of relative RU.

Table III. *Comparison of immunoassay sensitivity for the detection of anti-panitumumab monoclonal Abs*

Hybridoma Clone Designation	ELISA Sensitivity (μg/ml)	Biacore Assay Sensitivity (μg/ml)
2.2.1	0.016	15.382
3.7.1	0.016	2.173
18.2	0.986	0.650
19.1	31.989	2.000
12.2	11.588	4.742

did not detect any transient anti-panitumumab Ab responses whereas the Biacore assay detected twelve. The ELISA detected seven predose positive subjects one of whom also tested positive postdose. The Biacore assay detected 18 predose positive subjects, nine of whom also tested positive postdose. When determining the rate of immunogenicity, predose anti-panitumumab Ab positive subjects were excluded if they also tested positive postdose. Using the ELISA, 0.3% (2/612) of patients with postdose samples developed nontransient anti-panitumumab Ab response (Table I). Neutralizing Abs were detected in 1 of the 612 (0.2%) subjects with a postdose sample. Using the Biacore assay, 4.1% (25/604) of patients with postdose samples developed a nontransient anti-panitumumab Ab response. A total of 1.6% (8/604) of the patients with postdose samples tested positive for neutralizing Abs. There was no correlation between neutralizing potency and binding activity detected in the Biacore assay.

To rule out the possibility of an IgG4 subclass-restricted response, the 25 subjects developing nontransient anti-panitumumab Ab responses detected using the Biacore assay were subsequently tested for the IgG1, IgG3, and IgG4 subclasses. IgG4 subclass Abs were detected in two subjects, both simultaneously developed even higher levels of IgG1.

Characterization of anti-panitumumab mAbs

The dissociation rates of anti-panitumumab mAbs in 26 hybridoma culture supernatants were measured using the Biacore assay to identify Abs with a range of affinities (data not shown). Sixteen hybridomas distributed evenly throughout the range of dissociation rates measured were expanded in culture to provide purified mAbs for Biacore kinetic analyses. After kinetic analysis using a low-density Biacore surface, five of the 16 mAbs were selected providing a range of Ab affinities for use in evaluating ability of the ELISA and Biacore assays (Table II). The anti-panitumumab mAb affinities that were selected ranged over 4 logs from 1.1×10^{-6} to 8.4×10^{-10} M. The five selected mAbs had association rate constants (K_a) ranging from 1.5×10^4 to 3.2×10^5 M^{-1} min^{-1} and dissociation rate constants (K_d) ranging from 5.6×10^{-2} to 1.3×10^{-2} min^{-1}.

Epitope mapping of mAbs

The Biacore was used to map the epitopes bound by the five mAbs relative to each other. Monoclonal Ab 2.2.1 completely inhibited binding of mAb 12.2, completely inhibited binding of mAb 19.1, and partially inhibited binding of mAb 18.2; mAb 3.7.1 completely inhibited mAb 12.2 and partially inhibited mAbs 19.1 and 18.2; mAb 12.1 mapped to the same epitope as mAbs 2.2.1 and 3.7.1; mAb 19.1 mapped to the same epitope as mAb 2.2.1. The binding of mAb 18.2 was not completely inhibited by any of the other mAbs and thus mapped to a different, but overlapping, epitope.

Evaluation of mAbs in immunogenicity screening assays (sensitivity and drug interference)

The five mAbs were serially diluted in pooled normal human serum and tested in the ELISA and the Biacore assay. The resulting titration data was fit to a sigmoidal dose-response curve equation (version 4.01 GraphPad Prism) (Figs. 1 and 2). The sensitivity of detection for each mAb in both assays was determined by regression analysis at the assay threshold value for each assay (Table III). The sensitivity of the ELISA for the individual mAbs ranged from 16 ng/ml to 32 μg/ml and correlated directly with the affinity (K_D). The highest affinity mAbs were detected at the lowest concentrations in the ELISA. The sensitivity of the Biacore assay for the individual mAbs ranged from 650 ng/ml to 15 μg/ml and partially correlated with the association rate constant (K_a). The mAbs with the highest association rate constants were generally detected at the lowest concentrations in the Biacore assay.

The extent of drug interference in the detection of the five anti-panitumumab mAbs was evaluated by spiking serial dilutions of panitumumab into serum samples containing the individual mAbs at concentrations 2- to 4-fold above the previously determined assay sensitivities for the individual mAbs in both the ELISA and Biacore assays. The tolerated concentration of panitumumab was determined using interpolation at the assay threshold following a spline curve fit (version 4.01 GraphPad Prism) of the panitumumab titration data (Table IV). The tolerated concentration of panitumumab was divided by the anti-panitumumab Ab concentration to determine the molar excess of drug tolerated in the assays. The

Table IV. *Evaluation of drug interference in the detection of anti-panitumumab mAbs by ELISA and Biacore assay*

Hybridoma Clone Designation	Ab Concentration (μg/ml)	Concentration of Drug Tolerated[a] (μg/ml)	Molar Excess of Drug Tolerated	K_D (M)
ELISA drug tolerance				
2.2.1	0.064	24.3	380.0	8.4×10^{-10}
3.7.1	0.064	15.0	234.2	3.2×10^{-9}
18.2	3.96	137.4	34.7	3.8×10^{-8}
19.1	63.98	472.1	7.4	6.5×10^{-7}
12.2	46.36	2157.7	46.5	1.1×10^{-6}
Biacore assay drug tolerance				
2.2.1	40.0	13.8	0.3	8.4×10^{-10}
3.7.1	8.0	4.8	0.6	3.2×10^{-9}
18.2	2.6	2.1	0.8	3.8×10^{-8}
19.1	8.0	49.0	6.1	6.5×10^{-7}
12.2	19.0	36.0	1.9	1.1×10^{-6}

[a] Concentration of drug reducing signal in the assay to the threshold value.

molar excess of drug tolerated in the ELISA ranged from 7.4- to 380.0-fold and correlated with the mAb affinity. The highest affinity mAbs had the highest molar drug tolerance in the ELISA. The molar excess of drug tolerated in the Biacore assay ranged from 0.3- to 6.1-fold and was inversely correlated with the mAb affinity. The highest affinity mAbs had the lowest molar drug tolerance in the Biacore assay.

Evaluation of mAbs in a bioassay for neutralizing antibodies

All five mAbs were serially diluted and analyzed in the bioassay for neutralizing Abs. Only the two highest affinity mAbs (clones 2.2.2 and 3.7.1) were capable of neutralizing panitumumab activity in the bioassay.

Discussion

In support of the biological license application, the immunogenicity of panitumumab was evaluated in eight clinical studies using the ELISA and Biacore assays. The incidence of anti-panitumumab Ab development was >10-fold higher in the Biacore assay when considering all subjects with postdose samples. The Biacore assay also detected transient Ab responses not detected by the ELISA. During validation of both assays using the same positive control Ab, the ELISA was shown to be considerably more sensitive (10 ng/ml) than the Biacore assay (1 μg/ml). The ELISA was also shown to tolerate higher drug levels than could be tolerated in the Biacore assay. The acid dissociation step resulted in a <10% reduction of signal throughout the titration range of the positive control Ab (data not shown) and is comparable to observations in a previously described assay (11). The validation experiments did not predict the clinical performance of the assays. The explanation put forward was that the multiple incubations and wash steps in the ELISA cause it to fail to detect low affinity Abs detected in the Biacore assay. To test this hypothesis, both assays were evaluated using anti-idiotypic anti-panitumumab mAbs that were selected to cover a wide range of affinities.

The sensitivity for each mAb in the ELISA and Biacore assay formats was determined using the validated assay threshold for each assay set at the 95th percentile above background. The mAbs were serially diluted in pooled normal serum and analyzed in both assays, and the assay sensitivity based on interpolation at the assay threshold was determined. The sensitivity of mAb detection in the ELISA ranged from 16 ng/ml to 32 μg/ml and was directly proportional to the affinity of the mAb. The ELISA was most sensitive detecting the highest affinity mAbs. It should also be noted that despite a nearly 4-fold difference in affinity, the two highest affinity mAbs provided nearly identical sensitivities suggesting that Abs above a specific affinity can be expected to behave similarly in ELISA. The sensitivity range of mAb detection in the Biacore was narrower (650 ng/ml to 15 μg/ml) and the association rate constant (K_a) was a better predictor of sensitivity than the overall affinity. Despite being less sensitive for the detection of two highest affinity mAbs, the Biacore had better sensitivity for the three lowest affinity mAbs.

The performance differences are explained by the impact of binding kinetics on the two assay platforms and may best be compared through mAbs 2.2.1 and 19.1. Although having the highest affinity of the five mAbs, mAb Ab 2.2.1 has the lowest association constant. In the ELISA, where a long incubation can minimize the impact of a low association constant, mAb 2.2.1 is detected with the greatest sensitivity of the five mAbs. In the Biacore assay, where binding is measured in real time and association constants can have an impact, mAb 2.2.1 is detected with the least sensitivity of the five mAbs. Despite having the second lowest affinity, 19.1 is detected with the second greatest sensitivity of the five mAbs in the Biacore assay. Monoclonal 19.1 is detected with the least sensitivity in the ELISA where it's binding may be reversed during long incubation periods.

Ab therapeutics are often used at very high concentrations and typically have long half lives in serum. Drug interference as a consequence, is a considerable challenge when evaluating the immunogenicity of Ab therapeutics. To assess the impact of Ab affinity on the detection of anti-panitumumab Abs in both screening assays, serum was spiked with the anti-panitumumab mAbs and serial dilutions of panitumumab to determine how much drug is tolerated in both assays for the detection of all five mAbs. To allow comparison between the different mAbs and the different assays the molar excess of drug tolerated was also determined. The ELISA incorporates an acid dissociation step to improve resistance to drug interference and, as expected, tolerated the presence of the highest molar excesses of drug. The highest affinity mAbs also tolerated the highest molar excess of drug in the ELISA. The Biacore assay, in contrast, tolerated the highest molar excesses of drug with low-affinity Abs. The difference in performance between the two assays can be explained both in context of the binding kinetics and in format of the assay. Low-affinity Abs are more likely to bind and release due to their typically faster dissociation rate constants. In the Biacore assay, low-affinity Abs tolerate higher molar excesses of drug than high-affinity Abs because they are more likely to dissociate from free drug, thereby freeing an Ag binding site to bind to the immobilized drug on the solid phase. The ELISA in contrast is designed to allow the drug on the solid phase and the labeled drug to compete more effectively with soluble drug. The ELISA favors high-affinity Abs which are more likely to maintain a successful bridge formation between immobilized drug and labeled drug. Low-affinity Abs are more likely to dissociate from the solid phase immobilized drug and return to the soluble phase where binding to labeled drug or free drug is favored, resulting in decreased sensitivity and increased drug interference.

It was noted that the bioassay for neutralizing Abs detected more positive subjects than the ELISA. Despite this, bioassays are considered impractical for screening purposes due to the constraints of tissue culture and their inability to detect clinically relevant binding Abs that do not neutralize the drug's activity. Bioassays are also more susceptible to nonspecific serum interference and require a screening assay result to confirm any drug neutralizing effect is due to the presence of an Ab. To determine whether Ab affinity impacts detection of neutralizing Abs, the five mAbs were titered in the bioassay. Only the two highest affinity Abs (2.2.1 and 3.7.1) were shown to neutralize the bioactivity of panitumumab. Despite binding to the same epitopes recognized by either 2.2.1 or 3.7.1, mAbs 19.1 and 12.2 were not neutralizing suggesting that the bioassay was not capable of detecting low-affinity Abs that bind to neutralizing epitopes. Monoclonal Ab 18.2 bound to a slightly different epitope from 2.2.1 or 3.7.1, thereby preventing any interpretation of the impact of its affinity on its inability to neutralize panitumumab.

Although the overall incidence of anti-panitumumab Abs in clinical trials is low, clear differences in the incidence of Abs detected by the ELISA and Biacore screening assays were observed. Despite being less sensitive for detection of the polyclonal positive control Ab, the Biacore assay identified more anti-panitumumab Ab positive subjects than the ELISA. The results suggested that screening immunoassays were detecting different types of Abs, and the hypothesis put forward was that the Biacore assay detects more positive samples because of its ability to detect low-affinity Abs. The results support this hypothesis by demonstrating low-affinity Abs were detected at lower concentrations in the Biacore assay. Drug interference experiments revealed further differences

in assay performance showing that the highest molar excess of drug tolerated occurs with the highest affinity Abs in the ELISA and with the lowest affinity Abs in the Biacore assay. The possibility that monovalent IgG4 subclass restricted Ab responses could account for the subjects not detected by ELISA due to its bridging approach was eliminated by Ab subclass determinations in the Biacore assay where no IgG4-restricted responses were observed.

The higher incidence of anti-panitumumab Abs detected by the Biacore assay is likely due to patients generating mostly low-affinity Abs, and although some had neutralizing activity, there was a lack of correlation between Ab development to clinical sequelae or loss of efficacy (panitumumab prescribing information). The results also clearly demonstrate that the ELISA is better suited than the Biacore assay for the detection of higher affinity Abs in the presence of high concentrations of drug. It should be noted that in the course of immune response maturation, in addition to increasing affinity, generally the concentration of specific anti-drug Abs also increases. The use of both the Biacore assay and the ELISA for screening purposes in clinical studies to support panitumumab development is justified not only because both screening assays detected samples positive in only one assay type, but also because additional neutralizing Abs not detected in the ELISA were detected by the Biacore assay.

Characterization of the ability of the ELISA and the Biacore assay to detect Abs with different affinities has helped to explain the observed clinical results. The results also suggest careful consideration be given to the assay formats and procedures applied to immunogenicity testing in support of drug development. To provide the best chance of detecting low-affinity Abs, consideration should be given to reducing the numbers of incubations and wash steps in a proposed protocol. Use of the Biacore assay platform assures that low-affinity Abs will be detected, although steps must be taken to ensure that adequate drug clearance has occurred to enable sensitive detection. For detection of anti-drug Abs in the presence of high drug concentrations, assay choice may be limited to methods, similar to ELISA, that are capable of incorporating acid dissociation procedures to separate drug-antibody immune complexes before analysis. To date, the Biacore assays are not directly compatible with acid dissociation procedures as used in the ELISA, but an alternative approach could conceivably include a drug removal sample processing step as previously described (12).

Acknowledgments

We are grateful to Dr. David Chang and Aaron Winters of the Department of Protein Science, Amgen, for contributions in providing anti-panitumumab mAbs.

Disclosures

The authors have no financial conflict of interest.

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