

Multiplexed Serum Measurement of IgG, IgA, IgM, and IgE Antibody Responses to Therapeutic Biologicals

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Analytical methods characterizing the immunogenicity of therapeutic proteins are useful for monitoring, characterizing and predicting reactions to biopharmaceuticals. A multiplexed assay capable of isotyping the specific IgG, IgA, IgM and IgE (IgGAME) antibody responses against a biotherapeutic was demonstrated in a hyper-immunized cynomolgus monkey, over a 15-month period. The quantitative range of the antibody measurements was determined to be 15 ng/ml to 50 ng/ml in 10% serum. By the use of any biotinylated or fluorescently tagged therapeutic as a detector, this multiplexed isotyping assay can be broadly applied to human and non-human primate IgG, IgA, IgM and IgE immunogenicity studies.

Keywords Multiplex, Isotype, Immunogenicity, Cynomolgus, IgG, IgA, IgM, IgE.

INTRODUCTION

The immunogenicity of protein therapeutics leading to the production of specific antibodies has been correlated with altered therapeutic pharmacokinetics, potency, and adverse events (Biagini et al., 1988). Assays screening for antibodies against therapeutic biologicals is a requirement during most clinical trials, and may be permanently implemented for long-term patient

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monitoring in cases where a significant risk has been demonstrated. When a subject develops antibodies to a therapeutic, follow-up characterization of the antibodies can include measurements of titer, specificity and cross-reactivity, affinity, and isotype (Mire-Sluis et al., 2004).

In studies of vaccine efficacy and infectious diseases, the beneficial protective effects of particular antibody isotypes has been reported (Quan et al., 2004). However, the clinical significance of antibody isotypes in unwanted immunogenicity to therapeutic products is not well understood. Each of the IgG, IgA, IgM, and IgE antibody classes has its own time course of appearance, circulating serum concentration, half-life, structure, and interactions with other molecules and cells of the immune system, such as complement and effector cells. IgA pre-dominants in mucosal immunity, IgE is associated with allergic reactions, and the presence of IgM suggests a less mature immune response. Factors that could each affect the magnitude and class of an antibody response include: the route of therapeutic administration, whether it be oral, inhaled, intravenous, or intramuscular injection; the site of therapeutic accumulation; the target tissue; the mechanism and site of therapeutic clearance in vivo; and the patient population.

Because the volume of patient serum available for characterization assays can be limiting, it is advantageous to have a multiplexed assay able to measure multiple parameters in one small aliquot. The IgGAME isotyping assay was developed in a 4-plex format, able to report the anti-therapeutic IgG, IgA, IgM, and IgE antibody responses in a single well using 5 μ l of serum. In order to develop the assay, a source of positive controls for anti-therapeutic IgG, IgA, IgM, and IgE serum was needed. Cynomolgus monkeys are physiologically similar to humans, and thus are commonly used as an animal model for therapeutic safety and efficacy studies. Cynomolgus monkeys are also frequently hyper-immunized with biological therapeutics to generate polyclonal antibody positive controls for use in human anti-therapeutic antibody screening assays. Immunization bleeds were identified from such hyper-immune serum containing anti-therapeutic IgGAME antibodies. This sera was then used as a source of positive controls on which to develop the IgGAME assay.

MATERIALS AND METHODS

Antibodies

For in-house coupling of microspheres, mouse anti-human IgA antibody, clone IgA5-3B, was purchased from Custom Monoclonals International (Sacramento, CA), and rat anti-human IgE, clone LO-HE-17, was purchased from BioSource (Camarillo, CA). Purified IgA, IgM, and IgE human myeloma

immunoglobulins for testing assay specificity were purchased from Calbiochem (San Diego, CA) and purified serum IgG was purchased from Bethyl Laboratories (Montgomery, TX). Antibodies biotinylated for use as detectors, included anti-human kappa light chain (Upstate Inc., Charlottesville, PA), anti-human lambda chain (clone LO-hL-2, Upstate Inc.), and various recombinant humanized therapeutic monoclonal antibody, (rhuMAb), IgG molecules (Genentech, Inc. South San Francisco, CA).

Anti-Therapeutic Hyper-Immune Serum

A cynomolgus monkey was administered every 2 weeks with 1 mg in 0.5 ml of Raptiva, (a humanized recombinant human monoclonal antibody directed against human CD11a), plus adjuvant, subcutaneously in the neck. For the primary immunization TiterMax was used, and Freund's Incomplete Adjuvant was used for the subsequent boosts. The immunization period was 15 months.

Preparation of Capture Microspheres

Protein G (bead #38) and anti-human IgM (bead #19) capture beads were purchased from Upstate Inc. (Charlottesville, VA). Microspheres were purchased from Luminex Corp. (Austin, Texas) for the in-house coupling of anti-human IgA (bead #20) and anti-human IgE (bead #77). Methods for in-house microsphere protein conjugations were developed from protocols provided by Luminex.

For coupling antibody to microspheres, all reagents were warmed to ambient temperature. The antibody was diluted to 31.25 µg/ml in PBS, pH 7.3. Using an analytical balance, 5 mg of Sulfo-NHS (N-hydroxysulfosuccinimide, sodium salt) and EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide-HCl), (Pierce Biotechnology Inc., Rockford, IL) were weighed in Eppendorf tubes. Immediately before use (see below), 50 mg/ml Sulfo-NHS and 10 mg/ml EDC solutions were prepared in 0.1 M Sodium Phosphate, pH 6.1.

Microsphere stock vials were centrifuged, and the beads dispersed by a brief vortex and then sonication for 1 minute in a microbead sonicator (Upstate Inc., Charlottesville, VA). Then, 1.25×10^7 microspheres were dispensed from the stock into an eppendorf tube (USA Scientific Inc., Ocala, FL). The beads were centrifuged 1 minute, the supernatant aspirated, and the microspheres dispersed by a brief vortex and sonication in 400 µl of 0.1 M Sodium Phosphate, pH 6.1. The beads were activated by adding, 50 µl of 50 mg/ml Sulfo-NHS, followed by gentle vortexing. Immediately, 25 µl of 10 mg/ml EDC was added, followed by gentle vortexing. The suspension was incubated in the dark for 20 ± 2 minutes. The activated microspheres were

washed $2 \times 500 \mu\text{l}$ with PBS, pH 7.3, in a 96-well filter plate (Millipore Multi-screen-BV, MABVN1250, Millipore, Bedford, MA) using a vacuum manifold (Millipore, Bedford, MA). Beads were removed from the filter plate with $125 \mu\text{l}$ PBS into an eppendorf tube. Coupling was immediately initiated by adding 1 ml of $31.25 \mu\text{g/ml}$ monoclonal antibody. The coupling solution was mixed by vortexing, and sonicated for 1 hour in the dark. The protein-coupled microspheres were centrifuged for 1 minute and the supernatant carefully aspirated. The beads were washed $2 \times 500 \mu\text{l}$ with PBS, 0.05% polysorbate-20, pH 7.3, with centrifugation and aspiration. Microspheres were then blocked by adding 1 ml of PBS containing 1 mg/ml BSA, followed by a brief vortex, 1 minute of sonication, and a 30 minute incubation in the dark. The coupled and blocked microspheres were centrifuged for 1 minute, the block buffer removed, and 1 ml of fresh block solution added. The beads were enumerated using a hemocytometer, and stored at 4°C for up to 1 year in the dark. Working stock solutions of 5×10^5 beads/ml in PBS, 0.5% BSA, 0.05% polysorbate-20 and 0.05% ProClin300, pH 7.3, were prepared and stored at 4°C before use.

Preparation of Monoclonal Antibody Reporters

A reporter system of biotinylated recombinant humanized mAb, (rhu-mAb), therapeutic and Streptavidin-PE (Molecular Probes, Eugene, OR) was used for all anti-therapeutic antibody measurements.

Monoclonal antibodies were biotinylated using the NHS-Biotin, PIERCE EZ-link product (Pierce Biotechnology Inc., Rockford, IL). The antibody was dialyzed into 0.1 M NaHCO_3 , pH 8.0–8.6, for 2 hours at room temperature and made to a final concentration of 1 mg/ml. NHS-Biotin, 2 mg, was dissolved in 1 ml DMSO. Biotin was added to the antibody solution at a volume equal to $100 \mu\text{g}$ biotin/ mg antibody, vortexed gently and allowed to react at room temperature for at least 2 hours. The biotinylation reaction was dialyzed into PBS, overnight at 4°C , with one buffer change after 2 hours. The final protein concentration was determined using a protein assay (BioRad, Hercules, CA) and aliquots stored at -70°C .

Preparation of Affinity-Sepharose for Purification of Serum

Polyclonal anti-therapeutic antibodies were purified from the cynomolgus monkey serum using a Sepharose 4B matrix coupled with Raptiva, (Activated CH Sepharose 4B, Amersham Biosciences AB, Uppsala, Sweden). Specific antibodies were eluted in 0.1 M glycine, pH 2.3, neutralized in 1 M Tris, pH8, and dialyzed into PBS, pH 7.3.

Multiplex IgGAME Assay

The multiplex IgGAME assay was performed at ambient temperature in 96-well filter plates purchased from Millipore (Multiscreen-BV, MABVN1250, Millipore, Bedford, MA). The wells were pre-wet with 15 μ l of assay diluent (PBS, 0.5% BSA, 0.05% polysorbate-20 and 0.05% ProClin300, pH 7.3). A master mix of the four beads (Protein G, anti-human IgA, anti-human IgM, and anti-human IgE) was made by mixing equal volumes of working stock bead solutions of 5×10^5 beads/ml in assay diluent. The master mix was vortexed and sonicated for 1 minute and 40 μ l of bead mix added to each well. Next, 40 μ l of samples and controls were added at a dilution of 1/10 in assay diluent and incubated with agitation for 2 hours, in the dark. If samples needed to be diluted more than 1/10 the dilutions were made in 10% naïve serum of the same species. The plate was washed 3 $\times$ with PBST (PBS, 0.05% polysorbate-20) using a vacuum manifold (Millipore, Bedford, MA). Biotinylated Raptiva was added to each well at 5 μ g/ml in 80 μ l and incubated for 2 hours with agitation, in the dark. The plate was washed 3 $\times$ with PBST and 160 μ l of streptavidin-PE (Molecular Probes) was added at 0.25 mg/ml, for 30 minutes, in the dark, with agitation. With no further washing, the plate was fixed by adding 40 μ l of 5% formaldehyde in PBS. The plate was run on a Luminex 100 instrument, software v1.7 (Luminex Corp., Austin, TX), 100 events per bead were acquired and median RFU reported.

Total Antibody Assay

Total antibody responses, irrespective of isotype, were captured using 5×10^5 Raptiva-conjugated beads/ml in assay diluent. The assay was performed, using the biotinylated Raptiva reporter system, as described for the multiplex IgGAME assay (above).

Competition Studies

The specificities of the total and IgE antibody responses to Raptiva were confirmed by competition studies. To test the specificity of the total antibody response, immune serum at a dilution of 1/10 in assay diluent, was pre-incubated with 10 μ g/mL of a panel of recombinant humanized monoclonal IgG antibodies, a commercially available human IgG or F(ab')₂ for 2 hours at room temperature. Capture beads (Raptiva for total assays, or anti-IgE beads for IgE assays) were then added and the assay performed as usual. The IgE specificity was confirmed using 3 approaches. First, to check the specificity of capture, 100 μ g/mL of anti-IgG, anti-IgA, anti-IgM, and anti-IgE monoclonal antibodies were pre-incubated with immune serum at a dilution of 1/10 in assay diluent, overnight at room temperature. Second, to test the antigen

specificity, immune serum at a dilution of 1/10 in assay diluent, was pre-incubated with 10 $\mu\text{g/mL}$ of several recombinant humanized monoclonal IgG antibodies, a commercially available human IgG or F(ab')_2 for 2 hours at room temperature. Third, capture beads were blocked with 12 $\mu\text{g/mL}$ of human IgG, human IgE, or human IgM, overnight at room temperature before adding immune serum.

RESULTS

Development of Capture Beads

Capture beads specific for the IgG, IgA, IgM, and IgE isotypes were qualified with the added criteria of cross-reactivity to cynomolgus monkey immunoglobulins. In the absence of purified monkey immunoglobulins, cross-reactivity was assessed by comparing signal to noise ratios in naïve versus therapeutic-immune monkey serum, using the biotinylated Raptiva as a detector (Figure 1). Immune serum from days 166–210 were selected to develop the assay, because these timepoints were found to contain relatively high levels of all four anti-Raptiva antibody isotypes. Cynomolgus monkey IgG was captured using Protein G because commercially available anti-human and anti-monkey IgG antibodies were found to be less sensitive in detecting immune responses over background and many antibodies were also cross-reactive with cynomolgus IgA and IgM (data not shown).

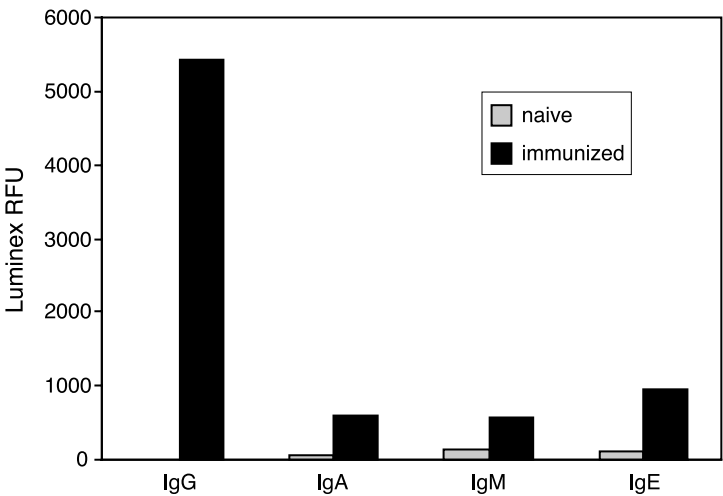


Figure 1: Development of capture reagents cross-reactive to cynomolgus monkey immunoglobulins. Day 210 Raptiva -immune serum was tested against naïve serum, using biotinylated Raptiva as a detector. The capture beads showing the best median RFU signal to noise ratio were selected.

Table 1: Specificity of capture beads measured with purified human immunoglobulins.

Immunoglobulin	Median RFU for capture bead			
	Protein G	Anti-IgA	Anti-IgM	Anti-IgE
Human IgG	15811	0	23	11
Human IgA	0	3905	34	0
Human IgM	0	29	4879	7
Human IgE	0	0	0	12118

Purified human myeloma immunoglobulins were used in assay diluent at 2 µg/ml IgG, 0.5 µg/ml IgA and IgM, and 50 ng/ml IgE, to test bead capture specificity. These concentrations were selected proportional to serum levels, and to fall within the high end of the quantitative range for the anti-light chain detectors used. For the Protein G and anti-IgM beads, the reporter used was biotinylated anti-human kappa light chain. For anti-IgA, and anti-IgE beads, the reporter used was biotinylated anti-human lambda light chain. Minimal to no cross-reactivity was observed after subtracting buffer background, and the data is reported in median RFU (Table 1). Because the recovery and dynamic range of each capture bead is unique, the magnitude of median RFU values cannot be quantitatively compared between different capture beads. Typically in multiplex analysis, a multiplexed standard cocktail is run to give standard curves for each analyte. In this case, we were unable to purify sufficient material to generate anti-therapeutic IgA, IgM or IgE standard curves. However, in order to enable inter-assay comparisons, positive controls could be prepared using the hyper-immune cynomolgus monkey serum.

Optimization of Bead Coating and Bead Input Number

The coupling of capture antibody to the bead is an important step for optimizing sensitivity. The optimal antibody coupling concentration was decided based on the capture beads that gave the maximum signal from the hyper-immune serum, using biotinylated Raptiva as a detector, as described in the methods (Figure 2). Using this approach, a coupling value between 2.5–5 µg antibody/million beads was most sensitive. Also, the number of beads input to each assay well was examined. It was found that 5000 beads/well/analyte (i.e., 25,000 beads total for an IgGAME 4-plex), maximizes sensitivity, while minimizing bead carryover well-to-well and sample acquisition time (Figure 3).

Assay Performance in 4-Plex Versus Single-Plex Assays

The performance of the capture beads used in single plex, or in multiplex with the other three bead sets was compared. Day 210 hyper-immune serum

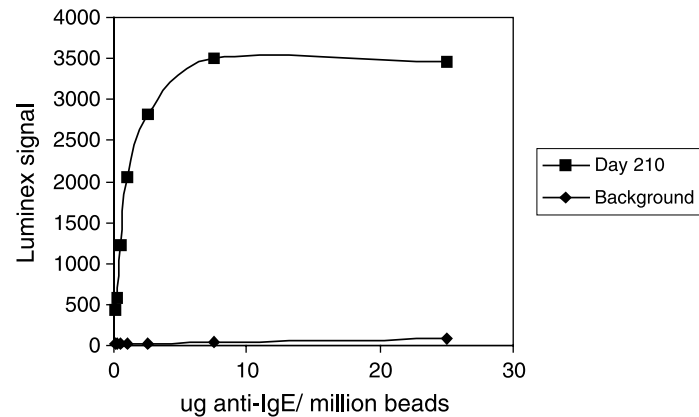


Figure 2: Optimization of bead coupling. The amount of anti-IgE antibody coupled/million microspheres was varied from 0–25 μ g. The sensitivity was tested by measuring the median RFU signal from day 210 hyper-immunized or naïve serum (for background) at a dilution of 1/10 and the biotinylated Raptiva detector.

or naïve serum (for background) at a dilution of 1/10 was run single-plex and multiplex. No significant difference in signal to background was observed (Table 2).

Sensitivity Evaluation of IgGAME Assay

Standards for isotype-specific anti-Raptiva antibodies were not available. Affinity purification of the hyper-immune serum on sepharose covalently linked with Raptiva, yielded polyclonal antibody of the IgG class, with very

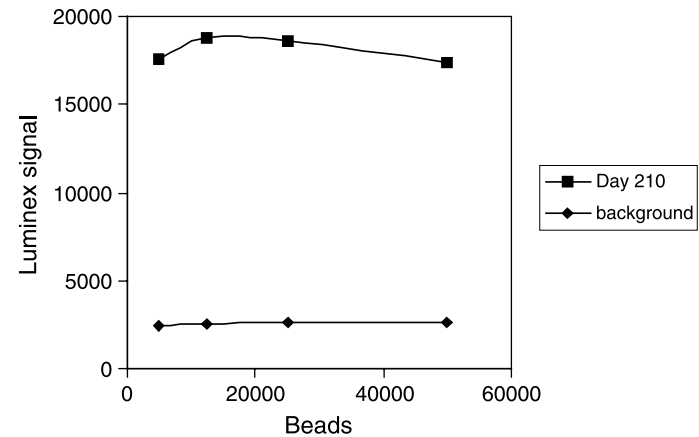


Figure 3: Optimization of bead input number. The number of beads input into a sample well was varied from 5,000–50,000. The median RFU signal from day 210 hyper-immune serum or naïve serum (for background), at a dilution of 1/10, using biotinylated Raptiva as a detector, was measured. No significant increase in signal was observed above the 5,000 bead input.

Table 2: Single-plex versus multiplex performance measured using Raptiva, day 210 hyper-immune or naïve (for background) cynomolgus monkey serum.

Microsphere	Single-plex RFU (background)	Multiplex RFU (background)	% Difference (after background subtracted)
Protein G	19,541 (3,894)	20,316 (4,387)	2
Anti-IgA	885 (40)	881 (46)	1
Anti-IgM	126 (60)	131 (59)	9
Anti-IgE	3,206 (93)	3,224 (109)	0

low levels of specific antibodies of the IgA, IgM, or IgE classes. A standard curve was generated by spiking known concentrations of the purified anti-Raptiva pAb into 10% naïve cynomolgus monkey serum. Data was acquired using single-plex assays with Protein G beads, or with Raptiva-coupled beads. The sensitivity of the total antibody assay (using Raptiva beads and a biotinylated Raptiva detector), was in the range of 50 ng/ml (Figure 4). The sensitivity of the IgG antibody assay (using Protein G beads and biotinylated Raptiva detector), was interfered with by the capture of non-specific IgG. The

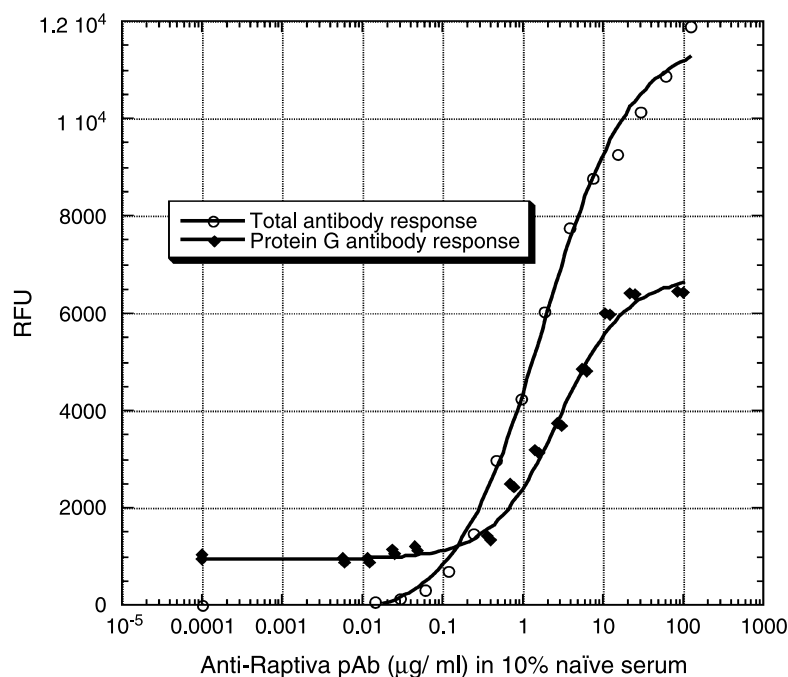
**Figure 4:** Sensitivity of the total and IgG antibody assays. Affinity purified pAb to Raptiva was spiked at known concentrations, into naïve, 10% cynomolgus monkey serum. Anti-Raptiva pAb (µg/ml) in 10% naïve serum.

Table 3: IgGAME intra-assay and inter-assay precision over 4 days using day 166, Raptiva hyper-immune cynomolgus monkey serum.

Day 166 serum (1/10) IgGAME data: intra and inter-assay						
Assay		IgG	IgA	IgM	IgE	Total
1	RFU	3430	351	133	232	4140
	Intra- %CV	4.1	1.6	3.2	3.7	2.8
2	RFU	3793	420	74	319	5465
	Intra- %CV	1.6	2.2	1.0	2.6	0.4
3	RFU	3722	389	144	298	5402
	Intra- %CV	4.0	4.2	1.4	1.4	4.3
4	RFU	3837	445	86	368	4945
	Intra- %CV	2.0	7.8	1.6	1.5	1.1
Mean	RFU	3695	401	109	304	4988
	Inter- Std dev	183.2	40.6	34.3	56.4	611.0
	Inter- %CV	5.0	10.1	31.4	18.4	12.2

total IgG levels in the cynomolgus monkey average 15 mg/ml (Shearer et al., 1999). Specific antibody, in 10% serum, could be detected in the range of 0.2 µg/ml, representing about 0.02% of total serum IgG.

Assay Precision and Analyte Stability

The reliability of the IgGAME assay was tested by measuring a single hyper-immune bleed over 4 days and comparing median RFU signals. Intra- and inter-assay precision were generally observed to be below 10%, with the exception of IgM which showed higher variability (Table 3). The development

Table 4: Stability of IgGAME measurements of day 166, Raptiva hyper-immune cynomolgus monkey serum during –70°C thaws and 4°C storage.

Day 166 Serum (1/10) IgGAME data: analyte stability						
Storage		IgG	IgA	IgM	IgE	Total
1 × thaw	RFU	3837	445	86	368	4945
	Intra-%CV	2.0	7.8	1.6	1.5	1.1
	% difference	0	0	0	0	0
2 × thaw	RFU	3880	436	93	383	5173
	Intra-%CV	1.2	2.3	0.8	4.8	2.0
	% difference	1.1	– 2.0	8.1	4.1	4.6
3 × thaw	RFU	3849	462	89	417	5173
	Intra-%CV	1.1	2.6	7.2	0.5	2.5
	% difference	0.3	3.8	3.5	13.3	7.9
4°C	RFU	4196	978	177	87	11590
4 weeks	Intra-%CV	3.0	3.0	8.5	3.9	0.0
	% difference	9.4	119.8	105.8	– 76.4	134.4

of this assay provided a good means of testing the stability of the various anti-Raptiva antibody isotypes during freeze thaws or at 4°C (Table 4). Here, the % difference in the median RFU signal was compared using the median RFU signal from a freshly thawed aliquot. Stability of signal was excellent for all isotypes over three –70°C freeze and room temperature thaw cycles. Storage at 4°C, was acceptable for 1–2 days, but storage beyond 2 days led to over-recovery of the IgA and IgM isotypes.

Time Course of Anti-Therapeutic IgGAME and Total Antibodies

The appearance of anti-therapeutic antibodies in the cynomolgus monkey hyper-immunized with Raptiva was monitored over time using the multiplex IgGAME and total antibody assays (Figure 5). Serum was measured at a 1/10 dilution using time points from day 14 to day 489, and data was reported in median RFU. Total and IgG specific antibodies were measured from day 14 and increased to a peak level at day 166. Anti-drug IgA antibodies appeared after day 28, peaked at day 166 and then decreased. Anti-drug IgM and IgE levels were first detected at day 166 and continued to increase over time. The absence of a measured primary IgM response may be due to pre-exposure of the animal to human IgG, giving rise to a secondary, pre-dominantly IgG early immune response to the humanized IgG therapeutic.

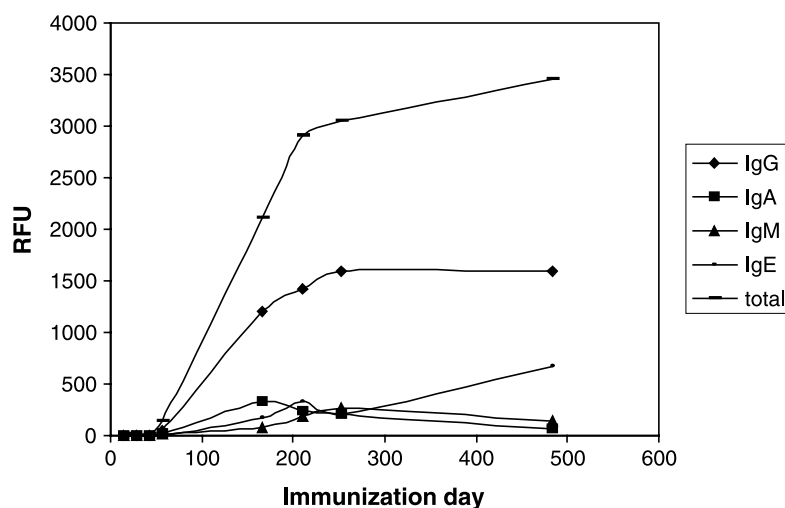


Figure 5: Time course of the anti-Raptiva IgGAME response. Serum from day 14 to day 489 of a cynomolgus monkey hyper-immunized with therapeutic was measured at a 1/10 dilution in the IgGAME and total antibody assays.

Specificity of the Anti-Therapeutic Antibody Response

The specificity of the total and IgE anti-therapeutic antibody response was tested by competing with a panel of recombinant humanized IgG therapeutics (with distinct CDR regions but near identical framework sequences), as well as generic commercial human IgG, and F(ab')₂ molecules.

Results for competitions of the total anti-therapeutic antibody response (using Raptiva both for capture and detection) are shown in Figure 6. The signal in the assay was reduced by 50% by any human IgG, including molecules identical to the immunizing therapeutic outside the CDR region. Human F(ab')₂ alone competed the assay signal by 30%. This data indicates that 50% of the antibody response was directed to framework regions, common to all human IgG molecules, and the remaining 50% would be specific to the idiotype of the Raptiva, largely determined in the CDR regions.

In contrast to specificity of the total antibody response, the anti-therapeutic IgE response (using anti-IgE capture and Raptiva detection) appeared to be directed entirely to framework (Figure 7). The assay signal was

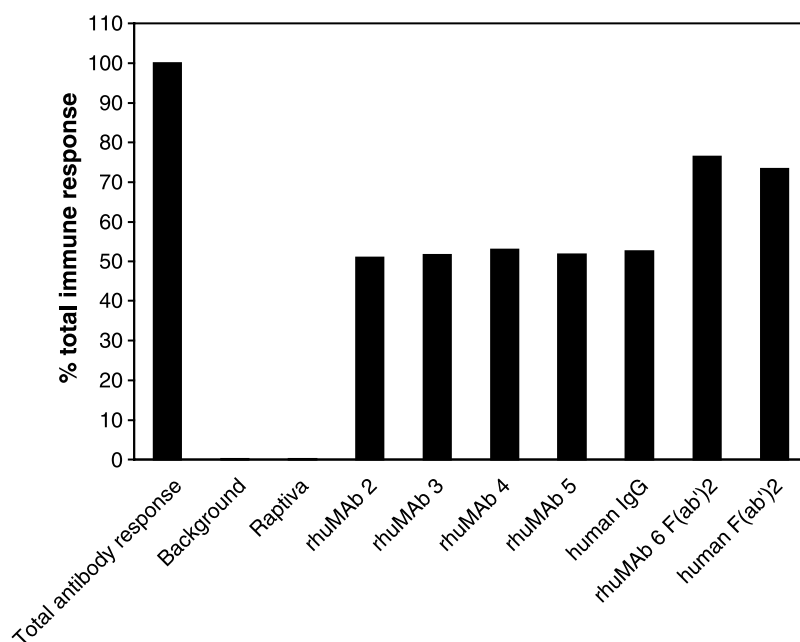


Figure 6: Specificity of the total anti-Raptiva antibody response. A single-plex, bridging format was used to measure the total antibody response, irrespective of isotype, wherein Raptiva was used both on the capture bead and as a detector. Therapeutic-specific total immunoglobulin was reported as a percentage of the day 210 median RFU. Anti-Raptiva hyper-immune serum was pre-incubated for 2 hours at room temperature, at a dilution of 1/10, with 10 µg/mL of the immunizing humanized mAb (Raptiva), other humanized mAbs of differing CDR (rhuMAb2-5), commercially available human IgG or F(ab')₂.

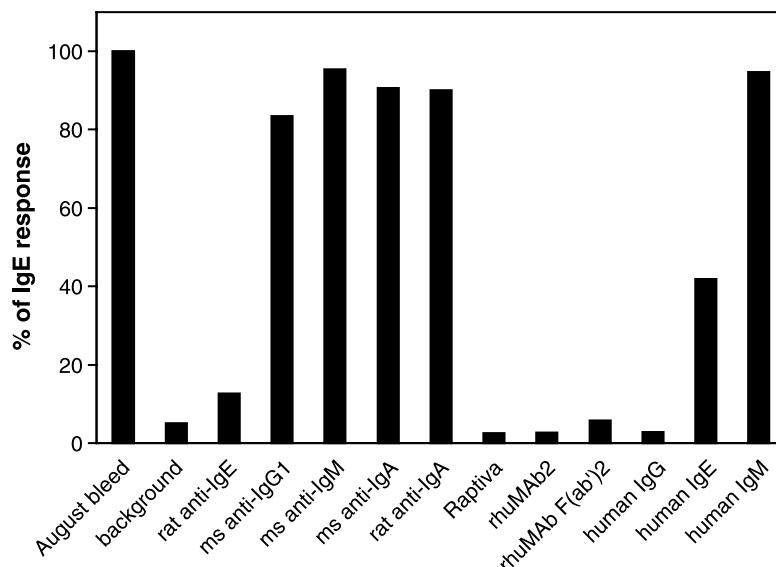


Figure 7: Specificity of the IgE anti-Raptiva antibody response. Total cynomolgus monkey IgE antibodies were captured from August, (day 210), hyper-immune serum, with anti-IgE beads. Therapeutic-specific IgE was reported as a % of the day 210 median RFU, using biotinylated Raptiva followed by Streptavidin-PE. The IgE capture specificity was confirmed using 100 µg/mL of anti-IgG, anti-IgA, anti-IgM, and anti-IgE monoclonal antibodies, incubated with immune serum at a dilution of 1/10 in assay diluent, overnight at room temperature. The IgE framework specificity was shown by pre-incubation of immune serum with 10–12 µg/mL of a several humanized monoclonal IgG or F(ab')₂ antibodies, or commercially available human IgG, IgM, or IgE, for 2 hours at room temperature.

competed to background levels by any human IgG, and to near background by generic F(ab')₂ alone.

The anti-IgE antibody used on the capture bead was a rat monoclonal antibody, while the capture of IgM and IgA were done using mouse monoclonal antibodies. Therefore, competitions were also done to confirm the anti-drug antibodies captured on the anti-IgE beads were not false positive due to the presence of cynomolgus monkey anti-rat antibodies. Only rat anti-IgE and human IgE competed for the antiserum binding to the capture bead (Figure 7). Rat anti-IgA did not compete the signal, nor did mouse anti-IgA, IgG, or IgM. Also, purified human IgE competed while human IgM did not. Human IgG did show competition, but this result reflects the anti-therapeutic framework IgE anti-IgG response.

DISCUSSION

The assay described uses a four-plex approach to characterize the anti-therapeutic antibody response in human and non-human primates. Immunological

reagents were developed with the ability to specifically and simultaneously measure serum anti-therapeutic IgG, IgA, IgM, and IgE antibodies in a 5 μ l sample volume. The assay could be adapted to isotyping antibodies to any antigen of interest by changing only the biotinylated reporter.

Purified anti-therapeutic polyclonal antibody was used to estimate the capture sensitivity in the range of 50 ng/ml for total antibody and 0.2 μ g/ml for specific IgG antibodies. The sensitivity of the total antibody assay is greater, as expected because only antibodies specific to the immunizing therapeutic are captured. In the IgGAME capture, total immunoglobulins are captured, of which a fraction is specific to the therapeutic. Our results indicate that the Protein G capture step is able to detect IgG representing about 0.02% of total serum IgG.

The intended use of existing multiplexing technologies, such as the Luminex system used here, is to specifically capture up to 100 multiple analytes from a complex mixture. The 4-plex assay used in this study, could be readily expanded to a 7-plex assay, including IgG_{1,2,3,4}AME, although the IgG₃ subtype may be absent in the macaque species (Wadhwa et al., 2003). Technology aimed at multiplexing analysis at the detection level, an approach that may improve sensitivity in an antigen-specific isotyping assay, could be developed, but would currently be limited to 4- or 6-color flow cytometric analysis.

Although the assay was developed using hyper-immune serum, we also were able to sensitively measure anti-drug antibodies (IgG) in cynomolgus monkey serum samples dosed with therapeutic at levels (0.05–10 mg/kg) normally used during non-human primate drug safety and efficacy studies (data not shown). Time points in the hyper-immunized cynomolgus monkey were identified containing all anti-drug isotypes. These bleeds would be a potential source of positive controls for qualified assays isotyping anti-therapeutic antibodies in pre-clinical and clinical studies. Measurements of RFU in immune serum exceeding the dynamic range of the assay, can be linearly titrated when diluted in equivalent, naïve serum (data not shown).

The predominant anti-therapeutic antibody isotype measured against humanized IgG monoclonal antibodies, in the cynomolgus monkey, appears to be IgG (unpublished data). In this study, the total antibody response was shown, by competition studies, to be 50% CDR-directed and 50% generic human IgG framework-directed. Interestingly, the anti-therapeutic IgE antibodies were directed exclusively against the humanized IgG framework, and were nearly completely inhibited by generic human F(ab')₂. Consistent with the competition specificity, alignments of sequences for Raptiva, (P_ADF11670, GENESEQ patent database) and cynomolgus monkey (NCBI AAA18103) gamma chains, reveal the hinge region, exposed in the F(ab')₂, to be the framework area of highest inter-species sequence divergence.

The clinical significance of characterizing the isotype of unwanted antibodies generated against a biological therapeutic is not well understood. Having assays in place, able to provide this information, with a minimum use of sample and resources, and in the absence of specific positive controls, has been challenging. The present study illustrates how non-human primate samples collected during pre-clinical studies can be leveraged as controls in human isotyping assays. The application of this approach will provide a deeper understanding of the profile and significance of antibody responses and how they relate to the product, patient status, mode and duration of therapy, and desired clinical response.

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