



Contents lists available at SciVerse ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

Biomarkers for non-human primate Type-I hypersensitivity: Antigen-specific immunoglobulin E assays

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ARTICLE INFO

Article history:

Received 3 November 2012

Received in revised form 14 March 2013

Accepted 18 March 2013

Available online xxxx

Keywords:

IgE

Anti-drug antibody

Immunogenicity

Hypersensitivity

Non-human primate

ABSTRACT

Immunoglobulin E (IgE) is the least abundant immunoglobulin in serum. However, development of an IgE immune response can induce IgE receptor-expressing cells to carry out potent effector functions. A reliable antigen-specific IgE biomarker method for use in non-human primate studies would facilitate (i) confirmation of Type-I hypersensitivity reactions during safety toxicology testing, and (ii) a better understanding of non-human primate models of allergic disease. We cloned and expressed a recombinant cynomolgus monkey IgE molecule in order to screen a panel of commercially available detection reagents raised against human IgE for cross-reactivity. The reagent most reactive to cynomolgus IgE was confirmed to be specific for IgE and did not bind recombinant cynomolgus monkey IgG1–4. A drug-specific IgE assay was developed on the MSD electrochemiluminescent (ECL) platform. The assay is capable of detecting 10 ng/mL drug-specific IgE. Importantly, the assay is able to detect IgE in the presence of excess IgG, the scenario likely to be present in a safety toxicology study. Using our ECL assay, we were able to confirm that serum from cynomolgus monkeys that had experienced clinical symptoms consistent with hypersensitivity responses contained IgE specific for a candidate therapeutic antibody. In addition, a bioassay for mast cell activation was developed using CD34⁺-derived cynomolgus monkey mast cells. This assay confirmed that plasma from animals identified as positive in the drug-specific IgE immunoassay contained biologically active IgE (i.e. could sensitize cultured mast cells), resulting in histamine release after exposure to the therapeutic antibody. These sensitive assays for Type-I hypersensitivity in the NHP can confirm that secondary events are downstream of immunogenicity.

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1. Introduction

IgE is the least abundant serum immunoglobulin (~0.04 µg/mL) in healthy individuals, yet has significant functions in the response to pathogens and toxins (Palm et al., 2012). However, attention to IgE is most-often in the context of allergy, where its essential role has been demonstrated by the success of

the anti-IgE therapeutic omalizumab (Holgate et al., 2005). This work focuses on detection of IgE in immediate hypersensitivity responses to biotherapeutics in the non-human primate (NHP). Administration of a biotherapeutic can result in immunogenicity (an anti-drug antibody (ADA) response in the host) involving IgM, IgG, IgA, and IgE antibody isotypes, and in the context of a toxicology study, has the potential to impact assessment of drug safety and exposure. In nonclinical models, analysis of immunogenicity assists in the interpretation of study results, and should be evaluated when there is “evidence of immune-mediated reactions (immune complex disease, vasculitis, anaphylaxis, etc.)” (International Conference on Harmonisation of Technical

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Requirements for Registration of Pharmaceuticals for Human Use (ICH), 2011; Swanson and Bussiere, 2012).

The physiologic effects of IgE depend on its two receptors. IgE binds each of the three lectin-domains of CD23 with moderate affinity ($K_a \approx 10^8\text{--}10^9\text{ M}^{-1}$), and FcεRI with high affinity ($K_a \approx 10^{10}\text{ M}^{-1}$) (Gould and Sutton, 2008). The 'bent' confirmation of soluble IgE becomes more acute and rigid during FcεRI binding, resulting in dissociation ($k_{\text{off}} \approx 10^{-5}\text{ s}^{-1}$) that is much slower than for IgG:receptor interactions (Holdom et al., 2011). As a result, mast cells and basophils that express high levels of FcεRI are sensitized by IgE production, and are ready to release their granules to initiate an immediate response to antigen-mediated receptor crosslinking. Granule contents include endopeptidases (tryptase), biogenic amines (histamine), lipid mediators (PAF) and cytokines (IL-4). These constituents contribute to both the immediate (bronchoconstriction, vascular leak, intestinal motility) and extended (inflammation and positive Th2 feedback) consequences of basophil and mast cell activation (Gould and Sutton, 2008). In addition, basophils express MHC class II and can capture and present antigens derived from IgE immune complexes to accentuate Th2/IgE responses (Yoshimoto et al., 2009).

Due to the safety risk associated with type I hypersensitivity responses in humans, regulatory agencies may request measures of drug-specific IgE when hypersensitivity-like phenomena occur in the clinic (European Medicines Agency Committee for Medicinal Products for Human Use, 2007; Food and Drug Administration (FDA), 2009). The best-known clinical example of type I hypersensitivity is IgE reactive with the α-Gal carbohydrate component of cetuximab detected in patients who had suffered immediate hypersensitivity reactions (Chung et al., 2008). Unfortunately, symptoms associated with immediate hypersensitivity (including flushing, fainting, and vomiting) can also be due to other causes (European Medicines Agency Committee for Medicinal Products for Human Use, 2012) such as Type-III (immune-complex mediated) hypersensitivity, infusion reactions, tumor-lysis syndrome, and anaphylactoid responses. Anti-infliximab IgE has been found in patients with immediate hypersensitivity reactions in some studies (Vultaggio et al., 2010), but not others (Steenholdt et al., 2011; Steenholdt et al., 2012). This could reflect challenges in detection of drug-specific IgE. The development and application of antigen-specific IgE assays in any species is confounded by: (i) minute serum IgE levels in the presence of excess IgG, and (ii) lack of positive controls of the IgE isotype (Hamilton et al., 2011).

Hypersensitivity responses can also occur during nonclinical drug development. To demonstrate safety of a candidate protein therapeutic, one must show that if hypersensitivity symptoms exist in the non-human primate (NHP) they are an artifact, resulting from the immunogenicity of a human protein administered to non-human test species, rather than target-mediated activities of the candidate drug itself. Support for this hypothesis can come from a panel of NHP hypersensitivity biomarkers (examples include IgE, tryptase, complement, and circulating immune complexes (CIC) (Stubenrauch et al., 2010)).

While there is modest conservation of the IgE constant region among primates; with 84.6% (rhesus) and 83.4% (cynomolgus) identity to human, the absence of purified or recombinant NHP IgE precludes confirmation of the specificity

of reagents and complicates development of NHP assays for IgE. As a consequence, an experimental 'toolkit' for diagnosis of NHP Type-I hypersensitivity is lacking.

To address this issue, we cloned and expressed cynomolgus monkey IgE. The recombinant IgE molecule is targeted against human IgG, and thus serves as a positive control for development of NHP IgE immunogenicity assays supporting antibody therapeutic drugs. We first identified an IgE detection reagent reactive with both human and cynomolgus monkey IgE, then confirmed its specificity by showing lack of reactivity to cynomolgus monkey IgG subtypes. We developed and implemented two assays: one to detect antigen-specific IgE antibodies, and a second to demonstrate that biologically relevant IgE activity is present in plasma. The use of this tiered approach to first identify, then confirm biologic reactivity of anti-drug IgE antibodies allows investigators to generate data that can distinguish whether clinical observations consistent with anaphylaxis are in fact due to Type-I hypersensitivity or a target-mediated/direct pathophysiologic activity of a candidate therapeutic.

2. Materials and Methods

2.1. Cloning of Human and Cynomolgus Monkey IgE

PCR amplification of cynomolgus monkey IgE constant region sequence from bone marrow cDNA (Zyagen, San Diego, CA (USA)) was done using the following primers: 5'-CAGGG CCCATCTGTCTTCCCTTG-3' and 5'-TCATTACCGGGATTACAG AC-3'. These primers are derived from the GenBank sequence JH331133.1, hypothetical protein EGM_20642 partial [*Macaca fascicularis*]. Cynomolgus kappa light chain constant region cDNA was produced at Amgen (Jacobsen et al., 2011). cDNAs of human IgE constant region and kappa light chain constant region were produced at Amgen.

2.2. Cloning and Expression of Positive Control anti-Hs-IgG Human and Cynomolgus Monkey IgEs

The chimeric IgE molecules utilized the CDRs from a mouse monoclonal anti-human IgG (anti-huFc) produced internally at Amgen. The mouse antibody is directed against an epitope on the CH2 domain of human IgG and can thus bind to IgG1, IgG2, and Fc-containing peptibody-based biotherapeutics containing this region, but has no cross-reactivity to mouse, rat, or cynomolgus monkey serum. Construction of His-tagged chimeric anti-huFc-cynoIgE-His₆ and anti-huFc-huIgE-His₆ expression plasmids was done by overlap extension PCR of anti-huFc variable heavy (VH) sequence and the constant sequence of cynomolgus monkey or human IgE heavy chain respectively. Construction of the chimeric anti-huFc-cyno Kappa LC and anti-huFc-human Kappa LC expression plasmids was done by overlap extension PCR of anti-huFc variable Light (VL) sequence and the constant sequence of cynomolgus monkey or human kappa light chain respectively. The chimeric antibodies were expressed in HEK 293-6E cells by co-transfection of the heavy and light chain expression vectors and incubation for 6 days at 37 °C and 5%CO₂. Antibodies were purified using a HisTrap column (GE Healthcare, Piscataway, NJ (USA)). Both the cynomolgus monkey and human purified antibodies were ~98% pure as assessed by size exclusion chromatography. All

positive control antibodies used in the anti-drug antibody immunoassay were generated by Amgen Inc. and are proprietary materials.

2.3. Surface Plasmon Resonance Immunoassay (SPRIA) Characterization of anti-IgE Reagents

The clone names, catalog numbers, and suppliers for anti-IgE reagents are shown in Table 1. Recombinant cynomolgus monkey IgG1-4 was produced at Amgen (Jacobsen et al., 2011). Purified Rhesus IgA was the kind gift of Keith Reimann at the NIH non-human primate resource. Recombinant human IgG1 and IgG2 were produced at Amgen.

To screen commercial anti-human IgE reagents for binding to cynomolgus monkey IgE, human and cynomolgus monkey IgE molecules were coupled via amine chemistry to Biacore CM5 sensor chips on a Biacore 3000 instrument. The aim-for-immobilization feature was utilized, resulting in approximately 7500 Response Units (RU) of each molecule immobilized per surface. Anti-IgE antibody reagents were diluted in HBS-EP buffer (Biacore/GE Healthcare, Uppsala, Sweden) to 5 µg/mL. Sample binding was determined after a one minute exposure at a flow rate of 5 µL/min. Surfaces were regenerated with a 1 min pulse of 50 mM NaOH.

To confirm specificity of the MB10-5C4 clone for IgE, MB10-5C4 was coupled via amine chemistry to Biacore CM5 sensor chips. The aim-for-immobilization feature was utilized, resulting in approximately 6000–7500 RU per surface. Human and cynomolgus monkey immunoglobulins were diluted in HBS-EP buffer to 5 µg/mL. The positive control in this experiment was human hybridoma-derived IgE (Abcam, Cambridge, MA (USA)). Binding was determined after a one minute exposure, at a flow rate of 5 µL/minute. Surfaces were regenerated with a 1 min pulse of Glycine-HCl pH 1.7.

2.4. Immunoassay for Binding of Anti-drug IgG Antibodies

The anti-drug IgG assay utilized an indirect assay format on the MSD platform (Meso Scale Discovery, Gaithersburg, Maryland (USA)), and has been described elsewhere (Bautista et al., 2012). Briefly, a candidate antibody drug is coated on a blank standard-bind MSD plate. The coating solution is 1 µg/mL therapeutic diluted in DPBS. After blocking with KPL milk block solution (KPL Inc., Gaithersburg, Maryland (USA)), study specimens and controls are diluted to 1% in KPL milk block solution, and incubated in the capture plate at ambient room temperature. After washing to remove unbound molecules, a detection step utilizing ruthenium-conjugated anti-cynomolgus

monkey IgG [with no cross-reactivity to human IgG] occurs. After a final wash and addition of MSD 2X t-buffer, the plate is read. Controls for this assay include drug-naïve pooled normal cynomolgus monkey serum (PNCS, Bioreclamation LLC, Westbury, NY (USA)), and two concentrations of a recombinant anti-human cynomolgus monkey IgG1 spiked into PNCS.

2.5. Immunoassay for Binding Anti-drug IgE Antibodies

The anti-drug IgE assay was developed as a modification of the IgG assay. In this case the detection is now a two-step procedure. First, MB10-5C4 diluted in KPL milk-block solution is used to recognize cynomolgus monkey IgE molecules on the capture surface. After washing, a ruthenium-conjugated anti-mouse reagent is used to detect the MB10-5C4. Sequential incubations improved performance of the assay over simultaneous detection (*data not shown*). Controls for this assay include drug-naïve PNCS, and recombinant anti-human cynomolgus monkey IgE spiked into PNCS. To determine the assay sensitivity, a dose-response curve of positive control antibody in PNCS was analyzed, and the quantity of positive control at the assay cutpoint was interpolated. Likewise, drug-tolerance was determined by preparing PNCS containing 0 or 500 ng/mL positive control with various concentrations of drug (15.62 to 1000 µg/mL), and interpolating the resulting inhibition curve at the assay cutpoint.

2.6. Culture of CD34⁺-Derived Cynomolgus Monkey Mast Cells

Cynomolgus monkey CD34⁺ stem cells were cultured for 6 weeks at 37 °C, 5% CO₂, with weekly changes of medium (StemSpan® supplemented with human Stem Cell Factor (SCF, Millipore, Billerica, MA (USA)) and varying cytokines (Lappalainen et al., 2007).

2.7. In-vitro Bioassay for Mast Cell Activation

Differentiated cynomolgus monkey mast cells were washed once with Tyrodes buffer and cell number and viability determined using a Vi-Cell (Beckman-Coulter, Brea, CA (USA)). Mast cells were plated into a 96-well plate at 100,000 cells/well. 75 µL of plasma from study animals was incubated with the mast cells overnight at 37 °C, 5% CO₂. Following overnight incubation with plasma, the mast cells were pelleted (5 min × 300 g) and washed three times with Tyrodes buffer to remove any non-surface bound immunoglobulin. The mast cells were then challenged with 10 µg/mL of the therapeutic antibody for 30 minutes at 37°. The

Table 1
Summary of anti-IgE antibody reagents.

Supplier	Clone	Catalog #	Sub-type	Reactivity with Mf IgE
Miltenyi	MB10-5C4	130-093-127	Mm IgG1	+
MP biomedical	HP6029	634371	Mm IgG2a, k	—
Southern Biotech	B3102E8	9160-01	Mm IgG1, k	—
BD Pharmingen	G7-18	555894	Mm IgG2a, k	—
BD Pharmingen	G7-26	555857	Mm IgG2a, k	—
Biologend	MHE-18	325502	Mm IgG1, k	—
KPL	Goat poly	01-10-04	NA	—/+
Bethyl	Goat poly	A80-108	NA	—/+
Genentech	Omalizumab	NA	Humaniz. IgG1, k	+

positive control was Tyrodes buffer spiked with 10 µg/mL human hybridoma-derived IgE (Abcam, Cambridge, MA (USA)), cross-linked with 5 µg/mL MB10-5C4. After challenge, the plates were placed on ice for 5 minutes to stop degranulation. The plates were centrifuged (5 minutes × 300 g) and the supernatants from all wells collected and assayed for histamine using a quantitative ELISA (Neogen, Lansing, MI (USA)). The plates were read on a SpetraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA (USA)) using Softmax Pro which calculated histamine concentrations from a standard curve generated on each plate.

2.8. Study Design

This exploratory study was in compliance with the Animal Welfare Act, the Guide for the Care and Use of Laboratory Animals, and the Office of Laboratory Animal Welfare. The study used naïve male cynomolgus monkeys of Chinese origin. Two candidate compounds (in several dose groups) or vehicle

control were administered intravenously, once weekly, for 6 weeks. During the course of the study and recovery period, serum and plasma specimens were drawn for antibody and TK analysis respectively. A subset of the toxicokinetic plasma specimens were available for additional analysis in the mast cell bioassay.

3. Results

3.1. Cloning and Expression Strategy

By using primers homologous to the 5' and 3' end of the predicted sequence of the cynomolgus IgE constant region we obtained a sequence by PCR amplification of cynomolgus bone marrow cDNA. The translated protein sequence obtained is ~85% homologous to human IgE (Fig. 1). To screen reagents for reactivity with cynomolgus IgE, and to develop positive controls for cynomolgus monkey and human anti-drug IgE assays, we spliced the variable region of a mouse antibody

50	1	11	21	31	41
CONSENSUS	AS QSP VFP L CCK I S	NATSVTLGCL	ATGYTPEPVM	VTWD GSIN	
Homo sapien	T S	TR N P			T G
Macaca fascicularis	I F	IP H A			A R
100	51	61	71	81	91
CONSENSUS	TMTLPATT	T SGHYATIS	LITVSGAWAK	FTC V HT	PSS D N
Homo sapien	T L	L		QM R A	T WVD K
Macaca fascicularis	S F	P		ET H V	A KEV N
150	101	111	121	131	141
CONSENSUS	TF VCSR FT	PPTVKILOSS	CD GHFPPT	IQLLCL SGY	TPG IN TWL
Homo sapien	S D		GG	V	T I
Macaca fascicularis	G N		DD	I	A V
200	151	161	171	181	191
CONSENSUS	E QQVM V	T TOEGE	LASTOSE TL	QKHNLSDRT	YTCQVYQG
Homo sapien	D D DL S AST		L S		H
Macaca fascicularis	N K NS P PFA		F A		T
250	201	211	221	231	241
CONSENSUS	T DSTKKCA	DSNPRGVSAV	LSRPSPFDLF	I KSPTITCL	WDLAPSK T
Homo sapien	FE			R	G
Macaca fascicularis	YN			S	E
300	251	261	271	281	291
CONSENSUS	VNLTSRASC	KPV H	KQRNGTLTV	TS LPV T D	WIEGETYQCR
Homo sapien		N STIRKE	E	T G R	
Macaca fascicularis		P IPATG K		I V Q	
350	301	311	321	331	341
CONSENSUS	VTHPLPRAL	RS TKTSCP	RAAPEVY FA	TPE SRDK	RTLACLQNF
Homo sapien		M T	A	WFG	
Macaca fascicularis		V M	V	KLE	
400	351	361	371	381	391
CONSENSUS	MPEDISVQWL	H VOLPDAR	HS TOPRKTK	GSQFVFSRL	EVT AEWECK
Homo sapien		NE	T		R
Macaca fascicularis		SD	V		K
401	411	421	428		
CONSENSUS	DEFICRAVHE	AASPS VQ	AVSNPQK		
Homo sapien		QT R			
Macaca fascicularis		WI Q			

Fig. 1. Alignment of immunoglobulin epsilon heavy chain constant sequences from human and cynomolgus monkey.

specific for human IgG (with no reactivity to cynomolgus immunoglobulins) to the human and cynomolgus monkey IgE constant domains.

3.2. Screening antibody reagents for reactivity and specificity to cynomolgus IgE

Reagents for characterization of the human IgE response have been available for some time (Ishizaka et al., 1966), and are being utilized in a broad range of assays (Patelis et al., 2012). However, a robust validation of the specificity of anti-cynomolgus monkey IgE reagents has only been conducted for the therapeutic, omalizumab (Meng et al., 1996). Unfortunately, this is not a reagent easily accessible for immunoassay development. We set out to identify reagents specific for IgE from the cynomolgus macaque (*M. fascicularis*).

A surface plasmon resonance strategy was utilized to screen commercially available reagents for binding with human versus cynomolgus monkey IgE (Fig. 2). While all antibodies reacted with human IgE, only a subset had any binding to the cynomolgus monkey IgE. Omalizumab binding with cynomolgus monkey IgE was also confirmed (data not shown). The single research reagent with good reactivity was clone MB10-5C4. We have also observed that MB10-5C4 is capable of activating cynomolgus monkey basophils ex-vivo, in keeping with a reactivity for cynomolgus monkey IgE captured by Fc ϵ RI (data not shown). The isotype specificity of MB10-5C4 was characterized through an additional SPRIA experiment. High levels of MB10-5C4 were immobilized, and were not reactive with recombinant cynomolgus monkey IgG1, IgG2, IgG3, or IgG4 (Jacobsen et al., 2011). In addition, purified rhesus IgA, human IgG1 and IgG2 were not reactive (Supplemental Fig. 1). Consequently, we are confident that MB10-5C4 is specific for cynomolgus IgE and is well-suited for use in anti-drug IgE immunoassay development.

3.3. Development of an Anti-drug IgE Assay

We developed an anti-drug IgE immunoassay as a variant of an indirect MSD electrochemiluminescent assay to detect anti-drug IgG. A two-step detection method was applied, whereby IgE captured by the therapeutic-coated MSD surface

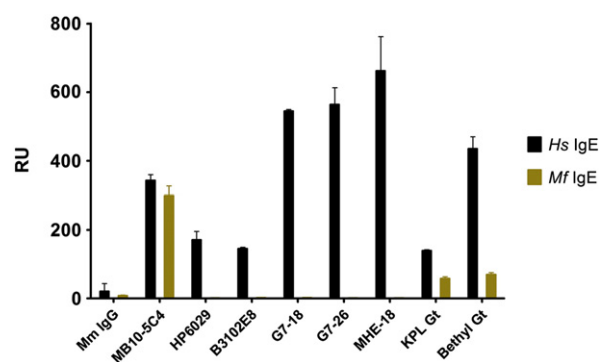


Fig. 2. Reactivity of anti-human IgE reagents with *Macaca fascicularis* IgE. Recombinant human and cynomolgus monkey IgE were immobilized to a Biacore CM5 sensorchip. 5 μ g/mL of the indicated reagents were passed over the Biacore surface (contact time = 1 min). Average reactivity $\pm$ S.D. of two runs over two flow cells is displayed here.

are bound by the murine MB10-5C4 mAb, which is detected by a ruthenium-conjugated anti-mouse antibody. According to a recent industry white paper, the issues most challenging to detection of drug-specific IgE include: (a) absence of good positive controls and (b) assay sensitivity in the presence of a robust IgG response (Hamilton et al., 2011). To characterize the sensitivity of our assay, IgE or IgG anti-drug positive controls were spiked into pooled normal cynomolgus monkey serum (Fig. 3). We found good binding with both human and cynomolgus IgE in pooled serum. The assay sensitivity for cynomolgus IgE was 10 ng/mL. At a moderate anti-drug IgE level (500 ng/mL), the assay was able to tolerate 78 μ g/mL circulating drug (data not shown). This drug tolerance is similar to that observed for the anti-drug IgG assay (173 μ g/mL at 500 ng/mL of anti-drug IgG) (Bautista et al., 2012). More importantly, the assay was able to detect μ g/mL quantities of anti-drug IgE in the presence of high anti-drug IgG (recombinant cynomolgus IgG1 of the same specificity as the IgE was spiked into the assay buffer at levels corresponding to an initial concentration of 1 mg/mL).

3.4. A Case Study with Binding IgG and IgE Analysis

During the course of an exploratory, multiple-dose intravenous study of a candidate human IgG2 antibody therapeutic in cynomolgus monkeys, clinical observations consistent with anaphylaxis were observed immediately after the third through sixth doses in animals from 1, 3, and 15 mg/kg dose groups. Vehicle-treated animals, and animals treated with an alternative candidate antibody (IgG2') did not exhibit symptoms. For all animals and for all subsequent doses, an H1-blocker was pre-administered, which largely ablated the symptoms. To complement an extensive set of experiments designed to demonstrate that the symptoms are not due to direct activities

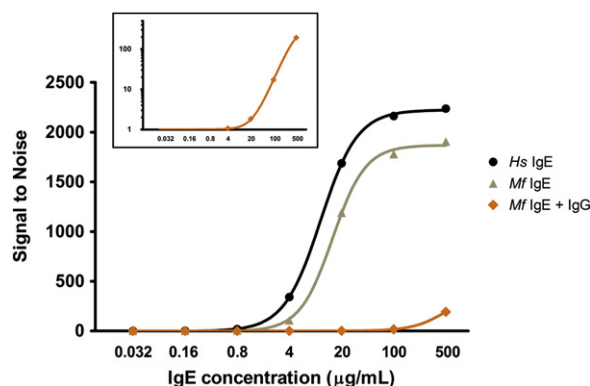


Fig. 3. An indirect MSD electrochemiluminescent assay for anti-drug IgE can detect antigen-specific cynomolgus monkey IgE, even in the presence of a robust IgG response. Human (Hs, circles) and cynomolgus monkey (Mf, triangles) recombinant IgE molecules were spiked into pooled normal cynomolgus monkey serum. An additional series (diamonds) represents cynomolgus monkey IgE spiked into PNCS, where the assay buffer contains 10 μ g/mL recombinant cynomolgus monkey IgG1 of the same specificity as the recombinant IgE molecule [equivalent to a starting concentration of 1 mg/mL IgG]. The inset shows the response of this IgG-spiked condition only. Data are expressed as signal to noise, where experimental specimens are normalized against ECL values from the pooled normal cynomolgus monkey serum. Average reactivity of four independent replicates is displayed here. %CV of all specimens run in the assay was less than 20%.

of the candidate antibody (*data not shown*), we utilized anti-drug IgG and IgE immunoassays to determine whether immunogenicity was a factor in the clinical observations.

As shown in Fig. 4, all animals within the 1, 3, and 15 mg/kg dose groups developed robust anti-drug IgG responses. In contrast, there were animals within the 100 mg/kg dose group that were positive for the presence of binding anti-drug IgG antibodies, but at levels that were significantly reduced compared to lower dose groups. This could be indicative of drug-interference in the anti-drug IgG assay. However, there were only low levels of IgG anti-drug antibodies detected during the recovery phase. High-zone tolerance may be a more likely explanation for the varied response among dose groups. Finally, a second human antibody drug (IgG2') had significantly reduced immunogenicity, and did not predispose towards anaphylaxis.

To determine whether Type-I (IgE-mediated) hypersensitivity was a contributor to the clinical observations, we utilized the anti-drug IgE immunoassay. A study-specific cutpoint (signal:noise = 1.14) for identification of positive specimens was derived by calculating the 99% upper bound of a one-sided distribution of 60 cynomolgus serum specimens (30 naïve donor sera and the 30 study specimens from the pre-study timepoint). As shown in Fig. 5, all five animals in the 1 mg/kg dose group, and 3 animals in the 3 mg/kg dose group are positive for development of anti-drug IgE. The magnitude of the drug-specific IgE signal increased in the drug-free recovery period (with the exception of animal no. 14). This may be a reflection of *a*) decreased drug-specific IgG (Fig. 4), and *b*) reduced serum drug levels (i.e. drug-tolerance of the assay).

In order to confirm that the signal from the anti-drug IgE immunoassay was not derived from minute cross-reactivity for cynomolgus monkey IgG, both IgG and IgE immunoassays were performed with exogenous recombinant cynomolgus monkey IgG spiked into the detection buffer(s). While the

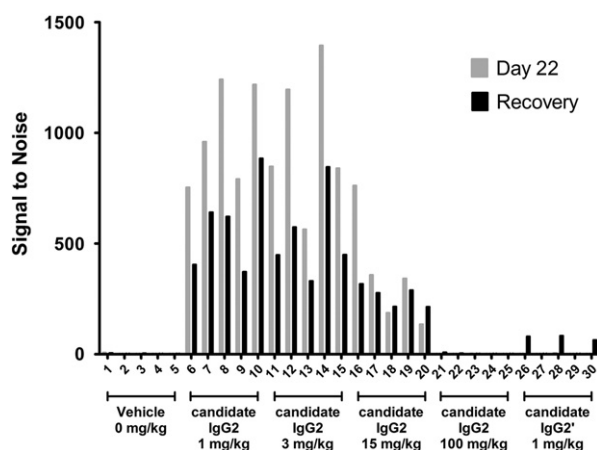


Fig. 4. An indirect MSD electrochemiluminescent assay for anti-drug IgG demonstrates a robust response in low-dose treatment groups. For a repeat dose exploratory toxicity study in cynomolgus monkey, serum specimens were analyzed from day 22 and recovery timepoints for anti-drug IgG. Treatment groups are shown below the x-axis. Data are expressed as signal to noise. Average reactivity of two independent replicates is displayed here. %CV of all specimens run in the assay was less than 20%.

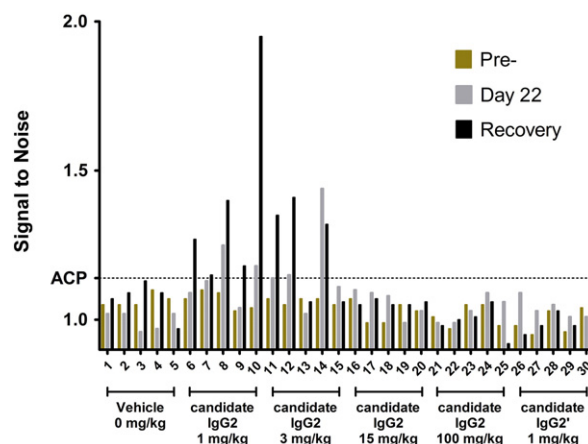


Fig. 5. Anti-drug IgE is present in a subset of animals in the 1 and 3 mg/kg treatment groups. Serum specimens from the pre-study, day 22, and recovery period were analyzed in an indirect MSD electrochemiluminescent assay for anti-drug IgE. An assay cutpoint (ACP) was determined based on the 99% upper-bound of a one-sided distribution derived from values of the 30 pre-study specimens, plus an additional 30 commercial naïve cynomolgus monkey serum specimens (total = 60). Treatment groups are shown below the x-axis. Data are expressed as signal to noise. The assay cutpoint (S/N: 1.14) is indicated as a dotted line. Average reactivity of two independent replicates is displayed here. %CV of all specimens run in the assay was less than 20%.

competition from exogenous IgG ablated drug-specific IgG signal, it had no effect on IgE signal (Supplemental Fig. 2).

Certainly a robust IgG response has the potential to block crosslinking of IgE::FcεRI on cell surfaces, especially when these responses are directed at a limited set of epitopes (Strait et al., 2006). It is possible that administration conditions (i.e. intravenous versus subcutaneous administration) could alter neutralization kinetics, and thereby influence the likelihood of a hypersensitivity response in the presence of both drug-specific IgG and IgE. The IgE immunoassay results and clinical observations in our case study suggest that there is enough drug-specific IgE present to drive mast cell or basophil degranulation after administration of the human antibody drug.

3.5. Differentiation of Cynomolgus Monkey CD34⁺-derived Mast Cells

To confirm that the anti-drug IgE detected in the serum of study animals was biologically active, i.e. capable of binding and cross-linking FcεRI to release mast cell granule contents upon drug challenge, we required non-human primate mast cells. Since there are no available cynomolgus monkey mast cell lines, and primary cynomolgus monkey mast cells are difficult to obtain, we derived mature cynomolgus monkey mast cells from bone marrow CD34⁺ cells. As shown by c-Kit expression (Supplemental Fig. 3) and IHC studies for mast cell granule markers (*manuscript in preparation*), these are phenotypically mature mast cells. In previous studies we have shown that these naïve, stem-cell derived mast cells degranulate and release histamine in response to both chemical agonists (48/80, *data not shown*), as well as IgE-mediated mechanisms (Fig. 6).

3.6. Sensitization of In-vitro Derived Cynomolgus Monkey Mast Cells to a Therapeutic Protein

Cynomolgus monkey mast cells were incubated with the plasma from study animals in the vehicle group and candidate therapeutic 3 mg/kg group. The only study specimens that were available to be analyzed in this assay were re-purposed toxicokinetic bioanalytical plasma specimens from these two groups of animals. Mast cells that were incubated with the plasma from 3/5 animals (animal no. 11, 12, and 14) in group 3 released robust quantities of histamine when challenged with the therapeutic antibody (Fig. 6). Mast cells incubated with plasma from animals in the vehicle group did not release histamine upon challenge with the therapeutic antibody. Importantly, all five animals in the candidate 3 mg/kg group had robust anti-drug IgG responses while only animals 11, 12, and 14 were positive in the anti-drug IgE assay. The mast cell bioassay complements the results of the IgE immunoassay, and confirms that anti-drug IgE was present and biologically active in the plasma of these animals. The ability to cross-link drug-specific IgE:FcεRI complexes (resulting in mast cell activation), may be sensitive to alterations in total IgE levels (either through production levels, or by altered FcεRI expression). As a consequence, it is unclear whether the change in histamine release in the day 15 vs. the day 29 plasma specimens is meaningful without additional data (total IgE and FcεRI expression levels). Finally, the mast cell bioassay demonstrates that the therapeutic antibody itself was not causing mast cells to degranulate (an anaphylactoid response).

4. Discussion

Nonclinical testing of biotherapeutics is an essential component in the drug-development pathway. Often, the 'immunization' of nonclinical species with a human protein results in an

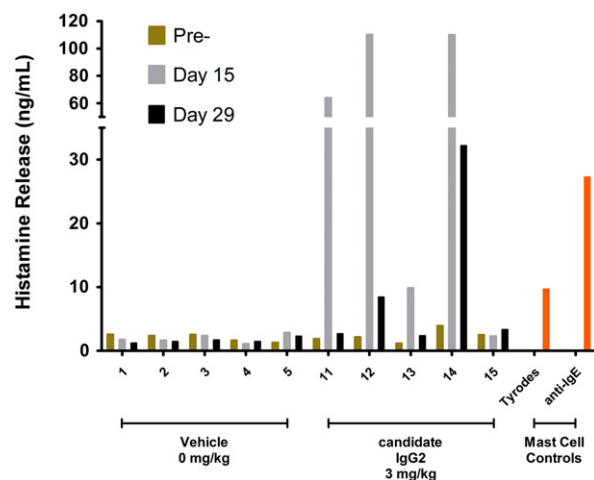


Fig. 6. An in-vitro cynomolgus monkey mast cell bioassay confirms biologic activity of specimens identified as 'IgE-positive.' CD34⁺-derived non-human primate mast cells were incubated overnight with plasma from the indicated animals and timepoints. Following extensive washes to remove unbound immunoglobulins, the mast cells were treated with the candidate therapeutic for 30 min, then histamine levels in the cell culture supernatant were measured by ELISA. Treatment groups are shown below the x-axis. Average histamine levels of two independent replicates are shown here.

anti-drug immune response. There are circumstances (when there is evidence of immune-mediated reactions for example) where the sponsor organization may choose to generate immunogenicity data to support interpretation of study data (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), 2011; Swanson and Bussiere, 2012). While the incidence of nonclinical hypersensitivity may not be high, our case study is one of several recent examples (Flaherty et al., 2012; Rohde et al., 2012). The work presented in this manuscript provides novel tools to confirm the presence and activity of cynomolgus monkey anti-drug IgE antibodies.

Both the IgE immunoassay and bioassay are susceptible to interference (the immunoassay is sensitive to levels of circulating drug and drug-specific IgG, while the bioassay may be sensitive to total IgE levels). Another analyte used to characterize Type-I hypersensitivity in the clinic is serum tryptase (Caughey, 2007). As with all nonclinical biomarkers, careful attention must be given to whether a particular non-human primate tryptase family member is an appropriate functional ortholog for human tryptase (Trivedi et al., 2008). We are developing an immunoassay to quantify cynomolgus monkey delta tryptase levels to corroborate nonclinical observations of hypersensitivity (*manuscript in preparation*). However, Type-I hypersensitivity represents only one immune-mediated path to hypersensitivity. Additional hypersensitivity biomarkers include complement activation (Type-II/III), CIC (Type-III) (Stubenrauch et al., 2010), and immune complex deposits in tissue (Type-III).

Importantly, from a drug development perspective the ability to discriminate among varieties of nonclinical hypersensitivity may not be necessary. In fact, work in the mouse demonstrates that both IgE and IgG can contribute to anaphylaxis-like symptoms (conventional and atypical anaphylaxis respectively) (Jonsson et al., 2011, 2012). There is good evidence that tryptase is a robust biomarker for conventional anaphylaxis (Khodoun et al., 2011), and antigen-specific IgE is a causative agent for Type-I hypersensitivity. However, it is unlikely that any panel of biomarkers will be able to rule out contributions from other 'flavors' of hypersensitivity. We advocate a weight-of-evidence based approach utilizing one or more of the aforementioned biomarkers to demonstrate involvement of an immune-mediated component. Furthermore, anaphylactoid responses may be prime suspects in the absence of data consistent with immune-mediated hypersensitivities, and ex-vivo models such as the CD34⁺-derived mast cells are an important platform to test this possibility.

The IgE immunoassay and mast cell bioassay presented in this manuscript allowed our product development team to conclude that anti-drug immune response contributed to the development of type-I hypersensitivity in an exploratory cynomolgus monkey toxicology study. However, several questions remain: (i) is there a threshold serum level of drug-specific IgE that correlates with pathologic events, and (ii) are there diagnostic changes in total IgE levels? It is clear that for food and seasonal allergies, antigen-specific IgE levels correlate with magnitude of the physiologic response (Benhamou et al., 2008; Rolinck-Werninghaus et al., 2008). Likewise, clinical total IgE levels are generally higher in atopic individuals (Zetterstrom and Johansson, 1981). In our case study, administration of an H1-antagonist precludes us from testing this correlation. Finally,

what were the conditions that 'primed' the immune response towards IgE? The most well-characterized signal upstream of IgE production is the cytokine IL-4, derived from CD4⁺ Th2 T cells (among others) that enables B cell class-switching to IgE (Del Prete et al., 1988; Finkelman et al., 1988). Infection with multicellular pathogens provides additional signals (including IL-25, IL-33, and TSLP) that drive the Th2 response (Paul and Zhu, 2010). It is unclear at present whether any of these effector molecules were induced in our case study.

A prerequisite for our work was isolation of cynomolgus monkey IgE. While a purification approach has proven successful in the presence of a robust anti-IgE reagent (Meng et al., 1996), we relied on generation of a recombinant cynomolgus monkey IgE control. There will always be challenges applying anti-human reagents to non-human primates, and specificity needs to be demonstrated. Our hypothesis was that the ~85% sequence identity between human and cynomolgus IgE sequences would result in significant cross-reactivity among reagents – this was clearly not the case. It is unknown whether there are functional consequences to the divergence in primary sequence between human and cynomolgus IgE constant regions. Our use of human hybridoma-derived IgE to sensitize cynomolgus monkey mast cells (positive control, Fig. 6) suggests that FcεRI expressed by those cells is able to recognize and respond to human IgE, but the magnitude of that response is not nearly as great as observed for cynomolgus polyclonal anti-drug IgEs. Future work will utilize our recombinant positive control molecules to examine this issue.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jim.2013.03.007>.

Disclosure Statement/Funding Information

The authors are employees of Amgen Inc. This work was funded exclusively by Amgen Inc.

Acknowledgments

We thank Keith Reimann and the NIH non-human primate resource for the gift of the purified *Macaca mulatta* IgA; Colin Gegg, Bram Estes, and Mark Guido for technical support; Cindy Afshari, Naren Chirmule and Mark Kroenke for suggestions and comments on the manuscript.

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