Short communication

Comparison of the ability of wild type and stabilized human IgG4 to undergo Fab arm exchange with endogenous IgG4 in vitro and in vivo

Kenneth B. Lewis a,*, Brent Meenga, Kent Bondensgaard d, Lay Chin b, Steven D. Hughes b, Birgitte Kjærd, Søren Lund d, Liping Wang c

a ZymoGenetics, Dept. of Biochemistry, 1201 Eastlake Ave. East, Seattle, WA 98102, USA
b ZymoGenetics, Dept. of Preclinical Development, 1201 Eastlake Ave. East, Seattle, WA 98102, USA
c ZymoGenetics, Dept. of Bioprocess Development, 1201 Eastlake Ave. East, Seattle, WA 98102, USA
d Novo Nordisk A/S, Biopharmaceuticals Research Unit, DK-2760 Måløv, Denmark

Article info

Article history:
Received 7 April 2009
Received in revised form 29 June 2009
Accepted 13 July 2009
Available online 14 August 2009

Keywords:
Human IgG4
IgG4S228P
Anti-IL-31
Fab arm exchange
Monovalent IgG
Monoclonal
In vivo
Cynomolgus
Mouse

A B S T R A C T

Fab arm exchange by a stabilized anti-IL-31 IgG4S228P monoclonal antibody (mAb) was studied using physiologically relevant antibody concentrations and thiol exchange conditions, and directly compared to that of matched wild type IgG4 (IgG4wt) and IgG1 control antibodies. In vitro arm exchange between the test mAbs and a purified IgG4wt exchange partner was monitored using capillary isoelectric focusing and a size-exclusion peak shift assay. Arm exchange between the test mAbs and IgG exchange partners with unknown specificity was monitored using only the shift assay. Studies were performed using single isotype human and mouse mAbs, unfractionated human, mouse, and cynomolgus monkey IgG, and human serum as the sources of the exchange partners. In vitro studies using human serum demonstrated that anti-IL-31 IgG4S228P did not undergo significant Fab arm exchange with endogenous human IgG4 whereas anti-IL-31 IgG4wt underwent rapid and extensive Fab arm exchange. The in vitro results were corroborated by in vivo studies in which mice were injected with a mixture of either form of the test mAb and an excess of non-specific human IgG4 exchange partner.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

Monoclonal IgG4 antibodies have been developed for a number of clinical indications in which effector functions mediated by the Fc region are not desired or necessary. Concurrently, a growing body of evidence demonstrates that human IgG4 molecules readily rearrange in vivo and exchange half molecule, heavy:light chain pairs (Fab arms) with other IgG4 molecules. Numerous studies have also demonstrated the unique lability of the inter-heavy chain disulfide bonds of human IgG4 and the ability of a single point mutation (S228P) in the IgG4 core hinge region to abolish this lability (Angal et al., 1993; Taylor et al., 2006; Schuurman et al., 2001; Virella and Parkhouse, 1973). Therapeutic monoclonal antibodies (mAbs) based on wild type human IgG4 (IgG4wt) would be expected to undergo arm exchange with endogenous IgG4. However, several therapeutic mAbs, including Clenoliximab and the IL-31 targeting IgG4S228P mAb described herein, contain the stabilizing substitution (Reddy et al., 2000; Salfeld, 2007). These are not expected to undergo arm exchange with endogenous IgG4. This study was undertaken to demonstrate the stability of an anti-IL-31 IgG4S228P mAb with respect to Fab arm exchange under physiologically relevant conditions relative to a matched IgG4wt control mAb.

The potential consequences of arm exchange between a therapeutic IgG4 mAb and endogenous IgG4 depend on its intended target. If the target is a monovalent soluble ligand, as for the anti-IL-31 mAb studied here, arm exchange should have little impact on either the pharmacokinetic or the functional properties of the mAb since the antigen binding properties will not change. In contrast, if the target is a cell surface antigen, and bivalent binding (using both Fab arms) is a necessary functional property of the mAb (e.g. to crosslink target molecules or to enhance binding through avidity), then arm exchange may alter both target-dependent clearance and potency. A recent study in rhesus monkeys comparing a matched pair of IgG4wt and IgG1 mAbs targeting the acetylcholine receptor provides an excellent example of this latter behavior (van der Neut Kolfschoten et al., 2007).

Endogenous human IgG4 undergoes Fab arm exchange in vivo resulting in a mixed population of bispecific IgG4 molecules in human serum (Schuurman et al., 1999; Aalberse and Schuurman, 2002). This phenomenon has been replicated both in vitro, in the...
presence of thiol exchange reagents, and in vivo using a pair of monoclonal IgG4wt antibodies with known specificities (van der Neut Kolfschoten et al., 2007). These published results provided the basis for using a matched IgG4wt mAb as the positive control, and a matched IgG1 mAb as the negative control, in this study to confirm that permissive exchange conditions were present both in vitro and in vivo. Since it was not possible to prove the physiological relevance of the in vitro exchange conditions, studies were also performed to assess the stability of the anti-IL-31 IgG4S228P mAb in vivo. A secondary objective of this study was to assess the relevance of the mouse and cynomologus monkey (Macaca fascicularis) as test species for investigating arm exchange in vivo.

In the published reports, pairs of IgG4wt mAbs with known specificity were tested, and the formation of bispecific mAbs resulting from arm exchange was monitored using bridging assay formats. In this study, one goal was to compare the extent of arm exchange of a matched set of anti-IL-31 mAbs with endogenous human IgG4. In this situation only the specificity of the test antibody (anti-IL-31) was known so bridging assay formats could not be used to monitor the extent of reaction. Additionally, since the population of exchange partners was heterogeneous, mAb-specific biochemical measurements were not useful. However, since the bispecific mAbs resulting from arm exchange are also functionally monovalent (with respect to IL-31 binding) a chromatographic shift assay was used to monitor extent of exchange based on the functional valency of the test mAbs. This assay was developed to tolerate complex sample matrices such as serum with minimal pre-analytical processing and to be sensitive enough to characterize therapeutically relevant concentrations of test mAbs.

2. Materials and methods

2.1. IL-31 specific and non-specific monoclonal antibodies

The cDNA for full-length anti-IL-31 human IgG4S228P antibody was synthesized at Geneart (Regensburg, Germany) and then subcloned into the pTT plasmid (Durocher et al., 2002). The heavy and light chains were subcloned to separate pTT plasmids. In a subsequent step wildtype human IgG4 was made according to the “QuickChange site-directed mutagenesis protocol” from Stratagen (AH Diagnostics A/S, Århus, Denmark). A non-depleting human IgG1 isotype containing several point mutations in the CH2 domain was also purchased from Geneart. Antibodies were expressed using HEK293 6E cells (WO 2006/096989 A2), purified using a Mabselect (GE Healthcare) column, and buffer exchanged into 25 mM phosphate pH 7.2, 0.1% PEG (PBS) (Fig. 5 and Supplemental Fig. 1), or a Sephadex 200 10/300 mm column. For use in the serum SEC shift assay, the PEG-IL-31 was additionally conjugated with amine reactive Alexa Fluor 647 (A647, Invitrogen) following the recommended protocol. The homogeneity of the PEG-IL-31 antigen preparations was confirmed using analytical SEC and concentrations were measured based on an extinction coefficient of 0.2 L/g cm.

2.2. Human, mouse, and cynomolgus monkey immunoglobulins and serum

Human myeloma IgG1, IgG2, IgG3, and IgG4 were obtained from Calbiochem (San Diego, CA). Mouse IgG1, IgG2a, IgG2b, and IgG3 were obtained from Southern Biotech (Birmingham, AL). Unfractionated human, mouse, and cynomolagus monkey IgG were obtained from Antibodies Incorporated (Davis, CA) and Rockland Immunochemicals (Gilbertsville, PA). The homogeneity of the antibody preparations was assessed using analytical SEC and concentrations were measured based on a nominal extinction coefficient of 1.4 L/g cm. As-received, many of the unfractionated IgG and specific isotype preparations contained significant amounts of aggregated protein and variable endotoxin levels. The impurities did not interfere with the arm exchange reaction, and no additionally processing was performed. Pooled human serum was obtained from in house donors (ZymoGenetics) and stored at −80 °C until use.

2.3. IL-31 antigen

IL-31 was produced in E. coli and refolded using standard dilution refolding techniques. Amino-terminal PEGylation was performed at pH 5.5, using NaCNBH3 and monomethoxy PEG-propionaldehyde, 20 kDa, (NOF, Japan). The PEG-IL-31 was purified by anion exchange chromatography using a Poros 50 HQ column. For use in the serum SEC shift assay, the PEG-IL-31 was additionally conjugated with amine reactive Alexa Fluor 647 (A647, Invitrogen) following the recommended protocol. The homogeneity of the PEG-IL-31 antigen preparations was confirmed using analytical SEC and concentrations were measured based on an extinction coefficient of 3 kV for 10 min. Electropherograms were aligned manually.

2.4. Isoelectric focusing

Imaged capillary isoelectric focusing (cIEF) was performed using an iCE280 Analyzer (Convergent Bioscience, Toronto, Canada). The separation cartridge, containing a 5 cm long, 100 μm i.d. × 200 μm o.d. separation capillary, was purchased from Convergent Bioscience. The anolyte was 80 mM phosphoric acid and the catholyte was 100 mM NaOH. A mixture of Pharmalyte pH 5–8 and 3–10, at a ratio of 4:1, was used as the carrier ampholyte. Urea (6 M) and methylcellulose (0.35%) were added to the sample matrix. Focusing was performed at 3 kV for 10 min. Electropherograms were aligned manually.

2.5. Size exclusion peak shift assay

The size exclusion based peak shift (SEC shift) assay was performed using three different columns: running buffer combinations: a TSK 3000SWxl 7.8 mm × 30 cm 5 μm column (Tosoh Bioscience) running at a flow rate of 0.8 mL/min with 100 mM (NH4)2SO4, 25 mM sodium phosphate, pH 7.2, 0.1% PEG-3350 (ICN) (Fig. 1), a Sephadex 200 10/300 mm column running at a flow rate of 0.5 mL/min with the same running buffer (Figs. 2–4 and Supplemental Fig. 1), or a Sephadex 200 10/300 mm column running at 0.5 mL/min with 100 mM NaCl, 25 mM sodium phosphate pH 7.2, 0.1% PEG (PBS) (Fig. 5 and Supplemental Fig. 2). The chromatographic performance was comparable with all three combinations, however the Sephadex 200:PBS combination was less prone to fouling by hemolyzed serum samples. The SEC shift assay characterized the valency of the IL-31 specific mAbs and resolved monovalent complexes (1 mAb + 1 A647-PEG-IL-31), bivalent complexes (1 mAb + 2 A647-PEG-IL-31), and uncomplexed A647-PEG-IL-31. The identity and elution position of the mAb:A647-PEG-IL-31 complexes was verified by titrating the binding sites of the parent mAb with A647-PEG-IL-31 (Supplemental Fig. 1). Shifts in peak position were initially monitored using the absorbance of the mAbs at 280 nm (Fig. 1). Subsequently, the detection wavelength was changed to 650 nm to increase sensitivity and minimize interference from serum proteins (Figs. 2–6; Supplemental Fig. 2).

2.6. In vitro studies

Unless otherwise specified, samples were prepared as matched pairs with and without the addition of reduced glutathione (ICN) to a final concentration of 0.5 mM and incubated for 8–20 h at 37 °C.
Fig. 1. (A) Capillary IEF electropherograms of anti-IL-31 IgG4wt, IgG4S228P, and IgG1 mAbs (140 μg/mL) incubated 20 hr at 37 °C with an equal concentration of non-specific recombinant human IgG4wt with (−) and without (----) 0.5 mM reduced glutathione. The pI range of the test mAbs is 7.8–8.3 for the anti IL-31 IgG4wt and IgG4S228P, and ~9.0 for the anti IL-31 IgG1. The pI range of the non-specific recombinant human IgG4wt is 6.3–7.0. The electropherograms were aligned manually. They are plotted using the same absorbance scale and offset for clarity. (B) Size exclusion chromatograms of the same samples using a detection wavelength of 280 nm. Uncomplexed mAb elutes at 8.6 min, uncomplexed PEG-IL-31 elutes at 7.7 min, monovalent complexes (1 mAb + PEG-IL-31) elute at 6.9 min, bivalent complexes (1 mAb + 2 PEG-IL-31) elute at 6.3 min. Prior to the chromatography the samples were mixed with a 10-fold molar excess of A647-PEG-IL-31. The chromatograms are plotted using the same absorbance scale and offset for clarity.

Fig. 2. Size exclusion chromatograms of (A) anti-IL-31 IgG4wt, IgG4S228P, and IgG1 mAbs (60 μg/mL) incubated for 18 h at 37 °C in human serum and (B) anti-IL-31 IgG4wt mAb (60 μg/mL) incubated for different times at 37 °C in human serum with (−) and without (----) 0.5 mM reduced glutathione. Prior to the chromatography the samples were mixed with a 10-fold molar excess of A647-PEG-IL-31.
Fig. 3. Size exclusion chromatograms of anti-IL-31 IgG4wt mAb (60 μg/mL) incubated 20 h at 37 °C with mouse, cynomolgus monkey, or human unfractionated IgG (10 mg/mL) with (−) and without (…) 0.5 mM reduced glutathione. Prior to the chromatography the samples were mixed with a 10-fold molar excess of A647-PEG-IL-31.

Fig. 4. Size exclusion chromatograms of anti-IL-31 IgG4wt mAb (60 μg/mL) incubated 20 h at 37 °C with a 5-fold molar excess of single isotype (A) human and (B) mouse IgG with (−) and without (…) 0.5 mM reduced glutathione. Prior to the chromatography the samples were mixed with a 10-fold molar excess of A647-PEG-IL-31.

Fig. 5. (A) Size exclusion chromatograms of serum samples from mice injected i.v. with 160 μg of anti-IL-31 IgG4S228P (−) or anti-IL-31 IgG4wt (…) 3 days following an i.p. injection of 1000 μg of non-specific human IgG4. Prior to the chromatography serum samples were mixed with an excess of A647-PEG-IL-31. (B) Plot of the sum of the peak areas of the monovalent and bivalent mAb:A647-PEG-IL-31 complexes vs. time.
No titration of the glutathione was performed and no attempt was made to limit oxygen exposure or to chelate metal ions. The pre-existing concentrations of low molecular weight thiols and mixed disulfide molecules in the serum samples were not measured. Samples for Fig. 1 were prepared with a final concentration for each test mAb of 140 μg/ml (0.9 μM). Otherwise, samples were prepared with a final concentration for each test mAb of 60 μg/ml (0.4 μM). In samples containing myeloma IgG as the exchange partner, the myeloma IgG was added at a 5-fold molar excess. In samples containing unfractionated IgG, the IgG was concentrated to ~10 mg/ml to approximate the concentration of total IgG in serum. In samples containing human serum, the test mixtures contained ~70% serum due to dilution accompanying the addition of the test antibodies. After incubation, samples were prepared for analysis by addition of N-ethylmaleimide (NEM; IBC) to a final concentration of 5 mM to inactivate potentially reactive thiol groups and prevent artifacts arising from thiol exchange during sample handling (Taylor et al., 2006). Samples were kept at 4 °C until analysis. Samples for the SEC shift assay were mixed with an equal volume of A647-PEG-IL-31 solution (62 μg/ml, 4 μM), and filtered using a pre-washed 0.2 μm Spin-X filter (Corning) prior to injection. The standard injection volume was 100 μL. The chromatograms were plotted using the same absorbance scale and offset for clarity.

2.7. In vivo studies

Two studies were conducted using BALB/c mice. Procedures involving animals were approved by the ZymoGenetics Institutional Animal Care and Use Committee. In the first study, two mice each were injected i.p. with 1000 μg of non-specific recombinant human IgG4 wt exchange partner. Three days later, they were injected i.v. with 160 μg test antibody (either anti-IL-31 IgG4S228P or IgG4 wt). Additionally, one mouse each was injected i.v. only with the test mAbs. For all survival bleeds, the target volume for the blood draw was 100 μL to yield 50 μL of serum. For the mice receiving the test mAbs in addition to the exogenous exchange partner, blood collection was staggered between the two mice to obtain serum immediately following injection and at 6 h, 2 days, 5 days, 8 days (terminal), and 15 days (terminal). For the mice receiving only the test mAbs, blood was collected on days 3 and 8 (terminal). Serum samples were frozen within an hour of blood collection and stored at ~80 °C prior to analysis. For analysis, serum samples were thawed at 37 °C for 10 min prior to staging at 4 °C. NEM was added to a final concentration of 5 mM, and the samples were mixed with an equal volume of PBS. Subsequently the serum samples were prepared for SEC shift analysis as described previously.

3. Results

The first experiment compared the extent of arm exchange between the test mAbs and a non-specific recombinant human IgG4 wt, and samples were analyzed using both cIEF and the SEC-shift assay (Fig. 1A and B). The cIEF demonstrated that arm exchange resulted in the formation of a new molecular species. The pI range of the non-specific IgG4 wt was 6.3–7.0. The pI range of the test result resulted in the formation of a new molecular species. The pI range were injected involving animals were approved by the ZymoGenetics Institutional shift assay were mixed with an equal volume of A647-PEG-IL-31 anti-IL-31 IgG4wt was incubated with the exchange partner and glutathione, the electropheretic profile showed only the two reacting mAbs. In contrast, when the anti-IL-31 IgG4 wt was incubated with the exchange partner and glutathione, a new cluster of peaks was observed in the pI 7.3–7.8 range corresponding to the expected pI of an IgG4 composed of 1 Fab arm from each reacting mAb (Fig. 1A). When the same samples were analyzed using the SEC shift assay (detection at 280 nm) (Fig. 1B), peaks at 8.6 min (uncomplexed mAb), 7.7 min (excess PEG-IL-31), and 6.3 min (bivalent complex: 1 mAb + 2 PEG-IL-31) were present in the chromatograms of all samples. When anti-IL-31 IgG4 wt was incubated with the exchange partner and glutathione, an additional peak at 6.9 min was observed. This was attributed to monovalent complexes (1 mAb + 1 PEG-IL-31). Consistent with published reports, no evidence of increased half-molecule formation was observed by non-reducing SDS-PAGE (results not shown) in any samples (Taylor et al., 2006).

A similar experiment was performed to compare the extent of arm exchange between the test antibodies and endogenous IgG4 in human serum. In accordance with the previous results, arm exchange was not observed in the samples containing either anti-IL-31 IgG4S228P or IgG1, or in samples incubated without glutathione. In contrast, exchange between anti-IL-31 IgG4 wt and endogenous IgG4 in human serum was clearly observed (Fig. 2A). Peaks at 26 min (excess A647-PEG-IL-31) and 20 min (bivalent complex) were present in the chromatograms of all samples (A650 was used for detection and uncomplexed mAb was not detected). When anti-IL-31 IgG4 wt was incubated in serum with glutathione, an additional peak at 22.5 min (monovalent complex) was observed. In a parallel experiment, anti-IL-31 IgG4 wt was incubated in serum and the reaction was stopped at various time points by the addition of 5 mM NEM (Fig. 2B). Significant exchange was observed as early as 1.5 h. At 22 h, approximately 80% of the test mAb was exchanged based on the peak areas of the 20 min and 22.5 min peaks (accounting for the difference in their A647 content). Shoulders at 23 min and 30 min and a peak at the void volume of the column ~17 min were present to varying degrees in the chromatograms of all samples, including the serum blanks. These peaks are not due to the anti-IL-31 mAbs.

The exchange of anti-IL-31 IgG4 wt mAb with endogenous IgG in unfractionated human, cynomolgus monkey and mouse IgG preparations was also investigated. Extensive exchange was clearly evident between the anti-IL-31 IgG4 wt mAb and all of the IgG preparations. The formation of a monovalent complex peak at 22.5 min was observed for all of the samples incubated with glutathione (Fig. 3). The monovalent complex formed as a result of exchange with mouse IgG consistently eluted later than the complexes formed as a result of exchange with human and cynomolgus monkey IgG. Human and mouse preparations from two different vendors were tested and the extent of exchange varied between them, particularly the human IgG preparations. Subsequent experiments were performed using single isotype preparations to identify the exchange partner in the unfractionated human and mouse IgG preparations. The formation of a monovalent complex was clearly evident after anti-IL-31 IgG4 wt incubation with human IgG4 and mouse IgG3 (Fig. 4A and B). A very small amount of monovalent complex was also observed after incubation with human IgG3. No single isotype preparations were available for cynomolgus monkey.

In vivo studies were performed to corroborate the results of the in vitro experiments. Comparison of the chromatograms of serum from the mice injected with exogenous non-specific human IgG4 wt followed by the anti-IL-31 mAbs taken immediately after injection and at 6 h, 2, 5, 8, and 16 days clearly showed the time-dependent formation of monovalent IgG4 in the serum from mice injected with anti-IL-31 IgG4 wt but not from mice injected with IgG4S228P (Fig. 5A). Exchange by the anti-IL-31 IgG4 wt was clearly evident at 6 h and the extent of exchange was similar at 2 and 5 days. The peak areas of the monovalent and bivalent mAb:A647-PEG-IL-31 complexes decreased rapidly and at 8 and 15 days were too small relative to the non-specific interfering peaks to quantify reliably. The sum of the peak areas of the mAb:A647-PEG-IL-31 complexes vs. time was plotted to estimate a circulating half-life of 1.2–1.6 days for the test mAbs in mice (Fig. 5B). The serum quality was variable and
peaks were observed at the void volume of the column (16 min) and ~32 min for many samples. These peaks were also present in the serum blank samples and were not due to the anti–IL-31 mAbs. The 32 min peak correlated with the degree of hemolysis. Note that the 6 h SEC sample contained a 10-fold rather than a 10-fold excess of A647-PEG-IL–31. No evidence for significant exchange by anti–IL-31 IgG4wt228P was observed. Serum samples from the mice injected with only anti–IL-31 IgG4wt or IgG4S228P showed no evidence of significant arm exchange at either 3 days or 8 days (Supplemental Fig. 2).

4. Discussion

Fab arm exchange produces antibodies that are both bispecific and monovalent with respect to single antigen binding. Either functional property can be used to monitor arm exchange, and it is assumed that the assays are measuring the properties of a properly formed, arm-exchanged IgG molecule. The SEC shift assay assesses the functional valency of the anti–IL-31 test mAbs. Using homogeneous mAb preparations and an IgG4wt exchange partner with a pi different from the pi of the test mAbs, cIEF was used to demonstrate that arm exchange resulted in the formation of a new molecular species with an intermediate pi. New cIEF peaks were observed only with the sample containing anti–IL-31 IgG4wt, and only when the mAbs were incubated with glutathione. The appearance of the new peaks in the electropherograms corresponded to the appearance of a peak attributed to monovalent (1 mAb + 1 PEG–IL-31) complexes in the SEC shift assay (Fig. 1A and B). A similar experiment using human myeloma IgG4 as the exchange partner, demonstrated similar behavior (results not shown). In a similar manner, van der Neut Kolfshoten et al. (2007) used mass-spectrometry to demonstrate that a new molecular species was forming in samples that also exhibited bispecific binding in a bridging assay. The extent of exchange was estimated from the change in peak areas of the cIEF electrophorograms (32%) and the SEC shift chromatograms (~37%).

In addition to being formed when arm-exchanged mAb was mixed with a saturating concentration of antigen, monovalent complexes were also formed by sequentially titrating the two binding sites of the un-exchanged mAb with sub-saturating concentrations of antigen (Supplemental Fig. 1). The monovalent and bivalent complexes produced by either method eluted at the same position. The routine SEC shift assay employs a 10-fold excess of antigen to ensure complete complex formation. Although arm exchange was readily observed using the SEC shift assay, varying degrees of matrix interference were observed with the serum samples in spite using A650 for detection. A small trailing shoulder on the bivalent complex peak and peaks at the void volume of the column were present to varying degrees in the chromatograms of test samples and serum blanks. These peaks did not correlate with presence of anti–IL-31 mAbs but added uncertainty to the measurement of peak areas, particularly when the proportion of monovalent complexes was low.

In addition to demonstrating a correlation between the results of the cIEF and the SEC shift assays, the in vitro experiments clearly demonstrated that the S228P replacement significantly stabilized the anti–IL-31 IgG4S228P with respect to Fab arm exchange (Fig. 1A and B). The same behavior was observed when the experiment was repeated using human serum as the source of endogenous exchange partner (Fig. 2A). Both experiments demonstrated that the in vitro thiol exchange conditions allowed arm exchange by IgG4wt to occur, but were not extreme enough to induce changes in IgG4. The serum experiments also demonstrated that the in vitro exchange conditions would not induce non-specific exchange with other serum proteins. Additionally, the kinetic experiment demonstrated that, when sufficient exchange partner was available, arm exchange of anti–IL-31 IgG4wt control occurred rapidly and that overnight incubation was sufficient for the exchange reaction to approach completion (Fig. 2B).

The concentrations of the exchanging antibodies will affect both the rate and extent of arm exchange. The serum concentration of therapeutic mAbs may reach several hundred μg/mL and will vary based on the dosing schedule and the pharmacokinetic properties of the mAb. The concentration of endogenous human IgG4 ranges from 0.2 to 1.2 mg/mL (Miles and Riches, 1994; Hamilton, 1987) and may also vary due to disease or prolonged exposure to various antigens. Consequently, circumstances may arise in which either a therapeutic mAb, or endogenous IgG4 will drive, or limit, the extent of arm exchange. For the majority of the in vitro experiments reported herein, 60 μg/mL test mAb and a 1–5-fold excess of exchange partner was used. This should reflect a physiologically relevant concentration of the test mAbs while simultaneously maintaining sufficient excess of exchange partner to allow the Fab arm exchange reaction to near completion. The unfraccionated human, mouse, and cynomolgus IgG preparations were concentrated to 10 mg/mL to approximate the concentration of total IgG in serum, however the concentration of minor immunoglobulin subfractions (e.g. human IgG4 and mouse IgG3) was not measured. The differences in the extent of exchange between preparations from different vendors may reflect differences in their concentration of exchange partner (Fig. 3).

To evaluate the potential relevance of cynomolgus monkey and mouse for in vivo studies, mixtures of anti–IL-31 IgG4wt mAb and unfraccionated IgG preparations were tested to determine whether human IgG4wt would exchange with endogenous IgG4 from either species. Unfraccionated human IgG4 was used as the positive control. Arm exchange was observed with all of the unfraccionated IgG preparations (Fig. 3). Subsequent single isotype experiments identified IgG3 as the mouse exchange partner and IgG4 as the human exchange partner. The trace amount of exchange observed with human IgG3 (Fig. 4A) is probably not physiologically relevant and may indicate that human IgG3 is susceptible to non-specific thiolphilic attack by the IgG4wt under the in vitro exchange conditions. Although anticipated, the results from the single human isotype experiments in conjunction with the serum experiments confirmed that there were no significant unidentified exchange partners for IgG4wt in human serum. A cynomolgus IgG4 ortholog has not been reported and the exchange between anti–IL-31 IgG4wt and unfractionated cynomolgus IgG was unanticipated. However, exchange between human IgG4wt mAb and an unidentified partner in rhesus monkey serum has been reported elsewhere (van der Neut Kolfshoten et al., 2007). Consequently cynomolgus monkey may be a relevant animal species for evaluating the effects of Fab arm exchange on the pharmacokinetic and pharmacodynamic properties of therapeutic human IgG4 mAbs.

Mice have been used for many studies involving human IgG4 and inter-species arm exchange has not been reported. Consequently, the exchange between anti–IL-31 IgG4wt and mouse IgG3 was also unanticipated. One can speculate that endogenous mouse IgG3 may also form bispecic molecules. Fab arm exchange is one possible explanation for the intermolecular cooperativity of mouse IgG3 described by Greenspan and Cooper (1992) and the similar behavior of mouse IgG3 and human IgG4 in binding assays (Greenspan and Cooper, 1992; Rispens et al., 2009). Exchange between human IgG4wt and mouse IgG3 may not be physiologically relevant, but it potentially complicates the interpretation of in vivo studies involving human IgG4. Based on the in vitro experiments, in which an excess of mouse IgG3 was present, extensive in vivo exchange between anti–IL-31 IgG4wt and endogenous mouse IgG3 was anticipated. However, no significant arm exchange was observed in the control in vivo experiment in which either anti–IL-31 IgG4S228P or anti–IL-31 IgG4wt was injected into mice without an exogenous exchange partner (Supplemental Fig. 2). It is probable that the
endogenous IgG3 was limiting in this animal model. In-house measurements (results not shown) and those of Lebrun and Spiegelberg (1987) indicate that serum IgG3 concentrations are low in BALB/c mice.

The control experiment confirmed that exchange between the test mAbs and mouse IgG1 would not interfere with the experiment in which anti-IL-31 IgG4S228P or IgG4wt were injected into mice with an exogenous non-specific human IgG4wt. In this experiment, no significant arm exchange by the anti-IL-31 IgG4S228P was observed. In contrast, SEC shift analysis clearly demonstrated a time-dependent formation of monovalent IgG4 by anti-IL-31 IgG4wt (Fig. 5A). This behavior was consistent with the results of the in vitro experiments. The exchange appeared to be nearly complete by 2 days, a rate of in vivo exchange that is consistent with that reported by van der Neut Kolfschoten et al. (2007). In addition to measuring the proportions of mono- and bispecific IgG4 in the samples, the SEC shift assay also provided a measure of the total serum concentration of the test mAbs. In this experiment, a circulating half-life of 1.2–1.6 days for both test mAbs was observed (Fig. 5B). Similar pharmacokinetic behavior has been reported elsewhere for human IgG4 in mice (He et al., 1998; Colcher et al., 1989; Zuckier et al., 1998). Since the concentration of the test mAbs decreased at a rate comparable to that of the arm exchange, a rate constant for in vivo Fab arm exchange was not determined. Even so, the increased stability of the anti-IL-31 IgG4S228P relative to the anti-IL-31 IgG4wt was significant.

5. Conclusions

Anti-IL-31 IgG4S228P did not undergo significant Fab arm exchange with either non-specific purified human IgG4 or endogenous IgG4 in human serum when incubated under in vitro thiol exchange conditions that allowed rapid and extensive Fab arm exchange by anti-IL-31 IgG4wt. Arm exchange by anti-IL-31 IgG4wt was observed as early as 1.5 h and was nearly complete after 22 h. In conjunction with the serum experiments, single isotype experiments confirmed that the relevant exchange partner in human serum is endogenous human IgG4. In vivo experiments in which mice were injected with a mixture of either form of the anti-IL-31 mAb and an exogenous human IgG4 exchange partner corroborated the in vitro results. No significant arm exchange by anti-IL-31 IgG4S228P was observed in contrast to rapid and extensive arm exchange by anti-IL-31 IgG4wt. Anti-IL-31 IgG4wt also underwent arm exchange in vitro with mouse IgG3 and an unidentified IgG from cynomolgus monkeys.

Acknowledgements

The authors would like to thank Hong Liu and Brian Reardon for preparing the conjugated PEG-IL-31 reagents used in the SEC assay.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2009.07.009.

References