Impact of Molecular Processing in the Hinge Region of Therapeutic IgG4 Antibodies on Disposition Profiles in Cynomolgus Monkeys

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ABSTRACT:

The IgG4 isotype antibody is a potential candidate for immunotherapy when reduced effector functions are desirable. However, antigen binding (Fab) arm exchange leads to functional monovalency with potentially reduced therapeutic efficacy. Mutagenesis studies suggested that the CH3 domain and not the core hinge is dominantly involved in in vivo molecular processing. This work investigated whether stabilization of the core hinge of a therapeutic IgG4 antibody by mutation of Ser228 to Pro (S228P) would be sufficient to prevent in vivo Fab arm exchange. In vitro experiments evaluated the influence of different levels of oxidation-reduction conditions in buffer and serum on Fab arm exchange (swapping) of wild-type (WT) IgG4 and IgG1 and of IgG4 S228P, which included a sterically neutral second mutation (Leu235 replaced by Glu). The objective of single-dose pharmacokinetic experiments in cynomolgus monkeys was to determine whether the mutation reduced IgG4 swapping in vivo. The results indicated that S228P mutation did not completely prevent Fab arm exchange in vitro in buffer under reducing conditions relative to IgG4 WT. The immunoassay findings were confirmed by mass spectrometry measurements. Results of the in vivo studies suggested that the therapeutic IgG4 WT antibody exchanged Fab arms with endogenous cynomolgus monkey IgG4, resulting in bispecific IgG4 antibodies with monovalency for the therapeutic target. In contrast, serum from cynomolgus monkeys dosed with the IgG4 mutant was virtually free of swapped IgG4. In conclusion, the results indicated that IgG4 swapping in vivo was markedly attenuated by S228P mutation.

Since the first approval of a therapeutic monoclonal antibody in 1986, more than 20 therapeutic antibodies were approved in the United States, and over 200 therapeutic monoclonal antibodies (mAb) were undergoing clinical evaluation in 2008 (Reichert, 2008). Therapeutic mAbs have become an increasingly important class of therapeutic compounds in a variety of diseases. A number of technologies have been successfully used to engineer mAbs (Lonberg, 2008). Of the 21 approved therapeutic mAbs, the majority (15) are of the IgG1 isotype, whereas three mAbs are IgG2 antibodies and three are IgG4 or hybrid IgG2/4 antibodies (Reichert, 2008).

The preference for the IgG1 isotype may be partially attributed to its ability to exert effector functions, such as antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity (Salfeld, 2007). Antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity are desirable properties in, e.g., cancer indications, for which most of the approved IgG1 antibodies are licensed (Jeferis, 2007). If the pharmacologic profile of a therapeutic antibody requires diminished antibody effector functions, mAbs of the IgG2 or IgG4 subclass are viable alternatives because of their distinct properties (Swann et al., 2008). Alternatively, IgG variants are described that impair the interaction with cellular Fc receptors (FcγR) via mutations in the Fc part or enhance effector functions via glycoengineering (Carter, 2006).

Recent data provided new evidence that antibodies of the IgG4 isotype undergo a kind of in vivo molecular processing by exchange of antibody light and heavy chains, thereby generating bispecific products that could have unanticipated effects in a therapeutic setting (van der Neut Kolfschoten et al., 2007). As a result of an intermolecular exchange reaction, bispecific IgG4 antibodies could be generated in vivo by swapping a heavy chain and attached light chain (“half-molecule of an antibody”) with a heavy-light chain pair of another IgG4 molecule. The mechanism of this post-translational modification is assumed to require the reducing environment in blood or at cell surfaces.

AABBREVIATIONS: mAb, monoclonal antibody; Fc, fragment, crystallizable (constant part of Ig); FcR, Fc receptor; Fab, antigen-binding fragment; Sple, S228P and L235E; redox, oxidation reduction; WT, wild-type; Oxo40L, Oxo40 ligand; IL13R, interleukin-13 receptor; IL1R, interleukin-1 receptor; BME, -mercaptoethanol; PBS, phosphate-buffered saline; Bi, biotin; SA-MTP, streptavidin-coated high-bind microtiter plates; Dig, digoxigenin; HRP, horseradish peroxidase; ABTS, 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); ELISA, enzyme-linked immunosorbent assay; AUC0-∞, area under curve from time 0 to infinity; CL, clearance; VdSS, volume of distribution under steady state; t1/2p, elimination half-life; ADA, anti-drug antibody.
surfaces to facilitate the shuffling of interheavy chain disulfide bonds located in the hinge region. An important second antibody heavy chain interface was located between the CH3 domains, which were shown to be critically involved in the antigen-binding fragment (Fab) arm exchange (van der Neut Kolfschoten et al., 2007). For the use of IgG4 as a therapeutic antibody, the loss of bivalency by Fab arm exchange may cause lower affinity and may reduce the function of the antibody in cases where avidity is part of the binding mechanism. In fact, human IgG4 was shown to be unable to precipitate purified antigen, which was not due to a difference in affinity but was caused by the inability of IgG4 to cross-link two antigens, illustrating that circulating human polyclonal IgG4 antibodies are functionally monovalent (Schaarman et al., 1999).

Until recently, the structural basis for this characteristic behavior of IgG4 molecules was regarded to be largely the result of a single amino acid change relative to human IgG1, which is the change of a proline in the core hinge of IgG1 to serine in IgG4 at position 228 (Aalberse and Schuurman, 2002). However, more recent work including mutagenesis studies suggested that the CH3 domain and not the core hinge is dominantly involved in the IgG4 Fab arm exchange (van der Neut Kolfschoten et al., 2007). To stabilize therapeutic IgG4 antibodies, variants of IgG4 with a reduced or eliminated potential of in vivo Fab arm exchange have been generated. The present studies investigated whether a variant of the IgG4 antibody against the undisclosed target AG (anti-AG), which was mutated within the hinge region by the amino acid substitution S228P and L235E (SPLE), was sufficient to prevent in vivo swapping. Specific immunoassays were developed to measure 1) total (swapped and unswapped), 2) only unswapped, and 3) only swapped antibody concentrations, respectively. The use of assays to differentiate between swapped and unswapped antibodies assured product stability and contributed to the appropriate assessment of immunogenicity (Weinberg et al., 2005). In addition, the influence of the oxidation-reduction (redox) conditions on IgG4 swapping using different buffer and serum conditions were evaluated.

Materials and Methods

Materials. The therapeutic antibodies used in the studies were recombinant mAbs manufactured by Roche Diagnostics GmbH (Penzberg, Germany): IgG4 wild-type (WT) anti-AG (lot no. JA032), IgG4 WT anti-AG (lot no. JA030), IgG4 double-mutant SPLE anti-AG (lot 013), IgG4 WT anti-Ox40 ligand (anti-Ox40L; lot no. KT 7.7), IgG4 WT anti-interleukin-13 receptor (anti-IL13R; lot no. 005T01), and IgG4 WT anti-interleukin-1 receptor (anti-IL1R; lot 006) antibodies. The anti-IL1R antibody was a deimmunized rat IgG4 antibody, whereas the anti-AG, anti-Ox40L, and anti-IL13R antibodies were IgG4. In series 1, the IgG4 WT mAb anti-Ox40L was incubated with each of the three IgG4 WT mAbs (anti-AG, anti-IL1R, and anti-IL13R) in the absence and presence of 50 mM BME. Furthermore, the IgG4 WT mAb anti-IL13R was incubated with each of the two IgG4 WT mAbs (anti-IL1R and anti-AG). The latter mAb was also incubated with the IgG4 WT mAb anti-IL1R. Control experiments incubated each of the four mAbs alone. The 1:1 antibody mixtures (final concentration of each antibody was 50 μg/ml) were incubated in a volume of 200 μl at 37°C under shaking conditions at 400 rpm. After washing the plates three times, 100 μl per well of each 1:20 diluted test sample was added in duplicate and incubated as described above. After washing three times, 100 μl of the respective Dig-labeled antigen solution at 0.5 μg/ml was added to each well and incubated. After washing three times, 100 μl of anti-Dig-HRP at 50 mM was added and incubated. After washing three times, 100 μl of the HRP substrate solution ABTS was added to each well and incubated for 15 min. The reaction was stopped by the addition of 100 μl of sodium dodecyl sulfate (1%). Absorbance at 405 nm was determined by using a plate reader with a reference filter of 490 nm.

In Vitro IgG4 Swapping in Buffer. Two series of in vitro experiments were conducted in a PBS matrix to systematically evaluate the influence of reducing conditions on the formation of swapped IgG4 antibodies. In series 1, the IgG4 WT mAb anti-Ox40L was incubated with each of the three IgG4 WT mAbs (anti-AG, anti-IL1R, and anti-IL13R) in the absence and presence of 50 mM BME. Furthermore, the IgG4 WT mAb anti-IL13R was incubated with each of the two IgG4 WT mAbs (anti-IL1R and anti-AG). The latter mAb was also incubated with the IgG4 WT mAb anti-IL1R. Control experiments incubated each of the four mAbs alone. The 1:1 antibody mixtures (final concentration of each antibody was 50 μg/ml) were incubated in a volume of 200 μl at 37°C under shaking conditions at 400 rpm for 1 day in the presence and absence of BME. Incubation of only 2 h did not show any reaction, whereas longer incubation times (48, 72, and 96 h) did not show incremental reactions. The antibodies were only mixed in equal amounts, because the in vitro serum and in vivo experiments were planned to reflect a higher ratio of endogenous IgG4. In series 2, varying levels of the oxidation-reduction conditions (expressed as the molar ratio of GSH/GSSG) on the quantitative formation of swapped IgG4 were tested by mixing the IgG4 WT anti-IL1R mAb 1:1 with each of the three anti-AG mAbs (IgG4 WT, IgG1 WT, or IgG4 SPLE). The 100-μl mixture had a final concentration of each antibody in the PBS medium of 25 μg/ml (corresponding to a total antibody concentration of 50 μg/ml) and was incubated with a final concentration of 1 mM GSH/GSSG for 1 day at 37°C. The ratios of GSH/GSSG were 1:0, 0:1, 1:20, 1:5, 1:1, 10:1, 20:1, and 200:1. To measure swapped (bispecific, monovalent) antibodies, samples were analyzed in an ELISA. Mass spectrometric analysis was performed for samples with 1:0 and 0:1 ratios of GSH/GSSG only.

Electrospray Ionization Mass Spectrometry Analysis of Antibodies from In Vitro Buffer Experiments. All samples were enzymatically deglycosylated and desalted by reverse-phase high-performance liquid chromatography before analysis of mass spectra in a Q-Star Elite mass spectrometer (Applied Biosystems, Darmstadt, Germany). Samples containing 50 μg of the respective antibody in a volume of 115 μl were incubated with 10 U/ml N-glycosidase F for 16 h at 37°C. The samples were desalted with a Sephadex G25 column (5 × 250 mm) (GE Healthcare, Munich, Germany). One hundred microliters (approximately 43 μg of antibody) was injected and eluted with a flow rate of 1000 μl/min. Eluent was 40% acetonitrile with 2% formic acid in purified water. Electrospray ionization mass spectra were recorded online in the mass spectrometer in the positive ion mode (calibrated with rexin). Mass spectra were deconvoluted by use of the Maximum Entropy algorithm.
In Vitro IgG4 Swapping in Serum. Two series of in vitro serum experiments were performed. The first series of experiments investigated the swapping tendency of IgG4 antibodies in vitro in human and various animal serum matrices. The recombinant fully human IgG4 WT anti-IL1R mAb was incubated in a volume of 200 μl with helium-degassed reagent water as carrier at a concentration of 1 μg/ml in 10% pooled serum from mice, rats, cynomolgus monkeys, and humans at 37°C overnight in the presence of 1 mM GSH or GSSG. The resulting samples were diluted with LowCross buffer to an antibody assay concentration of 15 ng/ml and analyzed in the ELISA using immobilized IL1R-Bi for capture and IL1R-Dig as detection agent to analyze bivalent unswapped anti-IL1R antibodies. The ELISA was performed as described above. The concentration of the antibody in the sample was calculated by using a calibration curve prepared by serial dilution of the stock solution of anti-IL1R IgG4 WT. The 10% serum concentration was preferred over undiluted or less diluted serum to avoid matrix effects in the assay, but it still provided an excess of serum IgG4 over the added antibody.

The second series of in vitro serum experiments evaluated the swapping tendency of the IgG4 antibody anti-IL1R in redox-buffer free, physiologic human, and cynomolgus monkey serum in comparison to GSH and GSSG over 4 days. The IgG4 WT anti-IL1R mAb was added to 10% human or cynomolgus monkey serum in comparison to GSH and GSSG over 4 days. The IgG4 WT anti-IL1R mAb was added to 10% human or cynomolgus monkey serum at a final concentration of 1 μg/ml. Five aliquots were prepared from the stock solution, and four of them were stored at −10°C until thawed after 1, 2, 3, and 4 days. Each aliquot was divided into three parts, which were incubated in the presence of PBS, 1 mM GSH, or 1 mM GSSG, respectively, at 37°C under shaking for the remainder of the 4-day period, i.e., incubation times were 96, 72, 48, 24, and 0 h. The samples taken from each of the 15 incubations were analyzed as previously described in the bivalent IL1R-specific ELISA.

In Vivo IgG4 Swapping in the Cynomolgus Monkey. The animal experiments described in the present article were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) as adopted and promulgated by the U.S. National Institutes of Health. Nine adult male and female cynomolgus monkeys (Macaca fascicularis) were assigned to one of two study groups. The anti-IL1R IgG4 WT antibody was intravenously administered at single doses of 0.5 mg/kg to six animals. In a second study, three animals received a single intravenous dose of the anti-AG IgG4 SPLE antibody at 1 mg/kg. Blood samples were taken from the femoral vein into a test tube before and repeatedly after administration of the therapeutic antibody and stored at room temperature for at least 30 min to allow clotting. Serum was prepared by centrifugation at 1000g for 15 min, and aliquots were taken, immediately frozen, and stored at −80°C until assayed.

Bioanalytical Assays. The serum samples obtained from the in vivo cynomolgus monkey studies were analyzed in four different ELISA formats. Figure 1b illustrates the principles of the four assay formats. Format A (to detect nonswapped and swapped antibodies) used AG or IL1R as coating and a digoxigenylated anti-human Fc mAb as detection agent. Format B (to detect only nonswapped antibodies) used pairs of AG-Bi and AG-Dig or IL1R-Bi and IL1R-Dig, respectively, as coating and detection reagents. Format C (to detect only nonswapped antibodies) used an anti-human Fc-Bi mAb and an anti-
human Fc-Dig mAb as coating and detection reagents. Format D (to detect only swapped antibodies) used an anti-human Fc-Bi mAb and an anti-cyno Fc-Dig mAb as capture and detection agents. The concentrations of the antibodies in the sample were calculated from calibration curves prepared by serial dilution of the stock solution of the respective standard anti-IL1R IgG4 WT and anti-AG IgG4 double-mutant SPLE for assay formats A, B, or C, respectively. For assay format D, as standard, a polyclonal anti-human IgG conjugated to polyclonal anti-cynomolgus monkey IgG was used.

**Pharmacokinetic Assessment.** Standard pharmacokinetic parameters were only calculated for total mAb (nonswapped and swapped) concentrations of format A using the WinNonlin Professional (version 3.2) software package (Pharsight, Mountain View, CA). Serum concentration-time data were calculated by using a model-independent approach based on the statistical moment theory. The parameters calculated included the area under curve from time 0 to infinity (AUC$_{0-\infty}$), clearance (CL), volume of distribution under steady state (Vd$_{SS}$), and elimination half-life ($t_{1/2}$).

**Results**

**In Vitro IgG4 Swapping in Buffer.** The Fab arm swapping tendency of WT IgG4 antibodies, mutants, and IgG1 controls was studied in buffered solutions in vitro. Incubation of an equimolar mixture of the wild-type IgG4 antibodies anti-AG and anti-IL1R in the presence of the disulfide-reducing agent BME induced swapping as indicated by a 7-fold absorbance increase in the AG/IL1R ELISA (Fig. 2). In the absence of BME, no swapping was observed as evidenced by a lack of absorbance increase. Each of the four antibodies alone as well as the five other antibody combinations when tested were negative. The same pattern of reactivity was found for three other bispecific ELISA configurations (Fig. 2). Only those antibody combinations showed an absorbance increase in the presence of BME for which the ELISA configuration was specific. For incubation of the antibody alone and the other five antibody combinations, no absorbance increase was seen in the absence of BME, which did not match the bispecific ELISA configuration. These results show that Fab arm exchange of WT IgG4 only occurs under reducing conditions.

A second set of in vitro experiments in PBS buffer evaluated the influence of varying ratios of GSH/GSSG (redox system) on the formation of swapped antibodies. Samples containing anti-IL1R IgG4 WT mAbs in a 1:1 ratio with anti-AG IgG1 WT, IgG4 WT, or IgG4 SPLE mAbs were analyzed in the IL1R/AG ELISA. The combination of the anti-IL1R IgG4 WT mAb with anti-AG IgG1 WT mAb did not enable swapping as demonstrated by an increased absorbance at 405 nm, whereas combination of the two IgG4 WT anti-IL1R and anti-AG mAbs showed an increase in absorbance dependent on the redox system applied (Fig. 3). If the anti-IL1R IgG4 WT antibody was incubated with the anti-AG IgG4 SPLE mutant mAb, then the Fab arm exchange was not completely prevented, albeit it was markedly diminished in comparison with the IgG4 WT mAbs (Fig. 3). To corroborate the findings obtained with the ELISAs, mass spectrometry was applied to analyze the same set of incubations (anti-IL1R IgG4 WT mAb with each of the anti-AG IgG1 WT, IgG4 WT, or IgG4 SPLE mAbs) at the selected GSH/GSSG ratios of 1:0 and 0:1 (presence and absence of reducing conditions).

Electrospray ionization mass spectrometry revealed that the molecular masses of each of the antibody combinations remained unchanged in the absence of reducing conditions (Fig. 4, bottom row). In the presence of GSH (Fig. 4, top row), molecular masses upon coincubation of anti-IL1R IgG4 WT mAb with anti-AG IgG1 WT mAb appeared to be the same as those in the absence of reducing conditions. However, a new peak appeared for the combination of anti-IL1R IgG4 WT mAb and anti-AG IgG4 WT mAb. This new mass corresponded to the expected mass of a bispecific (swapped) antibody. A less pronounced new peak was seen upon coincubation of anti-IL1R IgG4 WT mAb and anti-AG IgG4 SPLE mAb, indicating a less pronounced but still detectable swapping tendency.

**In Vitro IgG4 Swapping in Serum.** Simulating in vivo conditions, the anti-IL1R IgG4 WT mAb was incubated at a concentration of 1 µg/ml with 10% serum from humans and various animal species to evaluate species dependence of IgG4 swapping with polyclonal endogenous IgG4 of the respective serum. Figure 5 illustrates the recovery of a fortified concentration of 1 µg/ml of the therapeutic anti-IL1R IgG4 WT mAb under reducing or nonreducing conditions as detected by a IL1R/IL1R ELISA. The results show that in the absence of reducing conditions, the anti-IL1R IgG4 WT mAb added to 10% serum was recovered on average between 60 and 70% independent of the species, indicating little or no swapping in serum. In contrast, reducing conditions (1 mM GSH) led to reduced recovery of anti-IL1R IgG4 WT mAb to ≤10% in human and cynomolgus serum suggestive of swapping of the therapeutic antibody with human and cynomolgus IgG4. When the anti-IL1R WT IgG4 antibody was incubated in human serum free of redox buffer for up to 4 days, recovery of the added concentration of 1 µg/ml was at least 80%, which was
similar to the recovery in the presence of 1 mM GSSG (Fig. 6b). In contrast, in the presence of 1 mM GSH, approximately 60% of the added anti-IL1R mAb was not recovered. The same set of experiments performed in cynomolgus monkey serum confirmed the findings obtained in the human serum system, although at slightly lower levels of recovery (Fig. 6a).

**In Vivo IgG4 Swapping in the Cynomolgus Monkey.** Cynomolgus monkeys dosed with 0.5 mg/kg anti-IL1R WT IgG4 mAb revealed a serum concentration-time curve of total anti-IL1R mAb with an elimination half-life of 17 days (Table 1). Of the three cynomolgus monkeys dosed with 1 mg/kg anti-AG IgG4 SPLE mAb, one developed anti-drug antibodies (ADA) as measured in the immunogenicity screening ELISA (data not shown). The formation of ADAs was accompanied by a decrease of the serum concentration to undetectable levels, and the animal, therefore, was excluded from the evaluation.

The reasons for the sharp drop in serum concentrations in animal 7-1 are not clear, although an ADA response was not shown for this animal.

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**FIG. 5.** Recovery as percentage of an added concentration of 1000 ng/ml of the IgG4 wild-type antibody anti-IL1R IgG4 WT when incubated in 10% serum in the presence (1 mM GSH) or absence (1 mM GSSG) of reducing conditions at 37°C overnight and detected by a bridging ELISA for bivalent, monospecific anti-IL1R. Serum was from human, cynomolgus monkey, rat, and mouse. Data are the mean ± S.D. of n = 4 measurements from two experiments.

**FIG. 4.** Electrospray ionization mass spectrometry analysis of antibody swapping in PBS buffer samples of anti-IL1R IgG1 WT incubated in a 1:1 ratio with anti-AG variants IgG1 WT, IgG4 WT, and IgG4 double-mutant SPLE at 37°C overnight. The left column represents results obtained by incubation with 1 mM GSSG (absence of reducing conditions), and the right column shows results for incubation with 1 mM GSH (reducing conditions).

**FIG. 3.** Influence of varying ratios of 1 mM GSH/GSSG (redox system) on the formation of swapped antibodies by incubation of anti-IL1R IgG1 WT in a 1:1 ratio with anti-AG isotypes IgG1 WT, IgG4 WT, or IgG4 double-mutant SPLE at 37°C overnight. Analysis of swapping by bridging ELISA with capture and detection antigens IL1R + AG. Data are the mean ± S.D. of n = 4 measurements from two experiments.
animal (Fig. 8). Analysis of the samples of the two remaining animals 2-18 and 7-1 for total antibody concentrations also showed a biphasic elimination from serum (Fig. 8) with elimination half-life values of 13.3 and 8.3 days, respectively (Table 2). Analysis of swapping was performed for both studies by using samples of two animals each analyzed by four different ELISA formats as illustrated in Fig. 1 to determine swapped and nonswapped antibodies (“Total antibodies”), only nonswapped antibodies (as “Bivalent antibodies” and as “Double human Fc antibodies”), and only swapped antibodies (“Bispecific antibodies”).

The two animals dosed with anti-IL1R IgG4 WT showed very low serum concentrations of nonswapped therapeutic antibody, whereas concentrations of total and swapped IgG4 antibody were higher and decreased time-dependently as expected for single-dose administrations (Fig. 9). Figure 10 illustrates nearly identical serum concentration-time profiles measured for total antibody and nonswapped antibodies in animals dosed with the anti-AG IgG4 SPLE mAb, whereas virtually no swapped antibody was detectable. These results demonstrate that Fab arm exchange was observed in vivo for a therapeutic antibody of an IgG4 wild-type

![Fig. 6. Recovery of an added concentration of 1 μg/ml of the IgG4 wild-type antibody anti-IL1R when incubated in 10% cynomolgus monkey (a) or human (b) serum with PBS buffer and compared with incubation in the presence of 1 mM GSH or 1 mM GSSG at 37°C for up to 96 h and detected by a bridging ELISA for bivalent, monospecific anti-IL1R.](image)

![Fig. 7. Serum concentration-time profile of anti-IL1R IgG4 WT in cynomolgus monkeys after single intravenous administration of 0.5 mg/kg. Data are the concentrations of total antibody (swapped and nonswapped) obtained by measurement of the serum samples in a bridging ELISA, which detected all antibodies with at least one Fab arm for the specific antigen IL1R. This assay used biotinylated antigen IL1R immobilized on the streptavidin-coated microtiter plate to capture the therapeutic antibody in the sample and digoxigenylated anti-human Fc to detect specifically bound human antibody anti-IL1R via the constant region of human IgG. Data are the mean ± S.D. of six animals.](image)

![Fig. 8. Serum concentration-time profiles of anti-AG IgG4 double-mutant SPLE in two individual cynomolgus monkeys (2-18 and 7-1) after single intravenous administration of 1 mg/kg. Data are the concentrations of total antibody (swapped and nonswapped) obtained by measurement of the serum samples in a bridging ELISA, which detected all antibodies with at least one Fab arm for the specific antigen AG. This assay used biotinylated antigen AG immobilized on the streptavidin-coated microtiter plate to capture the therapeutic antibody in the sample and digoxigenylated anti-human Fc to detect specifically bound human antibody anti-IL1R via the constant region of human IgG.](image)

![Fig. 9. Serum concentration-time profiles of anti-IL1R IgG4 WT (swapped and nonswapped) in cynomolgus monkeys after single intravenous administration of 0.5 mg/kg. Serum concentrations were determined in a bridging ELISA, which detected all antibodies with at least one Fab arm for the specific antigen IL1R. Data are the mean ± S.D. of the pharmacokinetic parameters determined from six animals.](image)

### Table 1

<table>
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<th>Dose (mg/kg)</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (μg/ml)</th>
<th>AUC&lt;sub&gt;0-∞&lt;/sub&gt; (μg · h/ml)</th>
<th>CL (ml/h/kg)</th>
<th>Vd&lt;sub&gt;ss&lt;/sub&gt; (ml/kg)</th>
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![Table 1](image)
isotype, which could be prevented by introducing two mutations into the IgG SPLE mutant.

Discussion

The experiments described in the present report characterized the Fab arm exchange of IgG4 antibodies in vitro in buffer and in serum in dependence on the oxidation-reduction conditions (or disulfide isomerization promoting conditions). In addition, swapped antibodies were found in ex vivo samples from cynomolgus monkeys dosed with IgG4 antibodies. The results indicate that Fab arm exchange occurred under reducing conditions in vitro in buffer and in serum for four different therapeutic IgG4 WT antibodies directed against the targets AG, IL1R, IL13R, or Ox40L, but not for the IgG1 isotype anti-AG control. Substitution of the amino acid serine at position 228 by proline in the core hinge of IgG4 did not completely prevent Fab arm exchange in vitro in buffer, albeit it was lower than for the IgG4 WT experiments. Mass spectrometry measurements confirmed the appearance of swapped antibodies in buffer samples as an independent methodology. In vitro experiments in serum from human and various animals showed that IgG4 swapping occurred under reducing conditions equally in human and cynomolgus monkey serum, whereas the tendency decreased successively in serum from rats and mice, thereby confirming the selection of the cynomolgus monkey as an adequate and sensitive animal species to evaluate in vivo swapping of therapeutic IgG4 antibodies. Because the IgG4 mutant used in the present studies had a double mutation to enhance disulfide linkage in the core-hinge region (S228P) and to eliminate residual FcR binding (L235E), a contribution of the L235E mutation on diminishing swapping cannot be excluded by experimental data from the present work. However, experiments with an IgG4 S228P mutant in mice showed that the S228P mutation alone is capable of inducing the same effect as the double mutant used in the present studies (Labrijn et al., 2009). This result suggests that the influence of the second mutation L235E on swapping would be low or even negligible.

Although the limited number of sampling time points did not allow a full description of the time course of swapping in vivo and quantitate the extent, the available data qualitatively showed appearance of swapped antibodies in vivo. Results of the cynomolgus monkey
experiments suggest that the anti-IL1R IgG4 wild-type antibody underwent in vivo molecular processing, i.e., the antibodies exchanged one of their heavy-light chain moieties with another heavy-light chain moiety of endogenous polyclonal cynomolgus monkey IgG4. The Fab arm exchange resulted in a bispecific IgG4 antibody monovalent for the therapeutic target. In contrast to the in vitro buffer and serum experiments, ex vivo serum samples from cynomolgus monkeys dosed with the anti-AG IgG4 double-mutant SPLE were virtually free of swapped IgG4 molecules. Four differently configured ELISA assay formats were used to characterize IgG4 valency and specificity in ex vivo samples from the cynomolgus monkey. The complementary character of the assay formats for nonswapped and swapped antibodies cross-checked by the assay format for total antibody provided plausibility of the findings in the experiments. The use of standards in the assays for measurement of ex vivo samples allowed semiquantitative comparison of the back-calculated concentrations. However, care has to be taken when comparing concentrations of swapped antibodies with those of total antibodies or unswapped antibodies, because the assay for swapped antibodies used a nonspecific standard in contrast to the antibody-specific standards for the other three assay formats. The standards used in the assay for swapped antibodies were di- or tetrameric IgG conjugates of human and cynomolgus monkey IgG with a molecular mass of 300 to 600 kD in contrast to the in vivo-generated swapped IgG with an apparent molecular mass of 150 kD. This may lead to a 2- to 4-fold overestimation of the concentration and might explain the partially higher concentrations measured in the assay for the swapped antibody than for the total antibody.

The findings of the present in vivo studies, which suggest that mutation S228P in the core hinge is sufficient to prevent Fab arm exchange of IgG4 antibodies in the cynomolgus monkey, are in contrast to in vitro mutagenesis data obtained by van der Neut Kolfschoten et al. (2007). These investigators ascribed a dominant role to the IgG4 CH3 domains with their heavy chain interface. Indeed, the in vitro part of the present studies confirmed that the S228P core hinge mutation alone was not sufficient to completely abolish Fab arm exchange and that the CH3 domains could have mediated Fab arm exchange as suggested by van der Neut Kolfschoten et al. (2007). The in vitro experiments of the present work investigated IgG4 antibody concentrations similar to those found in vivo, i.e., 0.1 to 0.5 mg/ml (French, 1986). Concentrations of reduced glutathione and BME in the in vitro studies were in accordance with those used by van der Neut Kolfschoten et al. (2007). However, the oxidation-reduction system applied to the in vitro experiments might have been more reducing than that under in vivo conditions. Redox potential measurements in healthy volunteers indicated a rapid oxidation of GSH upon release into plasma with plasma GSH concentrations of 2.8 ± 0.8 μM (Jones et al., 2000), which is more than 300-fold lower than the mM threshold such as 100 μM measured in humans could be extrapolated to the cynomolgus monkey, then redox potential in the cynomolgus monkey experiments would have been considerably more oxidizing than that used in the present in vitro experiments (1 mM GSH). The mutagenesis experiments that described a dominant involvement of the CH3 domain in Fab arm exchange were conducted in the presence of 100 μM GSH (van der Neut Kolfschoten et al., 2007; Supporting Online Material). These data could suggest different threshold values of the redox system for Fab arm exchange: at a lower threshold of 2.8 ± 0.8 μM as found in vivo, stabilization of the core hinge region is enough to avoid IgG4 swapping, and at a higher threshold such as 100 μM, the CH3 domain interaction would become necessary for additional stabilization to prevent swapping. Although the anti-AG IgG4 mutant studied in the present experiments included a second mutation at L235E to reduce interaction with the Fc receptors, the site of the mutation is too far distant from the CH3 domain to suggest a possible involvement of the CH3 domain in the effect of the double mutation to prevent IgG4 swapping. The L235E mutation is also too far away sterically from the S228P mutation to affect disulfide bridge formation. It is noteworthy that the experiments conducted by Labrijn et al. (2009) showed that the S228P mutation alone was able to prevent Fab arm exchange in vivo, suggesting that the L235E mutation would not qualitatively influence the outcome of the present studies. The consequences of in vivo swapping of therapeutic IgG4 antibodies in man are not well characterized in human studies due to the lack of adequate assays and awareness of the problem. However, the functional monovalency might contribute to reduced efficacy due to loss of the ability for cross-linking of antigens such as receptors on cell surfaces in a 1:1 stoichiometric reaction and, thus, should be avoided to prevent unpredictable consequences in vivo. Swapping might also affect the disposition of the therapeutic antibody if target binding (“antigen sink”) plays a role. Monovalency for the target may influence the affinity and alter the disposition. The general metabolism of antibodies (FcRn receptor binding, recycling, lysosomal degradation) is less likely to be affected by swapping because the involved structures of the antibody are not located on the Fab arms. The present experiments in cynomolgus monkeys suggest that a single amino acid substitution in the core hinge of IgG4 (S228P) of the anti-AG antibody was sufficient to nearly fully abolish IgG4 swapping measured in ex vivo samples. The difference in the in vitro finding of van der Neut Kolfschoten et al. (2007) might be explained by the artificially too high oxidation-reduction conditions, leading to an understimation of the role of the core-hinge region for swapping and relative overestimation of the CH3 domain. Results from mice studies with an S228P IgG4 mutant antibody published by the same group (Labrijn et al., 2009) suggested that core-hinge stabilization alone prevents IgG4 Fab-arm exchange in vivo, thereby independently confirming, extending, and validating the findings of the present study.

References
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