Impact of molecular processing in the hinge region of therapeutic IgG4 antibodies on

disposition profiles in cynomolgus monkeys

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Abbreviations

ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); ADA, anti-drug antibody;

AU, arbitrary units; AUC, area under the serum concentration-time curve; Bi, biotin;

BME, β-mercaptoethanol; CL, clearance; Dig, digoxigenin; ELISA, enzyme-linked

immunosorbent assay; Fab, antigen-binding fragment; Fc, fragment, crystallisable

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(constant part of immunoglobulin); FcR, Fc receptor; h, hour; HRP, horse radish

peroxidase; Ig, immunoglobulin; IL1R, interleukin-1 receptor; IL13R, interleukin-13

receptor; mAb, monoclonal antibody; i.v., intravenous; OD, optical density; Ox40L,

Ox40ligand; PBS, phosphate-buffered saline; Vd, volume of distribution; WT, wild-type.

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ABSTRACT:

The IgG4 isotype antibody is a potential candidate for immunotherapy when reduced effector functions are desirable. However, antigen binding fragment (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 the 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 wildtype (WT) IgG4 and IgG1 and of IgG4 S228P which included a sterically neutral second mutation (Leu235 replaced by Glu). Single dose pharmacokinetic experiments in cynomolgus monkeys studied 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 Ser228Pro mutation.

Introduction

Since the first approval of a therapeutic monoclonal antibody in 1986, more than 20

therapeutic antibodies were approved in the US 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

employed to engineer mAbs (Lonberg, 2008). Of the 21 approved therapeutic mAbs, the

majority (15) are of the immunoglobulin G1 (IgG1) isotype, while three mAbs are IgG2

antibodies and three are IgG4 or hybrid IgG2/4 antibodies (Reichert, 2008).

The preference for the IgG1 isotype may partially be attributed to its ability to exert

effector functions, such as antibody-dependent cellular cytotoxicity (ADCC) and

complement dependent cytotoxicity (CDC). ADCC and CDC are desirable properties in,

e.g. cancer indications for which most of the approved IgG1 antibodies are licensed

(Jefferis, 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, 2008). Alternatively, IgG

variants are described which 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

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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 to facilitate the

shuffling of inter-heavy 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 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

were 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 (Schuurman 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). More recent work including mutagenesis studies

suggested, however, 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 Ser228Pro and Leu235Glu (S228P, L235E: SPLE) were

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.

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Materials and Methods

Materials. The therapeutic antibodies used in the studies were recombinant monoclonal

antibodies (mAb) manufactured by Roche Diagnostics GmbH, Penzberg, Germany: IgG4

wild-type (WT) anti-AG (lot no. JA032), IgG1 WT anti-AG (lot no. JA030), IgG4 double

mutant SPLE anti-AG (lot no. 013), IgG4 WT anti-Ox40 ligand (anti-Ox40L; lot no. KT

7.7), IgG4 WT anti-interleukin-13 receptor (anti-IL13R; lot no. r05T01), and IgG4 WT

anti-interleukin-1 receptor (anti-IL1R; lot no. 006) antibodies. The anti-IL-1R antibody

was a deimmunized rat IgG4 antibody whereas the anti-AG, anti-Ox40L and anti-IL13R

antibodies were fully human antibodies created by transgenic mouse technology. All were

expressed in chinese hamster ovary (CHO) cell systems. The disulfide reducing agent β-

mercaptoethanol (BME) was purchased from Amresco, Monlucon, France. Reduced

glutathione (GSH) was bought from Sigma Aldrich, Taufkirchen, Germany, and oxidized

glutathione disulfide (GSSG) was obtained from Roche Diagnostics GmbH, Mannheim,

Germany. Pooled human serum was obtained from the serum bank of Roche Diagnostics

GmbH, Penzberg, Germany. Pooled serum from rats and CD1 mice were purchased from

Charles River, Wilmington, MA, USA. Batches of pooled cynomolgus monkey sera were

obtained from Bioreclamation Inc., Hicksville, NY, USA. The in vitro experiments used

phosphate buffered saline (PBS; 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, pH 7.4)

prepared at Roche Diagnostics GmbH, Mannheim, Germany. LowCross Buffer was

obtained from Candor Bioscience GmbH, Weissensberg, Germany. Streptavidin-coated

"high-bind" microtiter plates (SA-MTP) were purchased from MicroCoat Biotechnologie

GmbH, Bernried, Germany. The biotinylated mAb against the constant part of human IgG

(anti-human Fc-Bi, M-R10Z8E9) and the digoxigenylated mAb against the constant part

of cynomolgus monkey IgG (anti-cyno Fc-Dig, M-7.72.32) were from Roche Diagnostics

GmbH, Penzberg, Germany. The polyclonal anti-digoxigenin-horse radish peroxidase

(anti-Dig-HRP) and the chromogenic substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-

sulfonic acid) (ABTS) were from Roche Diagnostics GmbH, Mannheim, Germany. N-

glycosidase F was purchased from ProZyme, San Leandor, CA, USA.

ELISA for Detection of Antibodies. The principle of the bridging ELISA is illustrated in

Figure 1a. In short, biotin-labelled antigens (IL13R-Bi, AG-Bi or IL1R-Bi) were

immobilized to SA-MTPs to capture one antigen-binding fragment (Fab) arm of the

swapped antibody. For detection, a digoxigenin (Dig)-labelled antigen (Ox40L-Dig,

IL13R-Dig, IL1R-Dig, or AG-Dig) was used to bind to the other Fab arm (2nd specificity)

of the swapped antibody. The Dig-labelled antigens were detected by an anti-Dig-HRP

antibody. The HRP enzyme reacted with its substrate ABTS and absorbance was

measured at 405 nm. LowCross buffer was used for all assays for dilution of reagents,

sample preparation and washing. Capture reagents, plating conditions and dilutions of the

antibodies were optimized and shown to be stable in the presence of reducing equivalents

for all species tested. The MTPs were coated by adding 100 μL of the respective biotin-

labelled antigen solution at 0.5 μg/mL to each well and incubated for 1h at room

temperature under shaking conditions (400 rpm). After washing the plates three times,

100 μL per well of each 1:20 diluted test samples were added in duplicates and incubated

as above. After washing three times, 100 μL of the respective Dig-labelled antigen

solution at 0.5 μg/mL were added to each well and incubated. After washing three times,

100 μL of anti-Dig-HRP at 50 mM were added and incubated. After washing three times,

100 μL of the HRP substrate solution ABTS were added to each well and incubated for

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15 minutes. The reaction was stopped with addition of 100 µL of 1% sodium dodecyl

sulphate. Absorbance at λ 405 nm was determined using a plate reader with 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 37ºC under

shaking conditions at 600 rpm for one 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, as the in vitro serum and 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 one day at 37ºC. The ratios of GSH:GSSG were 1:0; 0:1; 1:20; 1:5;

1:1; 10:1; 20:1; 200:1. To measure swapped (bispecific, monovalent) antibodies, samples

were analyzed in an enzyme-linked immunosorbent assay (ELISA). Mass spectrometric

analysis was performed for samples with 1:0 and 0:1 ratios of GSH:GSSG only.

Electrospray Ionization Mass Spectrometry (ESI-MS) Analysis of Antibodies from

in vitro Buffer Experiments. All samples were enzymatically deglycosylated and

desalted by reverse-phase high performance liquid chromatography (RP-HPLC) 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 of N-glycosidase F for 16 h at 37 ºC. The

samples were desalted with a Sephadex G25 column (5 * 250 mm) (GE Healthcare,

Munich, Germany). 100 µl (about 43 µg of antibody) were 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 renin). 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 ºC over night 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 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 at a final concentration of 1 µg/mL. Five aliquots were prepared from the stock solution and four of them stored at -70ºC 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 37ºC under shaking for the remainder of the 4-day period , i.e. incubation times were 96 h, 72 h, 48 h, 24 h and 0 h. The samples taken from each of the 15 incubations were analyzed as described before in the bivalent IL1R-specific ELISA.

In vivo IgG4 Swapping in the Cynomolgus Monkey. The animal experiments described in the present paper were carried out in accordance with the Guide for the Care and Use of Laboratory Animals 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 i.v. dose of the anti-AG IgG4 SPLE antibody at 1

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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 1000 g for

15 min and aliquots were taken, immediately frozen and stored at -80ºC 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 non-swapped and swapped) used AG or

IL1R as coating and a digoxigenylated anti-human Fc mAb as detection agent. Format B

(to detect only non swapped 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 non

swapped 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 (non-swapped and swapped) concentrations of Format A using

the WinNonlin Professional (version 3.2) software package (Pharsight Corp., Mountain

View, CA, USA). Serum concentration-time data were calculated using a model-

independent approach based on the statistical moment theory. The parameters calculated

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included the area under curve from time zero to infinity ($AUC_{0-\infty}$), clearance (CL),

volume of distribution under steady state (Vd_{SS}) and elimination half-life ($t_{1/2\beta}$).

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 were tested negative. The same pattern of reactivity was found for each of 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. No absorbance increase was seen in the absence of BME, for incubation of the antibody alone and the other five antibody combinations 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 IgG1 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, 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 (ESI-MS) revealed that the molecular masses

of each of the antibody combinations remained unchanged in the absence of reducing

conditions (Fig. 4, lower row). In the presence of GSH (Fig. 4, upper row), molecular

masses upon co-incubation 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 co-incubation 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 dependency of IgG4 swapping with

polyclonal endogenous IgG4 of the respective serum. Figure 6 illustrates the recovery of a

fortified concentration of 1 µg/mL of the therapeutic anti-IL1R IgG4 WT mAb under

reducing or non-reducing 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. 6a). In

contrast, in the presence of 1 mM GSH about 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. 6b).

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 (swapped and non-swapped) which indicated a biphasic

pharmacokinetic profile (Fig. 7). The antibody was cleared from serum with an

elimination half life of 17 days (Table 1). Of the three cynomolgus monkeys dosed with 1

mg/kg of 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 (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 Figure 2 to determine swapped and non-swapped antibodies ("Total Antibodies"), only non-swapped antibodies (as "Bivalent antibodies" and as "Two human Fc antibodies") and only swapped antibodies ("Bispecific antibodies").

The two animals dosed with anti-IL1R IgG4 WT showed very low serum concentrations of non-swapped 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 separately for both animals dosed with the anti-AG IgG4 SPLE mAb nearly identical serum concentration-time profiles measured for total antibody and non-swapped antibodies 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 wildtype isotype which could be prevented by introducing 2 mutations into the IgG SPLE mutant.

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Discussion

The experiments described in the present report characterized the Fab arm exchange of IgG4 antibodies in vitro in buffer and in serum in dependency 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 as an independent methodology the appearance of swapped antibodies in buffer samples. 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. As 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 Fc-receptor 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 to induce the same effect as the double mutant used in the present studies (Labrijn, 2009). This

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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 to fully describe the time-couse 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 virtually were 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 non-swapped and for 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 semi-quantitative 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 MW of 300 – 600 kD in contrast to the in

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vivo generated swapped IgG with an apparent MW of 150 kD. This may lead to a two- to four-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 Ser228 Pro 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. 0.1-0.5 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 1 mM concentration used in the present in vitro experiments. If the plasma GSH concentrations of 2.8 ± 0.8 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

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mutagenesis experiments which 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 neccesary for additional stabilization to prevent swapping. Although the anti-AG

IgG4 mutant studied in the present experiments included a second mutation at Leu235Glu

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 sterically is also too far

away from the S228P mutation in order to affect disulfide bridge formation. Importantly,

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 stochiometric 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 alterate 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 (Ser228Pro) of the anti-AG antibody was sufficient to nearly fully abolish IgG4

swapping measured in ex vivo samples. The difference to the in vitro finding of van der

Neut Kolfschoten (2007) might be explained by the artificially too high oxidation-

reduction conditions leading to an underestimation 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, 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.

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Legends for Figures

Fig. 1 Schematic illustrations of the principle of the bridging ELISA for measurement of swapped antibodies with specificity for two different antigens (Fig. 1a) and of four different ELISA formats (fig. 1b) to measure swapped and unswapped antibodies ("Total Antibodies"), unswapped antibodies ("Bivalent Antibodies" and "Double human Fc antibodies") and swapped antibodies ("Bispecific antibodies") in ex vivo samples from cynomolgus monkey experiments.

Fig. 2 In vitro IgG4 swapping in PBS buffer in the absence or presence of the disulfide reducing agent BME and incubation for 1 day at 37ºC. Analysis of swapping by a) bridging ELISA with capture and detection antigens AG and IL1R; b) bridging ELISA with capture and detection antigens Ox40L and IL1R, c) bridging ELISA with capture and detection antigens IL13R and IL1R; and d) bridging ELISA with capture and detection antigens IL13R and Ox40L.

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 37ºC over night. Analysis of swapping by bridging ELISA with capture and detection antigens IL1R + AG. Data are the mean $\pm$ SD of n=4 measurements from two experiments.

Fig. 4 Electrospray Ionization Mass Spectrometry (ESI-MS) 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 37ºC over night. The left column represents results obtained by incubation with 1 mM GSSG (absence of

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reducing conditions) and the right column shows results for incubation with 1 mM GSH (reducing conditions).

Fig. 5 Recovery as percentage of an added concentration of 1,000 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 37ºC over night and detected by a bridging ELISA for bivalent, monospecifc anti-IL1R. Serum was from human, cynomolgus monkey, rat and mouse. Data are the mean $\pm$ SD of n=4 measurements from two experiments.

Fig. 6 Recovery of an added concentration of 1 µg/mL of the IgG4 wild-type antibody anti-IL1R when incubated in 10 % human (Fig. 7a) or cynomolgus monkey (Fig. 7b) serum with PBS buffer and compared to incubation in the presence of 1 mM GSH or 1 mM GSSG at 37ºC for up to 96 hours and detected by a bridging ELISA for bivalent, monospecifc anti-IL1R.

Fig. 7 Serum concentration-time profile of anti-IL1R IgG4 WT in cynomolgus monkeys after single i.v. administration of 0.5 mg/kg. Data are the concentrations of total antibody (swapped and non-swapped) 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 $\pm$ SD of six animals.

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 i.v. administration of 1

mg/kg. Data are the concentrations of total antibody (swapped and non-swapped)

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.

Fig. 9   Serum concentration-time profiles of anti-IL1R IgG4 WT in two cynomolgus

monkeys (Fig. 9a: animal 482KF03; Fig. 9b: animal 1691KF04) after single i.v.

administration of 0.5 mg/kg. Data are the concentrations of antibody measured in four

different bridging ELISA formats: "Total antibodies" (swapped and non-swapped

antibodies with at least one Fab arm for the specific antigen IL1R), "Bivalent antibodies"

(non-swapped only antibodies with bivalency and mono-specificity for IL1R), "Double

human Fc antibodies" (non-swapped only antibodies with two human Fc paratopes) and

"Bispecific antibodies" (swapped only antibodies with one human and one cynomolgus

monkey Fc).

Fig. 10 Serum concentration-time profiles of anti-AG IgG4 double mutant SPLE in two

cynomolgus monkeys (Fig. 10a: animal 2-18; Fig.10b: animal 7-1) after single i.v.

administration of 1 mg/kg. Data are the concentrations of antibody measured in four

different bridging ELISA formats: "Total antibodies" (swapped and non-swapped

antibodies with at least one Fab arm for the specific antigen AG), "Bivalent antibodies"

(non-swapped only antibodies with bivalency and mono-specificity for AG), "Double

human Fc antibodies" (non-swapped only antibodies with two human Fc paratopes) and

"Bispecific antibodies" (swapped only antibodies with one human and one cynomolgus

monkey Fc).

Tables

TABLE 1

Pharmacokinetic parameters for total anti-IL1R IgG4 WT (swapped and non-swapped) in

cynomolgus monkeys after single i.v. 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 ± SD of the
pharmacokinetic parameters determined from six animals.

Dose (mg/kg)	0.5
C_{max} (µg/mL)	16.6 ± 2.08
$AUC_{0-\infty}$ (µg·h/mL)	2890 ± 553
CL (mL/h/kg)	0.19 ± 0.03
Vd_{SS} (mL/kg)	96.6 ± 9.04
$t_{1/2\beta}$ (h)	401 ± 90.0

TABLE 2

Pharmacokinetic parameters for total anti-AG IgG4 SPLE (swapped and non-swapped)

in cynomolgus monkeys after single i.v. administration of 1mg/kg

Serum concentrations were determined in a bridging ELISA which detected all antibodies with at least one Fab arm for the specific antigen AG. Data are the individual pharmacokinetic parameters for two animals .

Animal	7-1	2-18
C_{max} (µg/mL)	20.1	28.5
$AUC_{0-\infty}$ (µg·h/mL)	5,656	5,550
CL (mL/h/kg)	0.17	0.18
Vd_{SS} (mL/kg)	40.8	35.2
$t_{1/2\beta}$ (h)	318	199

Figure 1

a)

SA-MTP   Antigen A-Bi   Sample   Antigen B-Dig   <Dig>-HRP
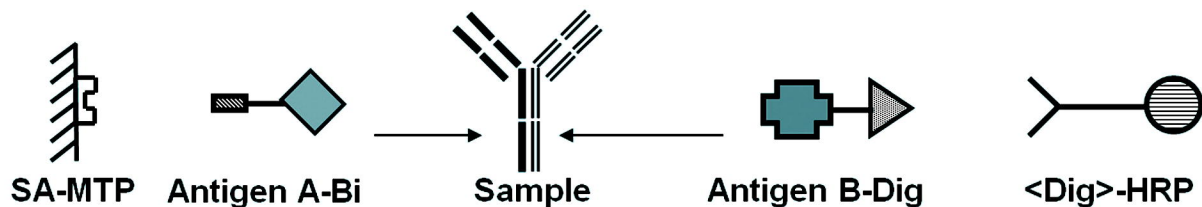
b)

Total antibodies: swapped and unswapped antibodies

SA-MTP   Antigen-Bi   Sample   <HumanFc>-Dig   <Dig>-HRP

Bivalent antibodies: unswapped antibodies

SA-MTP   Antigen-Bi   Sample   Antigen-Dig   <Dig>-HRP
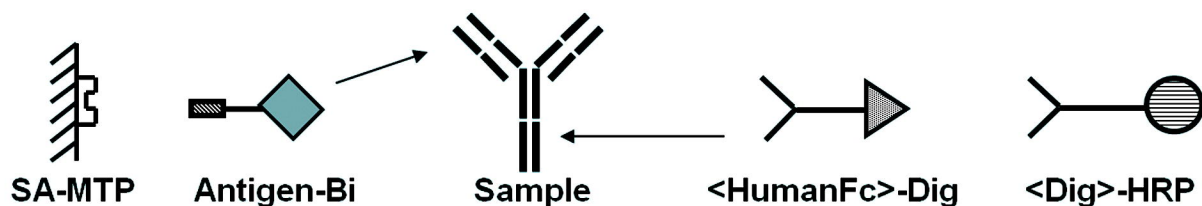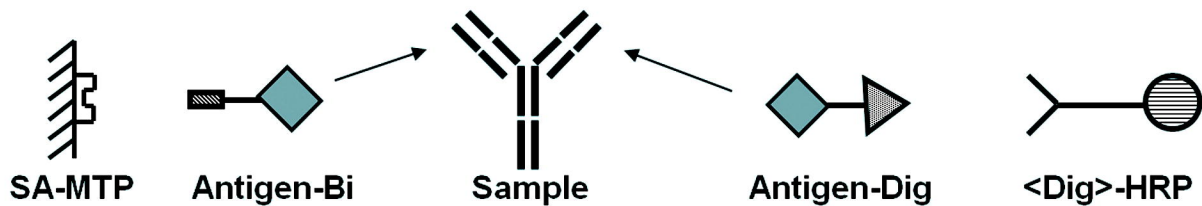
Double human Fc antibodies: unswapped antibodies

SA-MTP   <HumanFc>-Bi   Sample   <HumanFc>-Dig   <Dig>-HRP
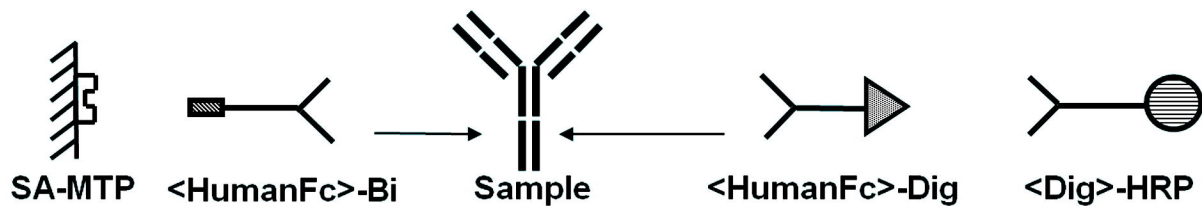
Bispecific antibodies: swapped antibodies

SA-MTP   <HumanFc>-Bi   Sample   <MonkeyFc>-Dig   <Dig>-HRP
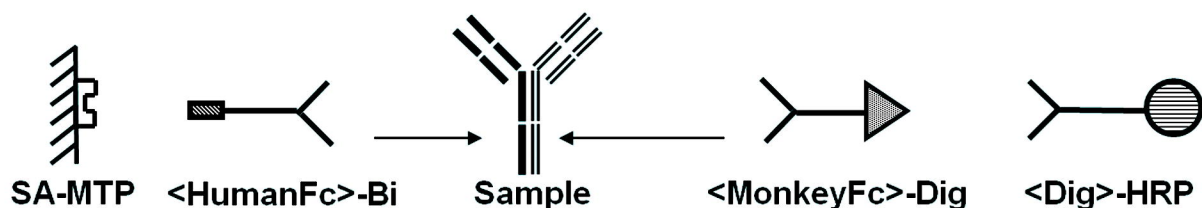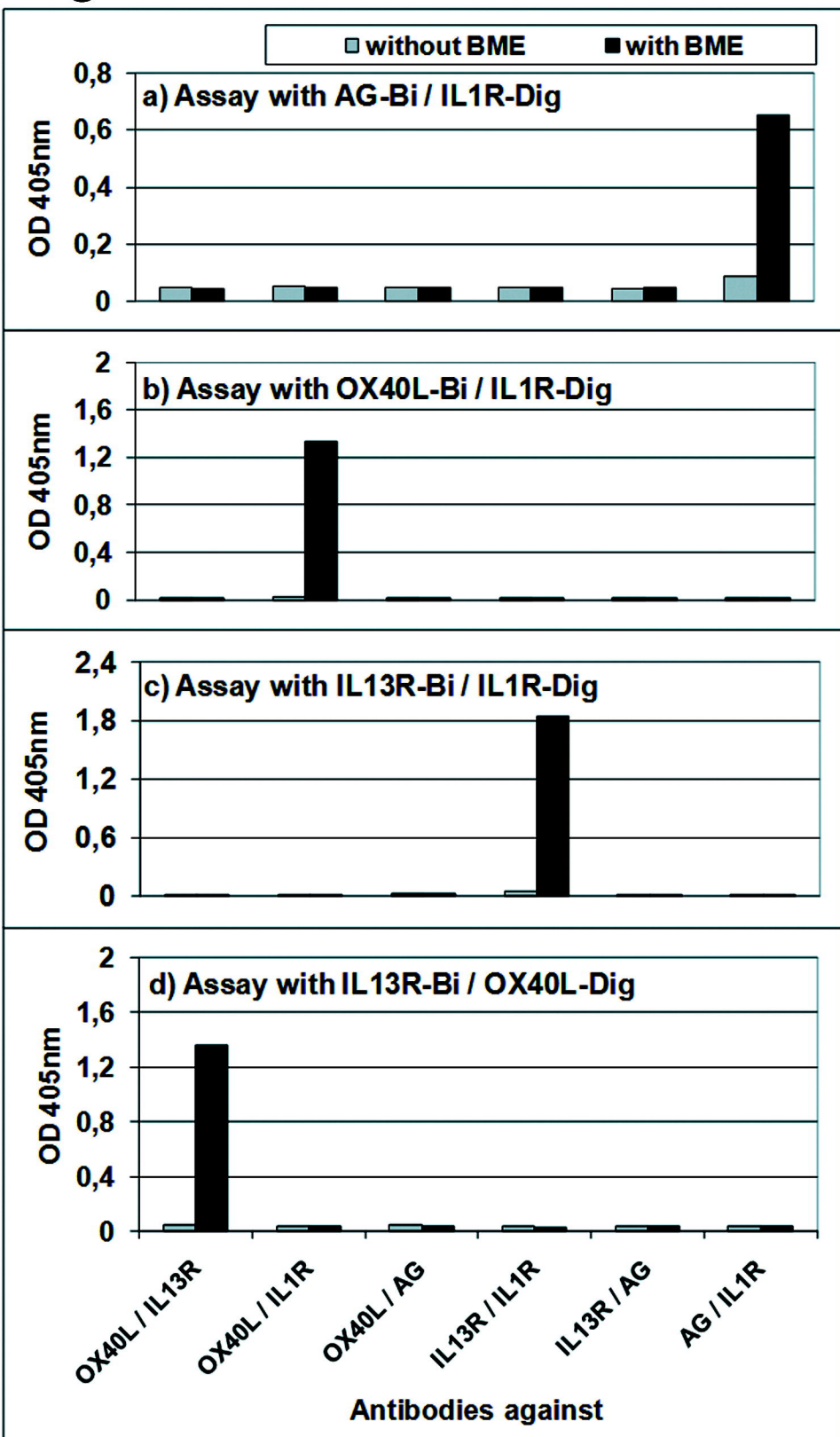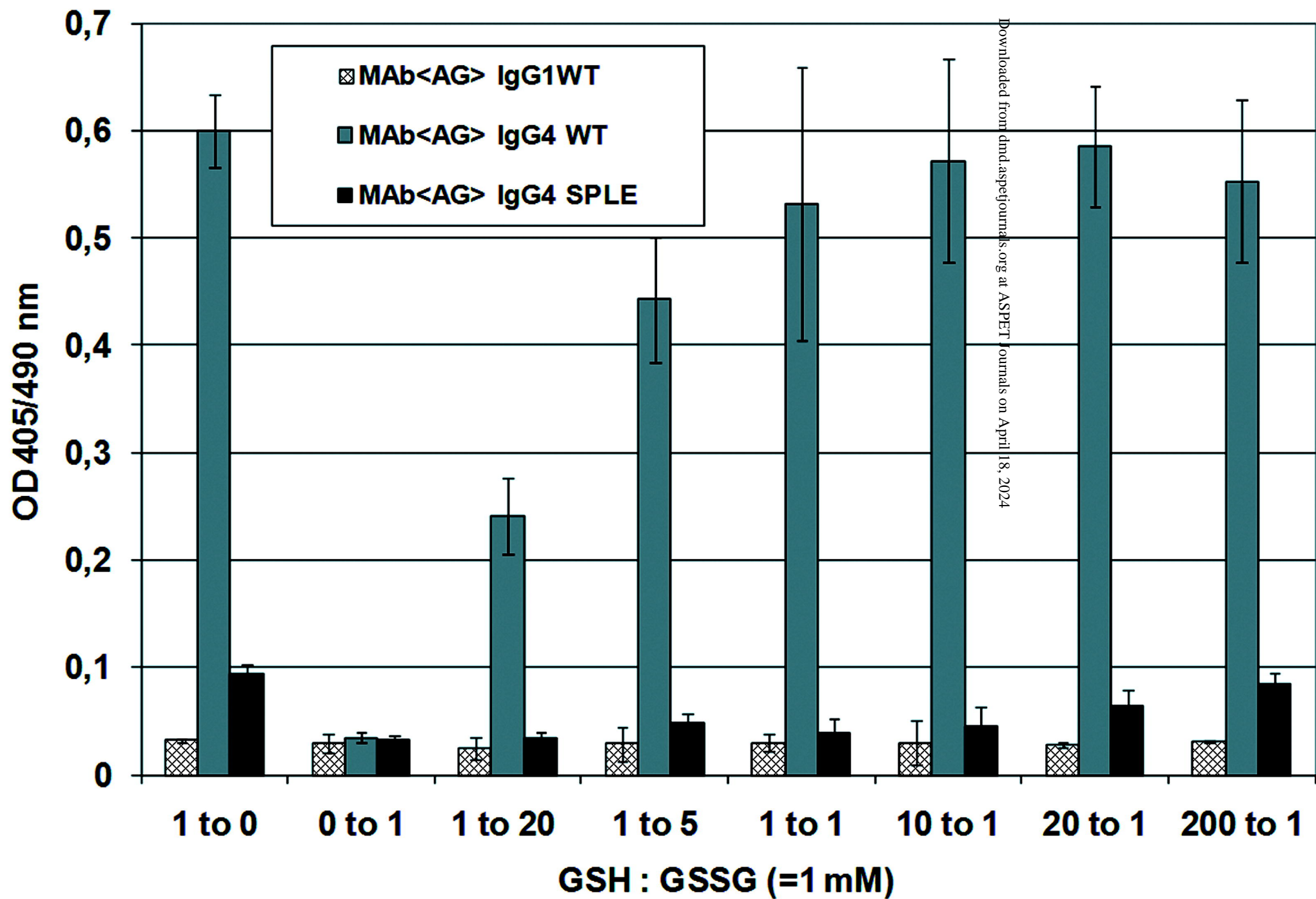
Figure 2

Figure 3

Figure 4
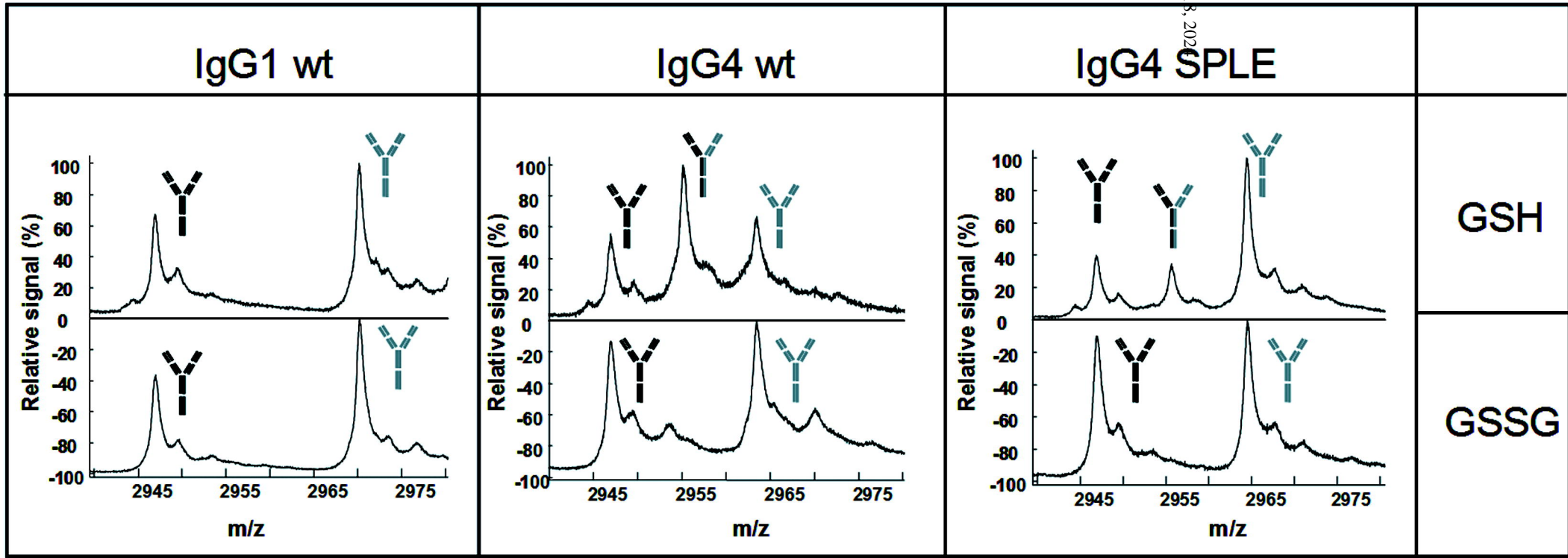
Figure 5

Figure 6

Figure 7

Figure 8

Figure 9

a)

482

b)

1691

Figure 10

a) 7-1

b) 2-18