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Human constant regions influence the antibody binding characteristics of mouse–human chimeric IgG subclasses

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SUMMARY

Although antibody affinity is primarily determined by immunoglobulin variable region structure human IgG antibodies of the four subclasses specific for the same antigen have been shown to differ in their affinity. To explore the influence of the immunoglobulin constant region on functional antibody affinity, a set of V region identical mouse–human chimeric IgG subclasses specific for TAG72 (tumour-associated glycoprotein) were studied. Biomolecular interaction analysis (BIA) was used to determine the binding kinetics of whole IgG subclasses and $F(ab')_2$ fragments. Despite identical V regions, binding kinetics differed for the four subclasses. The apparent dissociation rate constants of the intact immunoglobulins ranked IgG4 < IgG3 < IgG2 < IgG1. In contrast, analysis of the binding characteriztics of the $F(ab')_2$ fragments derived from IgG1, IgG2 and IgG4 revealed identical binding kinetics. The structure of the constant regions of the humanized IgG subclass antibodies clearly influenced functional antibody affinity, as has been described for the murine IgG subclasses. The exact mechanism for this phenomenon remains obscure but such differences should be taken into account when designing or choosing antibodies for therapeutic use.

INTRODUCTION

Antibody binding affinity is defined as the strength of the interaction between a monovalent antibody and monovalent antigen whereas the bivalent binding of an antibody to a complex antigen is referred to as functional affinity or avidity.[1] The affinity of an antibody is determined by the 'fit' of the antigen in the binding groove of the Fab portion of the antibody formed by the heavy- and light-chain variable regions. Following encounter with an antigen *in vivo* an increase in antibody affinity over time is observed and this is known as affinity maturation.[2] Affinity maturation has been shown to be due to a combination of clonal selection and somatic hypermutation,[3] the latter resulting in a high rate of mutation in the complementarity determining regions (CDR) of the variable region genes. The functional affinity of an antibody plays a crucial role in determining biological activity such as the ability to clear antigen, with high affinity antibody being superior.[4] In the assessment of the immune response to vaccines and natural infection it is important therefore to characterize an antibody in terms of functional affinity as well as titre.

Whilst antibody affinity is predominantly determined by variable region structure, human antibodies of different IgG subclasses which bind the same antigen have been shown to differ in functional affinity. This was first described for tetanus toxoid[5,6] but has also been shown for a variety of other antigens including hepatitis B surface antigen,[7] *Moraxella catarrhalis*[8] and the neo-antigen keyhole limpet haemocyanin.[9]

The underlying mechanisms for differences in the functional affinity of IgG subclasses specific for the same antigen are at present not understood, but may be due to differences in epitope specificity and consequently different variable (V) region gene usage or differences secondary to the constant region structure. The latter hypothesis is supported by recent evidence that murine antibody structure remote from the V region may influence affinity and function.[10–15]

To explore the role of the human constant region in influencing functional antibody affinity we have utilized a set chimeric antibodies having different human IgG subclass constant regions linked to identical murine V regions directed against a mucin-like glycoprotein (TAG72, tumour-associated glycoprotein) purified from the human colonic cancer xenograft, LS174T.[16] This molecule is found in large amounts on bovine submaxillary mucin which has been utilized as the target antigen in these studies. Avidity and binding kinetics of the mouse–human chimeric antibodies were investigated by biomolecular interaction analysis (BIA) using a BIAcore™ instrument (Biosensor AB, Uppsala, Sweden). This technology utilizes surface plasmon resonance to detect binding events at

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the surface of a sensor chip composed of a dextran matrix to which binding partners of interest may be immobilized, allowing binding to be observed in real time and rate constants to be calculated.[17,18]

MATERIALS AND METHODS

B72.3 monoclonal antibodies

B72.3 chimeric cell lines (IgG1, Mouse Myeloma Cell; IgG2, IgG3, IgG4, Chinese Hamster Ovary) were obtained from Celltech Therapeutics Ltd (Slough, UK). For antibody purification cells were allowed to grow to saturation in 0·5 l Dulbecco's modified Eagle's minimal essential medium (DMEM) (Gibco, Paisley, UK) supplemented with 10% fetal calf serum (FCS; Globepharm, Esher, UK), 50 U/ml of penicillin, 50 μg/ml streptomycin (Gibco) and 1% non-essential amino acids (Gibco). Culture medium was then centrifuged, the supernatant filtered through a 0·45 μm filter and 1 l concentrated to approximately 10 ml using stirred concentrator cells (Amicon, Stonehouse, UK). The antibodies were purified by affinity chromatography on a Sephadex–Protein G column using fast protein liquid chromatography (FPLC; Pharmacia LKB Biotechnology, Uppsala, Sweden). A buffer of 0·1 M glycine–HCl pH 2·7 was used to elute the bound antibody and the pH of this eluate was immediately neutralized with solid Tris. Antibodies were then concentrated and dialysed against phosphate-buffered saline (PBS) pH 7·2 containing 0·01% sodium azide using Centriprep-10 concentrators (Amicon). The presence of both heavy and light chains was confirmed using reducing 10% sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS–PAGE) under reducing conditions and the absence of aggregates confirmed by non-reducing SDS–PAGE. The antibodies had the expected molecular weight (including the expected earlier elution position of the IgG3 product) following size exclusion gel filtration employing a Superdex-200 column (Pharmacia) linked to an FPLC system. The subclass composition of each chimeric antibody preparation was then analysed by antigen-specific enzyme-linked immunosorbent assay (ELISA), employing bovine submaxillary mucin (Sigma) as a capture antigen and mouse anti-human IgG subclass monoclonal antibodies (mAb) (Zymed, San Francisco, CA) to detect specific subclasses, in order to exclude contamination. Finally, protein concentrations were determined by measuring absorbance at 280 nm and assuming a value of $A^{1\%}_{1cm,280}$ of 13·6.

Preparation of $F(ab')_2$ fragments

$F(ab')_2$ fragments were obtained from the IgG1, IgG2, IgG3 and IgG4 subclasses by pepsin digestion. Antibodies were dialysed against 0·2 M sodium acetate buffer pH 4·5 overnight and pepsin (Sigma) was added at the following previously determined optimal enzyme:substrate ratios; IgG1, 1:100 for 10 hr; IgG2, 1:500–1000 for 1 hr; IgG3, 1:100 for 2 hr; and IgG4, 1:500–1000 for 1 hr. All digestions were carried out at 37° and the reaction was stopped by neutralization with 2 M Tris. $F(ab')_2$ fragments were then separated using a Superdex-200 (Pharmacia) column linked to an FPLC system and appropriate fractions were used without further concentration. The protein concentration of each preparation was determined by absorbance at 280 nm assuming a value of $A^{1\%}_{1cm,280}$ of 15·0.

Measurement of binding kinetics by biospecific interaction analysis

Antibody binding kinetics were measured using the BIAcore™ system (Biosensor AB) which permits real-time biospecific interaction analysis (BIA) by allowing analyte to pass over a sensor chip, to which antigen is covalently bound. Binding is detected by a change in refractive index expressed as resonance units (RU) depicted as a sensorgram. Analysis of the sensorgram permits the determination of rate constants.

Immobilization and kinetic measurements

Bovine submaxillary mucin (Sigma) was immobilized onto the dextran matrix of a CM5 sensor chip (Biosensor AB) in 10 mM citrate buffer pH 3·8. The dextran layer was activated for 7 min at a flow rate of 5 μl/min using an amine coupling kit (Biosensor AB) containing *N*-ethyl-*N'*-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinamide (NHS). Approximately 900 resonance units (RU) of mucin were immobilized to the sensor chip by passing through the flow cell 35 μl of 200 μg/ml mucin in citrate buffer at a flow rate of 5 μl/min. Remaining active sites were then blocked with 1 M ethanolamine–HCl pH 8·5. Various concentrations of mAb were then passed over the immobilized mucin for kinetic analysis. After each measurement, bound antibody was washed out with 10 M NH_4SCN with no significant loss of immobilized mucin.

Kinetic calculations

Apparent rate constants k_a and k_d were calculated using BIAevaluation software from Biosensor AB. The interaction was treated as monovalent of the type antibody (Ab) + antigen (Ag) ↔ AbAg to simplify the mathematical treatment of the data, even though a portion of the AbAg complex is likely to have become an Ab2Ag complex. Detailed descriptions of the mathematical analysis have been reviewed extensively elsewhere.[19,20]

RESULTS

Kinetic measurements using real-time biospecific interaction analysis

The purity of the chimeric B72.3 mAb was checked by SDS–PAGE, specific ELISA and size exclusion gel filtration and found to be free of contaminating proteins, free of aggregates and to consist entirely of the relevant subclass (data not shown). Mucin-binding sensorgrams were obtained for all four B72.3 IgG subclasses and $F(ab')_2$ fragments of IgG1, IgG2 and IgG4 over a range of concentrations. The low yield of $F(ab')_2$ fragments from IgG3 precluded kinetic analysis. Each IgG subclass had different binding characteristics (Fig. 1). Maximal resonance units achieved for each subclass during the time-course of the injection differed with IgG4 having the highest maximal response. While antibodies at the same concentration might be expected to have similar maximal resonance units, the interaction curves observed here displayed a degree of heterogeneity. Furthermore, dissociation curves for the four antibodies showed subtle differences. IgG1 and IgG2 both demonstrated rapid initial dissociation phases while the dissociation curves for IgG3 and IgG4 appeared to be slower. These differences were observed at each concentration of antibody investigated.

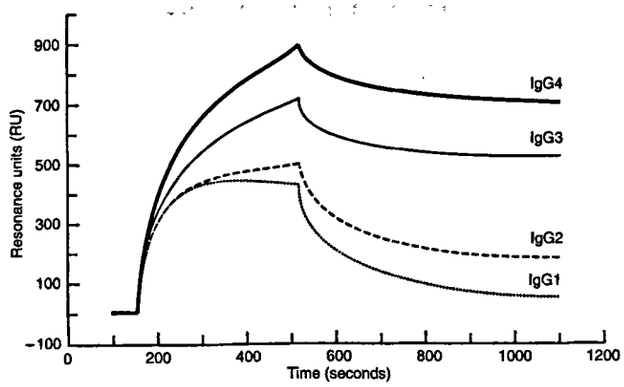

Figure 1. Sensorgrams for all four human IgG subclasses binding to mucin covalently attached to a sensor chip. Each preparation was analysed at a concentration of 170 nM.

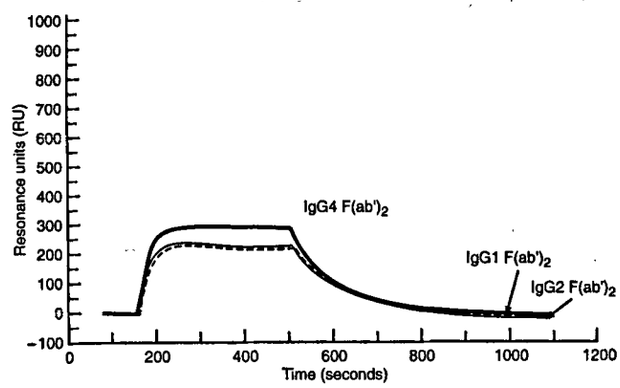

Figure 2. Sensorgrams obtained for $F(ab')_2$ fragments of human IgG1, IgG2 and IgG4 binding to mucin. All preparations were examined at a cocentration of 250 nM. The sensorgrams for the IgG1 and IgG2 fragments are virtually superimposable.

In contrast to the intact parent antibodies, binding curves for the $F(ab')_2$ fragments of IgG1, IgG2 and IgG4 were strikingly similar to each other, showing rapid association followed by rapid dissociation (Fig. 2). For IgG2 and IgG4 there was a significant difference in the pattern obtained using the intact antibodies compared with the patterns obtained with the $F(ab')_2$ fragments.

Apparent rate constants

Association and dissociation rate constants were calculated for each IgG subclass and $F(ab')_2$ fragment studied. A range of five different concentrations of each antibody were analysed. Apparent association rate constants were found to be similar for both intact antibodies and $F(ab')_2$ fragments (Table 1). However, apparent dissociation rate constants were found to differ between the IgG subclasses with IgG1 having the fastest dissociation rate and IgG4 the slowest dissociation rate. The $F(ab')_2$ fragments were found to have dissociation rates similar to each other and all were faster than the respective intact antibodies.

DISCUSSION

We have utilized V region identical humanized antibodies of the four IgG subclasses to show that despite possessing identical V regions such antibodies exhibit differences in apparent dissociation rate constants. Whilst standard techniques of affinity measurement permit the derivation of equilibrium constants, BIA provides detailed analysis of the association and dissociation phases of the antibody–antigen interaction. The antibodies studied here showed a degree of heterogeneity in their association phase, although the major differences in the antibody kinetics was in their respective dissociation rates. Such differences appear to be due to the influence of the constant region on antibody binding, as shown by their abolition following the removal of the constant region by pepsin digestion.

Morelock *et al.*, using the less sensitive technique of competitive binding ELISA, were able to show functional affinity differences between mouse–human chimeric, V region-identical IgG1, IgG2 and IgG4 antibodies specific for intracellular

Table 1. Apparent rate constants of intact chimeric IgG subclass proteins and peptic $F(ab')_2$ fragments studied*

IgG subclass	Antibody range (nM)	k_a M^{-1} second^{-1} ($\times 10^5$)	k_d second^{-1} ($\times 10^{-3}$)
IgG1	170–10	1·92 (±0·08)	3·21 (±0·13)
IgG2	170–10	1·98 (±0·13)	2·56 (±0·03)
IgG3	150–20	1·41 (±0·10)	1·16 (±0·06)
IgG4	170–10	1·68 (±0·18)	0·69 (±0·06)
IgG1 $F(ab')_2$	250–15	1·62 (±0·03)	4·01 (±0·15)
IgG2 $F(ab')_2$	250–15	2·09 (±0·08)	3·93 (±0·12)
IgG4 $F(ab')_2$	250–15	1·93 (±0·13)	3·71 (±0·10)

* Rate constants were calculated as the mean ± standard error of a range of concentrations of antibodies. The same portion of the binding curve was chosen for analysis for each antibody concentration. Results are expressed as the mean value obtained from five different antibody concentrations, molar values were determined assuming a molecular weight of 146 000 MW for IgG1, IgG2 and IgG4, 170 000 MW for IgG3 and 100 000 MW for $F(ab')_2$ fragments

adhesion molecule-1.[14] In their study the functional affinity of the antibodies was ranked IgG1 > IgG4 > IgG2 suggesting that the functional affinity differences could be explained by the respective subclass hinge flexibilities. The greater flexibility of the IgG1 hinge region may permit bivalent binding, in contrast to that of IgG2, the isotype thought to have the least flexible hinge region.[21–24] In our study, however, IgG1 and IgG2 had similar binding kinetics suggesting that hinge flexibility is unlikely to be the sole explanation for the differences noted above.

Differences in the functional affinity of IgG subclasses have been investigated more extensively in the murine system. Fulpius *et al.* showed that an IgG1 switch variant of an IgG3 parent lacked the expected rheumatoid factor activity despite having identical V regions.[12] Similarly, Schreiber *et al.* demonstrated functional affinity differences between an IgG1 switch variant of a V region-identical IgG3 parent specific for a *Pseudomonas* species, with IgG1 being of lowest avidity.[13] Cooper *et al.* have demonstrated differences in binding kinetics determined by BIA between mouse IgG1, IgG2b and IgG3 directed against *N*-acetyl-glucosamine (GlcNAc) of streptococcus group A carbohydrate with IgG3 being of the highest affinity.[15] The authors have suggested that the higher functional affinity of mouse IgG3 specific for GlcNAc is due to molecular co-operativity of IgG3, whereby IgG3 antibodies bound in close proximity to antigen undergo non-covalent Fc–Fc interactions stabilizing the complex. This might explain the consistent finding of higher IgG3 functional affinity in the murine literature although it is unlikely to explain differences in human IgG subclasses as they do not appear to display co-operative binding.[25] Furthermore, the human functional equivalent of murine IgG3 is IgG2 which is thought to have the least hinge flexibility[21–24] while human IgG3, in contrast to all the murine IgG subclasses, has a long flexible hinge. Such differences highlight the restricted structural and functional homology between the mouse and human IgG subclasses and underlines the danger in extrapolating data from the murine to the human IgG system.[26]

The functional significance of the differences in the apparent dissociation rate constants demonstrated here are as yet, unclear. However, the ability to analyse separately association and dissociation kinetics by BIA may prove crucial in the understanding of certain biological phenomena. Foote and Milstein have shown that the association constant of antibodies specific for the hapten 2-phenyl-5-oxazalone may be critical for B-cell selection and that concurrent with affinity maturation there is also kinetic maturation.[27] With regard to antibody function, the neutralizing capacity of a panel of antibodies reactive with the V3 loop of human immunodeficiency virus has recently been shown to correlate directly with dissociation rate.[28]

It appears likely that the differences observed here in the binding kinetics of chimeric mouse–human IgG subclasses may be due to structural differences in the human constant regions although the exact mechanism underlying such differences is at present unclear. In addition it remains to be established whether such differences exist between the different subclasses in a naturally occurring antibody response. Engineered antibodies may differ in their functional affinity and hence in their function, depending upon their subclass. Such consideration may be of importance when considering the choice of antibodies for therapeutic use.

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