



ANTIGEN-BINDING SITES DOMINATE THE SURFACE PROPERTIES OF IgG ANTIBODIES

CHRISTER WINGREN,* ULLA-BRITT HANSSON,*†

CARL G. M. MAGNUSSON‡ and M. OHLIN§

*Department of Biochemistry, University of Lund, S-221 00 Lund, Sweden; †Department of Clinical Immunology, Karolinska Institute, Stockholm, Sweden and §Department of Immunotechnology, University of Lund, Lund, Sweden

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Abstract—A new technique, liquid–liquid partition chromatography in an aqueous polyethylene glycol–dextran two-phase system, was used to detect differences in surface properties of antibodies with different antigen-binding sites. Employing well-characterized monoclonal IgG antibodies and Fab and Fc fragments thereof as well as chimeric IgG antibodies we found a remarkable relationship between structure of the antibody combining site and chromatographic behaviour. The surface properties of the IgG antibodies were dominated by those of its antigen-binding regions. In addition, our results indicated that the constant parts of the IgGs form similar scaffoldings, on to which CDRs of variable shapes and sizes are interspaced and constitute the major dominant differences in exposed surface properties.

Key words: antigen-binding sites, surface properties, IgG antibodies, LLPC, Partition chromatography.

INTRODUCTION

The examination of the properties of exposed surfaces of antibody molecules is of major interest as several structural features of importance in the biological function of the molecules are still not entirely understood. The antibody is a Y-shaped molecule in which the two Fabs are linked to Fc by the hinge region. The antigen-binding sites, located at the tips of the Fabs, are highly solvent-exposed structures formed by six hyper-variable polypeptide loops (Getzoff *et al.*, 1988; Poljak, 1991) which determine the specificity and affinity of the binding site (Colman, 1988; Davies and Chacko, 1993; Padlan, 1994). The hinge region and the Fc part, the structure of which varies in the different antibody classes and isotypes (Burton *et al.*, 1986; Jefferis, 1990), are responsible for triggering effector functions which eliminate antigens (Brüggemann *et al.*, 1987; Burton and Woof, 1992; Valim and Lachmann, 1991).

Both the antigen-binding properties and effector functions are dependent on the overall conformation of the intact molecule. However, the direct methods available for the determination of detailed molecular structures, such as NMR and X-ray crystallography, are restricted to the analysis of mainly fragments of individual monoclonal antibodies (Campbell, 1991; Padlan, 1994). Several indirect methods can, on the other hand, be used to examine the surface properties of intact antibody molecules, although most of these techniques

detect a single property such as charge, hydrophobicity or shape. In the case of partitioning in aqueous polyethylene glycol–dextran two-phase systems, the technique can also be designed to detect differences in the overall surface properties, i.e. three-dimensional structures and general surface properties (Albertsson, 1978, 1986; Diamond and Hsu, 1990; Johansson, 1985; Walter *et al.*, 1991).

A column chromatographic approach, liquid–liquid partition chromatography (LLPC), which increases the selectivity and sensitivity of the two-phase technique, has been developed (Müller, 1986, 1994; Hansson *et al.*, 1989; Hansson and Wingren, 1995; Wingren *et al.*, 1994). We have previously demonstrated LLPC to be a rapid analytical method with which even large and flexible biomolecules can be analysed (Andersson *et al.*, 1993; Wingren *et al.*, 1992, 1994). Conformational changes occurring upon the binding of a ligand by enzymes or antibodies may be detected (Andersson *et al.*, 1993; Wingren and Hansson, 1995) and our results have even indicated that the partition properties of antibodies are related to the structure of their antigen-binding sites (Andersson *et al.*, 1993; Hansson *et al.*, 1989).

The purpose of this study was to explore the possible relationship between the properties of exposed surfaces, as detected by LLPC, and the biological functions of well-characterized IgG antibodies and fragments thereof. We showed that there was indeed a relationship between the chromatographic behaviour and the structure of the antibody-combining site. The antigen-binding regions of IgG were thus very dominant structures and responsible for the major differences in surface properties of IgGs or Fab fragments thereof which were detected by LLPC.

†Author to whom correspondence should be addressed.

MATERIALS AND METHODS

Materials

LiParGel 650 was obtained from Merck AG (Darmstadt, Germany). Protein A-Sepharose 4 Fast Flow, protein G-Sepharose 4 Fast Flow, Sepharose CL-4B and Ultropac TSK G 3000 SW column (600 × 7.5 mm ID) were produced by Pharmacia Biotech Norden AB (Uppsala, Sweden). Dextran T 500 ($M_r = 500,000$) was obtained from Pharmacia, and polyethylene glycol 8000 (PEG) ($M_r = 6000$ –7500) from Union Carbide (New York, U.S.A.).

Chimeric mouse/human IgG 1, 2, 3 and 4 antibodies against 5-iodo-4-hydroxy-3-nitrophenacetyl (NIP) (Brüggemann *et al.*, 1987) and NIP conjugated to bovine serum albumin were gifts from Dr L. Hammarström (Clinical Immunology, Huddinge Hospital, Sweden). Human monoclonal IgG 1 antibodies against cytomegalovirus and tetanus toxoid were prepared as described previously (Ohlin *et al.*, 1993). Mouse monoclonal IgG1, 2a or 2b antibodies against thyroxine and tri-iodothyronine were purchased from Biodesign International (Kennebunkport, U.S.A.) and OEM Concepts (Toms River, U.S.A.), and against human albumin and different epitopes on human IgG from Sigma and The Binding Site (Birmingham, U.K.). Rat monoclonal IgG1, 2a and 2b antibodies against 2,4-dinitrophenol were obtained from Zymed Laboratories (San Francisco, U.S.A.).

Isolation and characterization of IgG antibodies

IgG was purified by affinity chromatography on protein G-Sepharose [chimeric IgG, polyclonal IgG (from normal human serum), myeloma IgG (from human myeloma sera)], protein A-Sepharose (anti-tetanus toxoid), IgG-Sepharose (anti-IgG) or on albumin-Sepharose (anti-albumin) as described elsewhere (Hansson *et al.*, 1985; Ohlin *et al.*, 1993; Wingren *et al.*, 1994). The purified fractions contained >95% IgG and no aggregates (<2%), as determined by SDS-PAGE (Laemmli, 1970), size-exclusion chromatography (HPLC-SE) on the Ultropac TSK G 3000 SW column, immunoelectrophoresis (Scheidegger, 1955) and by double immunodiffusion (Ouchterlony, 1962). The protein concentration was determined by absorbancy at 280 nm ($E_{280\text{ nm}}, 1\% = 12.5$).

The net charge of IgG was determined by agarose gel electrophoresis (0.8% agarose in 75 mM barbital buffer, 2 mM calcium lactate, pH 8.6).

The light chain isotype of human IgG was determined by immunofixation (Ritchie and Smith, 1976) and the heavy chain isotype was determined by competitive two-step ELISA (Lilja *et al.*, 1990).

The affinities of monoclonal anti-2,4-dinitrophenol antibodies for non-conjugated, unlabelled 2,4-dinitrophenol (Sigma) were determined by equilibrium dialysis (Steward, 1986). The other affinity constants of antibody for antigen were determined by the manufacturers (Ohlin *et al.*, 1993; data sheets from manufacturers).

Preparation of Fab and Fc fragments

Papain (Sigma), 1–5% (w papain/w IgG) was added to 0.5–1 mg IgG dialysed against 0.1 M sodium phosphate, 10 mM cysteine-HCl, 2 mM EDTA, pH 7.0, and the mixture was incubated for 12–24 hr at 37°C. The mixture was then dialysed against 75 mM Tris, 75 mM NaCl, 0.1 mM CaCl_2 , pH 8.1, and immediately analysed with LLPC. Immunoelectrophoresis, agarose gel electrophoresis, HPLC-SE and SDS-PAGE showed that more than 90% of the IgG had formed Fab and Fc fragments. The fragmentation was performed at least twice for each sample and the physico-chemical properties of these fragments, obtained at different occasions, were identical. Identical results were obtained using LLPC whether the fragments had been separated by affinity chromatography on immobilized anti-Fc or not prior to the analysis. The amount of papain present in the sample did not affect the LLPC elution profiles.

Liquid-liquid partition chromatography

The LLPC set-up is schematically illustrated in Fig. 1. All experiments were performed in a 4.4% (w/w) PEG 8000–6.2% (w/w) dextran T 500 two-phase system containing 50 mM sodium phosphate, 0.1 M NaCl and 0.1 M glycine at pH 7.0. The two-phase system was prepared by thoroughly mixing 110 g 40% PEG and 248 g 25% dextran with some of the water. Dry sodium chloride, glycine and sodium phosphate were added and pH was adjusted with 4 M HCl before the rest of the water was added until a 1000 g two-phase system was obtained. The system was equilibrated at 20°C for 72 hr and the clear phases were separated. The matrix, LiParGel 650, was equilibrated with the dextran-rich bottom phase

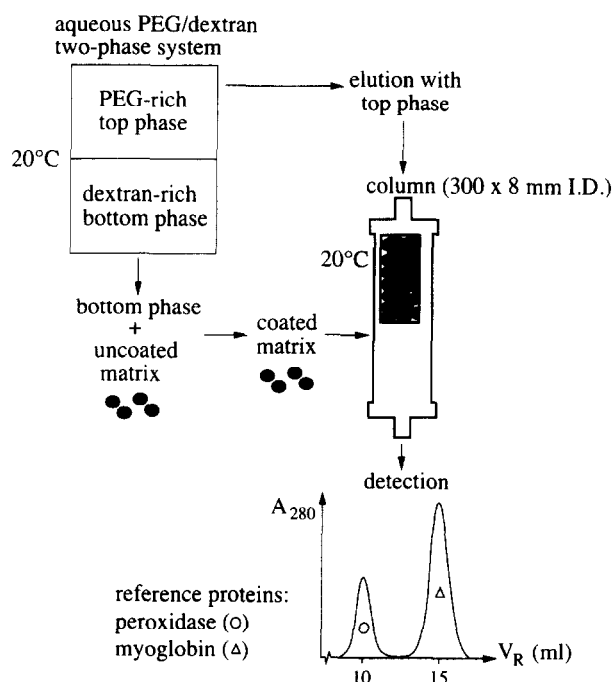


Fig. 1. Schematic illustration of the LLPC set-up. A typical LLPC chromatogram for the reference proteins, peroxidase and myoglobin is shown.

(stationary phase) overnight. The coated matrix was rinsed with the PEG-rich top phase (mobile phase) in order to remove excess stationary phase, suspended in mobile phase and poured into a thermostated (20°C) steel column (300 × 8.0 mm I.D.) with a filling reservoir. The column was first packed under gravitational sedimentation (≤30 hr) and then at flow rates ≤1.0 ml/min (≤25 MPa) using an HPLC pump (2248, Pharmacia) (Wingren *et al.*, 1994). Protein was applied to the column, 20–200 µg, in 0.1 or 0.2 ml mobile phase at a flow rate of 0.12 ml/min. Eluates were continuously monitored at 280 nm. The distribution of human IgG subclasses was determined in collected fractions using subclass specific competitive two-step ELISA (Lilja *et al.*, 1990) and the distribution of Fab and Fc fragments with immunoelectrophoresis. Chimeric anti-NIP antibodies were assayed in collected fractions with ELISA using solid-phase BSA–NIP conjugate as described elsewhere (Magnusson, 1995).

The performance and capacity of the LLPC columns were determined by daily application of two reference proteins, peroxidase (Merck) and myoglobin (Sigma) (20–40 µg), to the columns. The partition coefficients of the reference proteins, K_{batch} , were determined in batch experiments (Wingren *et al.*, 1992). $1/K_{\text{batch}}$ was used as K_C , the ratio of the concentration of a molecule in the two phases:

$$K_C = C_{\text{stationary phase}}/C_{\text{mobile phase}} \quad (1)$$

The volumes of the stationary and mobile phases, V_S and V_M , were calculated from the retention volumes for the references, V_R , using the relationship

$$V_R = V_M + K_C V_S \quad (2)$$

The plate number, N , was calculated from the peak width at half height (w_h) of the myoglobin peak according to

$$N = 5.54(V_R/w_h)^2 \quad (3)$$

The resolution of the peroxidase and myoglobin peaks, R_s , was calculated as

$$R_s = (\sqrt{N/4})(k/(1+k))(\alpha-1), \quad (4)$$

where k is the capacity factor ($k = (V_S/V_M) K_C$) and α is the ratio of the partition coefficients of the references ($\alpha = K_{\text{batch, peroxidase}}/K_{\text{batch, myoglobin}}$).

The parameters of the LLPC columns used were $V_S/V_M = 1.7 \pm 0.2$, $V_S/V_C = 0.42 \pm 0.02$, $V_M/V_C = 0.25 \pm 0.02$, $N = 720 \pm 100$ and $R_s = 3.7 \pm 0.3$, where V_C is the column volume. In order to facilitate the comparison of chromatograms obtained from columns with different parameters, retention volumes were expressed as K_C , according to eqn (2). The relative standard deviation (100 (standard deviation / mean)) of K_C was ≤3%. All LLPC analyses were run 2–5 times.

Statistical analysis of data

A two-tailed *t*-test was used to determine the existence of significant linear correlations [at the 95% significance

level ($P = 0.05$) and ($n - 2$) degrees of freedom] and to test whether two K_C values differ significantly [at the 95% significance level ($P = 0.05$) and ($n_1 + n_2 - 2$) degrees of freedom].

RESULTS

The purpose of this study was to explore the possible relationship between the partition properties of an IgG molecule and its biological functions. The partition properties (expressed as partition coefficients, K_C) of IgG and fragments thereof were examined in an aqueous polyethylene glycol–dextran two-phase system at pH 7, using a column chromatographic approach, liquid–liquid partition chromatography (LLPC) (Fig. 1). The relative standard deviation of K_C was ≤3%.

First, we determined whether differences in physico-chemical properties such as 'shape', net charge or light chain isotype affected the partition properties of IgG. We found no significant linear correlation between K_C and either 'shape' (Fig. 2A), net charge (Fig. 2B) or light chain isotype (Fig. 2C). This was the case not only for intact human and mouse monoclonal IgG1 as shown in Fig. 2, but also for Fab and Fc fragments from all the human IgG subclasses (data not shown). Our results thus showed that the observed differences in partition properties of intact IgG1s or of Fab and Fc fragments could not be exclusively assigned to differences in 'shape', net charge or light chain isotype.

The question was then whether differences in heavy chain isotype were reflected in the partition properties of IgG. The distribution of IgG isotypes was therefore determined within fractions collected upon LLPC analysis of human polyclonal IgG. The results, given in Figs 3A,B, showed that the elution profile of each of the four IgG subclasses was similar to that of the applied polyclonal IgG population. In Fig. 3C the partition properties of several mouse monoclonal IgG antibodies with different heavy chain isotypes are also compared. As for human IgG, the K_C values of the mouse monoclonal IgGs were also found to be independent of heavy chain isotype.

Next, we examined whether or not differences in the variable domains affected the partition properties of IgG (Table 1). Human and murine monoclonal antibodies with known affinities were analysed and were all, except for one anti-thyroxine antibody, eluted as single, homogeneous peaks in LLPC as demonstrated for the anti-CMV gp58 antibodies (Table 1). It was interesting to note that antibodies with identical affinity constants for a hapten (tri-iodothyronine or thyroxine) had identical K_C values. It was even more interesting to observe that antibodies with identical affinity constants for the same epitope on IgG(Fc) also had identical values of K_C . It should also be observed that IgGs with different affinity constants for the same antigen had significantly different K_C values and that the differences in K_C could be large, even when the differences in affinity constants were small, or vice versa. This was the case not only for IgGs against a large antigen (cytomegalovirus) but also for IgGs

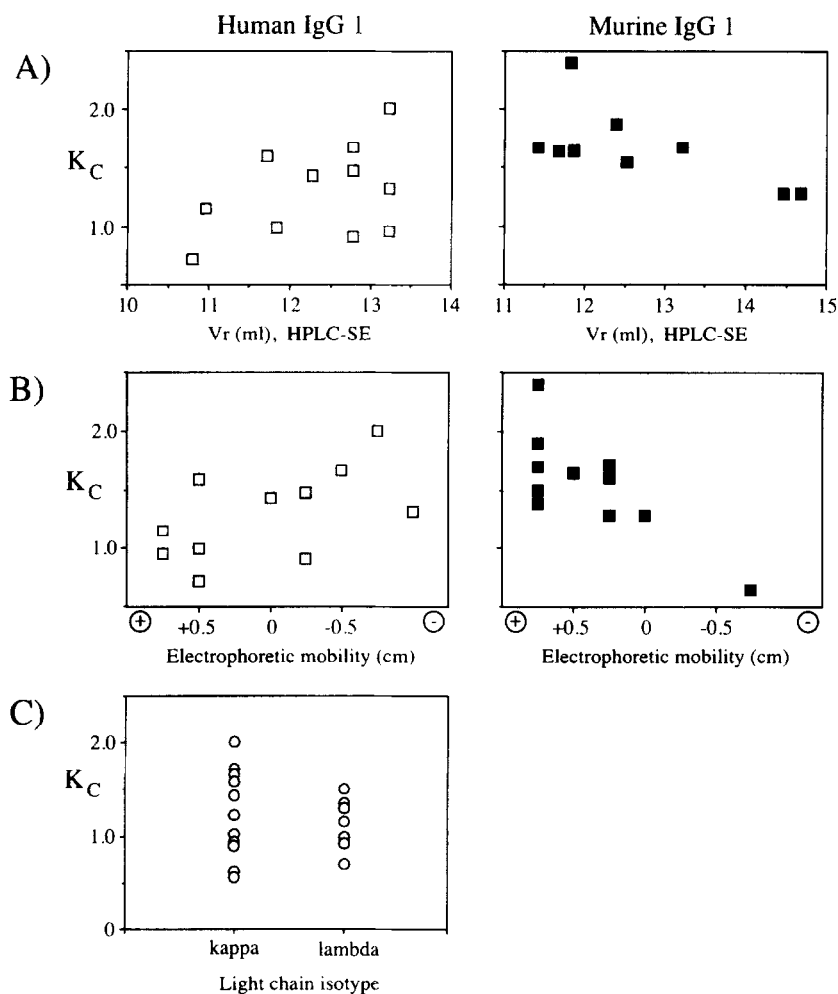


Fig. 2. The dependence of the partition properties of IgG on physico-chemical properties such as (A) 'shape', (B) net charge or (C) light chain isotype. Human and mouse monoclonal IgG 1 antibodies were employed. The partition properties were determined using LLPC (as described in Materials and Methods) and are expressed as K_C according to eqn (2). The 'shape' of the molecules was determined by gel filtration (HPLC-SE) and is expressed as a retention volume (V_r). The net charge of the IgGs was determined by agarose gel electrophoresis and is expressed as electrophoretic migration. The correlation coefficients for human and mouse IgG were (A) 0.18 and 0.42, and (B) 0.31 and 0.59, respectively, and none of them were significant.

against a hapten (2,4-dinitrophenol, tri-iodothyronine or thyroxine). It should be noted that the affinity constant for the anti-thyroxine IgG2b antibody represents the mean value for both the components detected by LLPC. Hence, our results clearly indicated a relationship between partition properties and structure of the antibody combining site, i.e. the surface properties of IgG appeared to be dominated by those of its antigen-binding regions.

The structures determining the partition properties of IgG were further localized by analysing Fab and Fc fragments from human myeloma IgG. A significant ($P < 0.05$) linear correlation was indeed obtained between the partition properties of intact IgG and their corresponding Fab fragments (Fig. 4). In contrast to the Fabs, Fc fragments from IgG1, 2 and 4 had identical K_C values (Fig. 4), again indicating that the differences detected by LLPC between intact IgGs were located on their Fabs. Despite the fact that the partition properties of Fc fragments from human IgG3s differed significantly

not only from those of the other subclasses but also from each other, the K_C of intact IgG3 correlated with that of its Fab fragments (Fig. 4). Thus, the indications that the surface properties of IgG were dominated by those of the antigen-binding regions was further substantiated.

The relationship between structure of an antibody's combining site and its partition properties observed with LLPC was confirmed when chimeric anti-NIP antibodies corresponding to the four human IgG subclasses were analysed (Fig. 5). These antibodies are assembled from identical mouse V_H , V_L and C_L domains but from different human C_H domains. The chimeric antibodies were all eluted as single peaks in LLPC and IgG 1, 2 and 4 had almost identical values of K_C , i.e. differences in the constant part of the heavy chains did not affect the partitioning. The K_C value of IgG 3 deviated significantly from those of the others, indicating the existence of a dominant structure, or structures, even on the Fc part. This unique property of Fc from IgG 3 had, however, only a minor influence on the partitioning of the intact

molecule (cf. Figs 4 and 5). The structure of the antigen-binding region thus appears to be dominant and responsible for the major differences in the surface properties of IgGs or Fab fragments that are detected with LLPC.

DISCUSSION

In this investigation, we have used liquid-liquid partition chromatography (LLPC) in an aqueous PEG-dextran two-phase system as a simple, quick and sensitive method of detecting differences in surface properties of antibodies with different antigen-binding sites. Partitioning in aqueous PEG-dextran two-phase systems is a mild method and as the polymers may even have a protective effect, the conformation of a partitioned protein is not likely to be disturbed (Albertsson, 1986; Walter *et al.*, 1991). The distribution of a molecule between the two phases depends mainly on the balance between its interactions with the phase-forming polymers (Albertsson, 1986; Brooks *et al.*, 1985). The composition of the two-phase system employed was chosen in order to

obtain a selective system in which the partition properties of IgG, soluble at <2 mg/ml mobile phase (Hansson *et al.*, 1989), reflected a combination of its three-dimensional structure and general surface properties (Albertsson, 1978, 1986; Diamond and Hsu, 1990; Johansson, 1985; Walter *et al.*, 1991). Although the support may influence the partitioning in LLPC (Walsdorf and Kula, 1991; Wingren *et al.*, 1992, 1994), the partition properties of the IgGs could still not be related to a single property such as net charge or 'shape' (Fig. 2). The observed differences in partition properties could neither be related to merely the light chain isotype (Fig. 2) nor the heavy chain isotype (Fig. 3).

We found a remarkable relationship between the structure of an antibody's combining site and its chromatographic behaviour, i.e. differences in the variable domains affected the partition properties of the IgGs. Chimeric anti-NIP antibodies, with identical specificity, corresponding to the human IgG 1, 2 and 4 subclasses were found to exhibit almost identical partition properties (Fig. 5). Identical partition coefficients were also obtained for mouse monoclonal antibodies with identical affinity constants for the same hapten or with

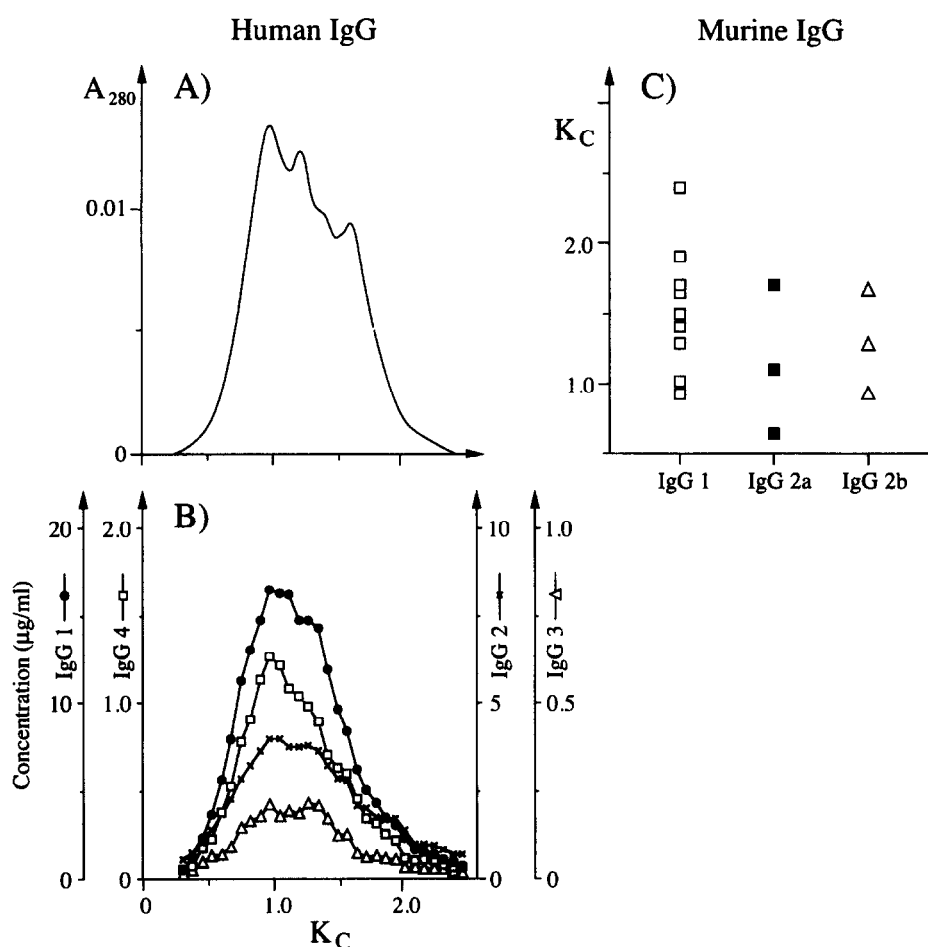
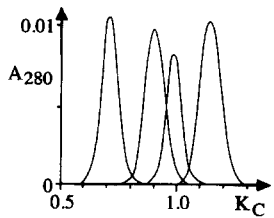


Fig. 3. The dependence of the partition properties of IgG on heavy chain isotype. The partition properties determined using LLPC (as described in Materials and Methods) are expressed as K_C according to eqn (2). (A) shows the LLPC chromatogram for human polyclonal IgG (66% IgG 1, 22% IgG 2, 4% IgG 3 and 8% IgG 4) and (B) shows the distribution of each of the four IgG subclasses determined by ELISA. (C) shows the partitioning of several mouse monoclonal IgG1, 2a and 2b antibodies.

Table 1. The relation between K_C , determined by LLPC (as described in Materials and Methods), and the affinity constant (K) for monoclonal antibodies against cytomegalovirus (CMV), IgG (Fc) or haptens [2,4-dinitrophenol (DNP), tri-iodothyronine (T3) or thyroxine (T4)]. Antibodies with identical affinity constants for the same antigen (epitope) are shaded. The 95% confidence limits of the K_C values are given

Antibody	Subclass	K (M^{-1})	K_C	LLPC chromatograms for anti-CMV gp58 antibodies
anti-CMV gp58	IgG1	$2 \cdot 10^9$	0.71 ± 0.01	
	IgG1	$9 \cdot 10^9$	0.90 ± 0.02	
	IgG1	$1 \cdot 10^9$	0.99 ± 0.01	
	IgG1	$3 \cdot 10^9$	1.15 ± 0.01	
anti-IgG(Fc)	IgG1	x*	1.70 ± 0.02	
	IgG2a	x*	1.70 ± 0.02	
anti-DNP	IgG1	$5 \cdot 10^6$	1.02 ± 0.01	
	IgG2a	$2 \cdot 10^6$	1.10 ± 0.01	
	IgG2b	$10 \cdot 10^6$	0.93 ± 0.01	
anti-T3	IgG1	$1 \cdot 10^{10}$	1.64 ± 0.01	
	IgG1	$1 \cdot 10^{10}$	1.64 ± 0.01	
	IgG2a	$5.2 \cdot 10^9$	0.64 ± 0.01	
anti-T4	IgG1	$1 \cdot 10^9$	1.28 ± 0.01	
	IgG1	$1 \cdot 10^9$	1.28 ± 0.01	
	IgG1	$4 \cdot 10^9$	1.67 ± 0.02	
	IgG2b	$1 \cdot 10^{10**}$	<u>1.28 ± 0.01</u> , 1.67 ± 0.02	

*The affinity constants (epitope specificities) are declared to be identical by the manufacturer although the numerical value is not given.

**The affinity constant represents the mean value for both the components ($K_C = 1.28$ and 1.67) detected by LLPC.

known identical epitope specificity (Table 1). The specificity and affinity of the antigen-binding site are governed by the structure of the complementarity-determining regions (the CDRs) (Colman, 1988; Davies and Chacko, 1993; Padlan, 1994) which are, at least in human and mouse species, limited to a few main-chain conformations (canonical structures) (Cothia *et al.*, 1989). Identical canonical structures do not imply identical antigen binding characteristics, as these properties are defined by the nature and orientation of the side-chains of individual amino acid residues (Cothia *et al.*, 1989; Givol, 1991; Milstein, 1990; Padlan, 1994). Using monoclonal antibodies, we thus confirmed our previous findings that polyclonal rabbit antibodies against different polyvalent antigens were eluted in overlapping but different K_C regions (Andersson *et al.*, 1993; Hansson *et al.*, 1989). Hence, we showed that LLPC could be used to detect differences in the surface properties of the antigen-binding regions.

Antibodies with apparently different specificities were sometimes found to have similar values of K_C . This was, for example, the case for antibodies against T3 and T4 (Table 1). As a high degree of complementarity is

exhibited between the interacting surfaces upon an antigen-antibody interaction (Davies and Chacko, 1993; Davies *et al.*, 1988; Mariuzza and Poljak, 1993; Padlan, 1994), similarities in K_C may reflect similarities in overall conformation of the epitopes that the antibodies are directed against. It was in this connection of interest to note that the haptens, T3 and T4, have similar conformation and that these antibodies against T3 discussed here have been reported to cross-react with T4, and vice versa (data sheets from the manufacturer).

The observed relationship between the structure of the antibody combining site and chromatographic behaviour implied that the surface properties of IgG were, as might be expected from a functional point of view, dominated by those of its antigen-binding sites. This new finding was further substantiated by the fact that the partition properties of intact IgGs correlated with those of their Fabs (Fig. 4). The minor deviations observed (Fig. 4) may be explained by reasoning that the fragmentation altered the conformation of Fab or led to the exposure of new dominant surfaces. Although they have been shown to be highly solvent-exposed (Getzoff *et al.*, 1988; Poljak, 1991), the dominant feature of the antigen-binding sites

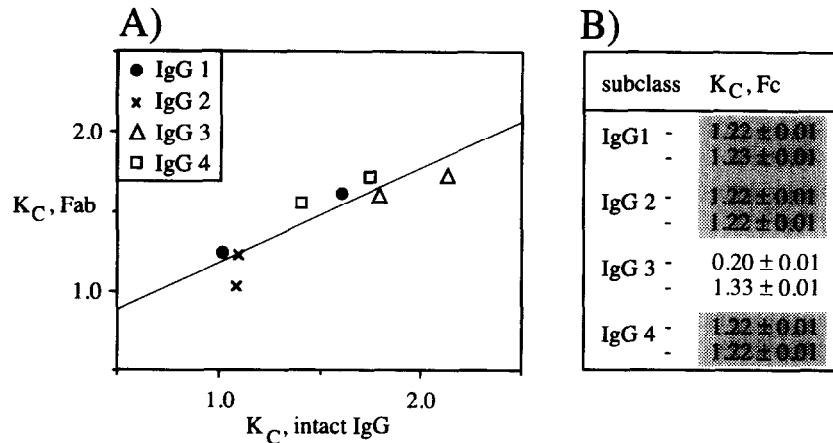


Fig. 4. Comparison of the partition properties of (A) intact IgG with those of the corresponding Fab fragments and (B) Fc fragments from one IgG subclass with those of the other subclasses. Two human myeloma IgG of each subclass were employed. The partition properties were determined using LLPC (as described in Materials and Methods) and are expressed as K_C according to eqn (2). The correlation coefficient was (A) 0.80 ($p < 0.05$). (B) The 95% confidence limits of the K_C values are given. Fc parts with identical K_C values are shaded.

is astounding since these sites constitute only a minor part (about 5%) of the molecule (Owen and Lamb, 1988; Padlan, 1994). Hence, the results obtained here for the IgGs confirmed our previous findings regarding enzymes (Wingren and Hansson, 1995), that LLPC can be used to detect differences in the surface properties of even small functionally important sites on large molecules.

Disregarding the regulatory implications, the fact that anti-idiotypic antibodies are produced *in vivo* may also be taken as yet another indication of the dominant properties of the antigen-binding regions. The fact that antibodies against the Fc part of IgG are produced mainly under abnormal conditions, for example, during some auto-immune diseases, may also strengthen our findings that the surface properties of Fabs are more dominant than those of Fc.

The partition properties of both intact IgGs and of isolated Fc fragments were found to be independent of

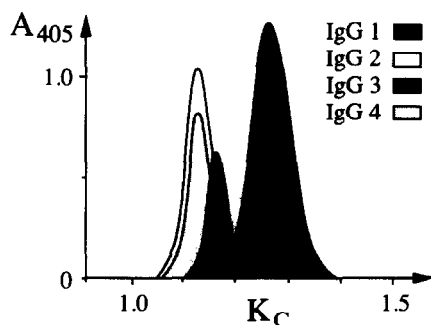


Fig. 5. Comparison of the partition properties of chimeric IgGs corresponding to the four human IgG subclasses having identical antigen specificity. The partition properties were determined using LLPC (as described in Materials and Methods) and are expressed as K_C according to eqn (2). Mouse-human chimeric anti-NIP antibodies were employed. K_C was 1.12 ± 0.02 for IgG 2 and 4, 1.16 ± 0.02 for IgG 1 and 1.26 ± 0.02 for IgG 3, respectively. The 95% confidence limits of the K_C values are given.

their heavy chain isotype (Figs 3, 4 and 5). Again, the less dominant properties of Fc were demonstrated and the results also indicated similarities in overall surface properties of Fc. It has also been shown by others, that the Fc parts from all human IgG isotypes have essentially the same structure (Padlan, 1994) and display a 95% sequence homology (Burton *et al.*, 1986; Jefferis, 1990; Padlan, 1994). However, it has also been shown that the ability to exert effector functions varies among the IgG subclasses (Brüggemann *et al.*, 1987; Burton and Woof, 1992; Valim and Lachmann, 1991). The binding motif for the complement factor C1q, is present on all the human IgG isotypes (Duncan and Winter, 1988) but the binding may be affected by sterical hindrance by the Fabs (Carayannopoulos and Capra, 1993; Isenman *et al.*, 1975) and/or by minor differences in residues near the binding motif (Greenwood *et al.*, 1993; Tan *et al.*, 1990; Tao *et al.*, 1991). The interaction between human IgG and its Fcγ receptors is complex and the binding is affected by minor differences in the multiple binding sites on Fc (Canfield and Morrison, 1991; Chappel *et al.*, 1991; Greenwood *et al.*, 1993; Jefferis *et al.*, 1990; Lund *et al.*, 1992; Sarmay *et al.*, 1992). Despite LLPC being a very sensitive method, we could not, perhaps with the exception for IgG 3, detect these differences, implying that the differences related to effector functions are small or negligible. In the case of human IgG3s, we found that the partition properties of their Fc fragments differed significantly not only from those of Fc from the other subclasses, but also from each other (Fig. 4). Moreover, the value of K_C for chimeric IgG3 deviated significantly from those of the other IgG subclasses (Fig. 5), despite the IgGs having identical specificities (Valim and Lachmann, 1991). Thus, Fc appeared to have another conformation and to be more dominant in human IgG3 than in the other isotypes. The hinge region may be responsible for these deviations as its conformation varies and is different from that of the other IgG isotypes (Gregory *et al.*, 1987; Kilár *et al.*, 1985; Michaelsen and Natvig, 1974; Ryazantsev *et al.*, 1990).

The exact nature, location and function of this dominating structure(s) on human IgG3 Fc remain to be elucidated.

The domains of IgG all form compact globular structures with a characteristic fold, the immunoglobulin fold (Padlan, 1994). The structural differences between the domains of different IgGs are, with the exception of the hinge, mainly confined to the CDRs (Burton *et al.*, 1986; Jefferis, 1990; Padlan, 1994). Hence, the constant parts of unliganded IgGs may form similar scaffoldings, on to which CDRs of variable shapes and sizes are interspaced and constitute the major dominant differences in exposed surface properties. The surface properties of Fc may, however, from a functional point of view, be expected to be more dominant after antigen binding. Several experiments have indicated that antigen-induced conformational changes occur in the Fc part of the antibody molecule (Brown and Bekisz, 1984; Chiang and Koshland, 1979; Harada *et al.*, 1992; Mota *et al.*, 1981; Schlessinger *et al.*, 1975), although the relevance of these changes in the elimination of immune complexes is still a matter of debate (Poljak, 1991). LLPC analysis of complexes formed by monoclonal antibodies and haptens did not confirm any theory about Fc dominans (Wingren and Hansson, 1995). The changes observed upon complexation appeared to be dependent not only on the IgG isotype, but also on the affinity of the antibody as well as on the nature of the hapten (Wingren and Hansson, 1995). Further experiments are required to unravel the relationship between surface properties of Fc and its effector functions.

In conclusion, we have shown that LLPC can be used to detect differences in surface properties of antibodies with different antigen-binding sites. We present the remarkable finding that the surface properties of IgG are dominated by those of its antigen-binding regions and that these regions constitute the major differences in surface properties of IgGs and Fab fragments that are detected by LLPC.

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