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Production and characterization of pepsin fragments of human IgA1 to determine domain-specificity of monoclonal anti-IgA antibodies

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SUMMARY

Eight human IgA1 myeloma proteins were analysed by SDS–PAGE. These experiments showed that purified IgA1 proteins comprise both fully S–S bonded and partly S–S bonded molecules. Pepsin digestion of the IgA1 proteins yielded three four-chain and two two-chain fragments. The four-chain fragments are likely to be derived from intact IgA through cleavage of its α chains at different sites: between the CH2 and CH3 domains or in the hinge region. The occurrence of F(abc) (ab′) fragments, with α chains of different lengths, showed that the α chains of IgA can be cleaved independently at the hinge region site. The two-chain pepsin fragments must originate from IgA molecules, which lack inter-assay-chain disulphide linkages. The fragments $F(abc)_2$ and Fabc tended to form dimers, probably through non-covalent interactions of their CH2 domains. An immunoblotting method was used to identify Fd-, CH2- and CH3-specific anti-IgA antibodies. The CH2-specific antibodies could be subdivided into antibodies recognizing an isotype present on both four-chain and two-chain molecules or on two-chain molecules only.

INTRODUCTION

Normal serum IgA is heterogenous because of its origin from multiple cell clones and because of differences in the carbohydrate content of the molecules. Structural differences between IgA molecules may also reflect whether they were produced by cells of the systemic or by cells of the mucosal immune system (Benner, Hijmans & Haaijman, 1981; Craig & Cebra, 1971; Husband & Gowans, 1978; Kutteh, Prince & Mestecky, 1982). Serum IgA, derived from cells of the systemic immune system, is mainly monomeric. Secretory IgA, synthesized at mucosal sites, is dimeric and contains a J chain and secretory component (Mestecky *et al.*, 1980).

Although a myeloma protein originates from a single cell clone, and is therefore homogeneous with respect to amino acid composition and sequence, some structural and functional heterogeneity has been detected among its molecules. For instance, portions of IgA myeloma proteins are able to form complexes with serum proteins such as albumin (Mestecky *et al.*, 1977), α_1-anti-trypsin (Tomasi & Hauptman, 1974), α-amylase (Levitt & Cooperband, 1968), or lactate dehydrogenase (Biewenga & Feltkamp, 1975), or with protein A from *Staphylococcus aureus* (Brunda, Minden & Grey, 1979; Biewenga *et al.*, 1982). Furthermore, IgA myeloma proteins produce multiple bands in isoelectric focusing gels (Elkon, 1984), indicating differences in the isoelectric point of the molecules.

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Present knowledge of the relationship between structure, function and antigenicity of IgA is poor, especially when compared to IgG (Dorrington & Klein, 1983). Such relationships can be studied with well-defined IgA fragments, and with monoclonal antibodies against different structures on the IgA molecule. IgA fragments have been produced by enzymatic cleavage with bacterial IgA proteases (Plaut, Wistar & Capra, 1974; Plaut *et al.*, 1975) and pepsin (Rivat *et al.*, 1977). The IgA proteases yield Fab and Fc fragments. However, they cleave IgA1 proteins only, except for a recently discovered IgA protease from *Clostridium* sp. This IgA protease also cleaves IgA2 of the A2m(1) allotype (Fujiyama *et al.*, 1985). Pepsin cleaves both IgA1 and IgA2 proteins. The CH3 domain is cleaved off first to yield $F(abc)_2$ fragments (see Discussion and Fig. 5). Further digestion yields $F(ab')_2$ fragments through cleavage in the hinge region (Rivat *et al.*, 1977).

The present study on human IgA was undertaken to characterize and separate different IgA fragments and to determine the domain-specificity of monoclonal anti-IgA antibodies in an immunoblotting assay.

MATERIALS AND METHODS

IgA1 proteins were purified from myeloma sera as described previously (Biewenga *et al.*, 1982). Contamination could not be detected by immunoprecipitation methods, and in SDS–PAGE gels of reduced samples only α and light chains were found. IgA1 was enzymatically cleaved with pepsin (Boehringer, Mannheim, FRG) in 0·05 M acetate buffer at pH 4·5 and 37°, at an enzyme to

substrate ratio of 1:50. IgA1 fragments were purified by gel filtration on Ultrogel AcA 34 (LKB Produktor, Bromma, Sweden) in 0·05 M Tris–HC1, 0·5 M NaCl, pH 8·0, and by ion exchange chromatography on DEAE–Sephacel (Pharmacia Biotechnology, Uppsala, Sweden) in 20 mM Tris–HC1, pH 8·0, with a stepwise increase of NaCl concentration. SDS–PAGE was performed in 1 mm thick 5% gels by flat-bed electrophoresis in 0·1 M phosphate, pH 7·1, and later in 1 mm thick 7–17% gradient gels by vertical electrophoresis in 0·25 M Tris, 0·19 M glycine at pH 8·8. Reduction of samples was affected with 0·1 M dithiothreitol (DTT). Gels were stained with Coomassie brilliant blue. The molecular weights of proteins were estimated from electrophoresis mobilities according to Laemmli (1970). For re-electrophoresis single protein bands were cut out of stained Coomassie brilliant blue obtained wet gels and transferred to the application slots of a fresh SDS–PAGE gradient gel. The proteins were then reduced with 50 μl of 0·2 M DTT in sample buffer and re-electrophoresed after 60 min of incubation. These gels were stained with silver staining reagents (BioRad Laboratories, Richmond, CA) according to Merril *et al.* (1981). Electrophoretic transfer of proteins in SDS–PAGE gels to nitrocellulose sheets (Western blotting) was performed according to Burnette (1981). Complete transfer of proteins from 10 × 11 cm SDS–PAGE gels was obtained in 1·5–2 hr at 4·5 mA 5°. The nitrocellulose sheets were washed in phosphate-buffered saline containing 0·1% Tween 20 and cut perpendicular to the protein pattern into narrow strips. These were incubated with the mouse monoclonal anti-IgA antibodies, or with rabbit polyclonal antibodies against human light chains (obtained from the Central Laboratory of the Dutch Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) according to Batteiger, Newhall & Jones (1982). Binding of antibody was detected by subsequent incubation with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin or swine anti-rabbit immunoglobulin antibodies (Dakopatts, Glostrup, Denmark) followed by staining for peroxidase activity with 3,3′-diaminobenzidine-tetra-HCl (Sigma, St Louis, MO) in 0·05 M Tris-HCl, pH 7·6, containing 0·01% H_2O_2. Immunoblotting experiments were performed in duplicate on fragments from different IgA1 proteins.

Fast protein liquid chromatography (FPLC) was performed on an anion exchange (Mono Q) column (Pharmacia Biotechnology) in 0·02 M diethanolamine–HCl at pH 8·4 with a gradient of NaCl, or on a Superose 6 gel filtration column (also from Pharmacia Biotechnology) equilibrated in 0·1 M Tris–HCl, 1 mM EDTA at pH 8·0.

Monoclonal antibodies were produced according to standard techniques (Dijkstra *et al.*, 1985) in BALB/c mice. These were immunized intravenously with human IgA myeloma proteins or human parotid saliva (HISA-43). Anti-IgA-producing cell lines were detected by the reaction of supernatants with IgA in haemagglutination-inhibition or by an ELISA method with IgA-coated Terasaki plates. Anti-IgA-containing ascites fluids were produced by intraperitoneal injection of 10^6 hybridoma cells in BALB/c mice which had been given 0·5 ml pristane (2,6,10,14-tetramethylpentadecane, Sigma) 1 week before. The antibodies did not react with IgG and IgM of κ or λ type.

RESULTS

Purified IgA1 myeloma proteins were analysed by SDS–PAGE. Non-reduced IgA1 proteins produced a double band at about 160,000 MW monomeric IgA1. Higher molecular weight bands were often seen and indicate the presence of polymers or aggregates. Weaker bands of lower molecular weight components were also detected (Fig. 1). Reduced samples showed prominent α- and light-chain bands. Amongst the lower molecular weight bands of unreduced IgA, an 85,000 MW band was usually the strongest, and was present in all IgA proteins tested. This 85,000 MW protein contained α and light chains according to re-electrophoresis after reduction. This finding was confirmed by immunoblotting experiments with monoclonal anti-IgA and polyclonal anti-light-chain antibodies. Therefore, the 85,000 MW protein was identified as a two-chain IgA1 half molecule. Additional bands were seen in several of the SDS–PAGE patterns at about 130,000, 60,000 and 46,000 MW. On the basis of molecular weight and composition as determined by SDS–PAGE and immunoblotting, these bands represented αL_2, α and L_2 molecules. A weak light chain band was always present at about 23,000 MW. In spite of the molecular heterogeneity in SDS–PAGE, the IgA proteins were not resolved into subfractions when subjected to gel filtration or ion exchange chromatography performed under non-denaturing conditions.

Four IgA1 myeloma proteins were cleaved with pepsin. The time of digestion was varied in order to produce predominantly $F(abc)_2$ or $F(ab')_2$ fragments. $F(ab')_2$ fragments obtained by overnight digestion were shown by SDS–PAGE analysis to contain some contamination with both higher and lower molecular weight products. This could be removed by repeated gel filtration on Ultrogel AcA 34. After short (1·5–4 hr) pepsin digestion, the IgA1 proteins produced more fragments, among them $F(ab')_2$ fragments. Separation of the fragments by gel filtration on Ultrogel AcA 34 (Fig. 2) was far from complete. Only two peaks were obtained. SDS–PAGE analysis of the material in the first small peak showed the presence of a single

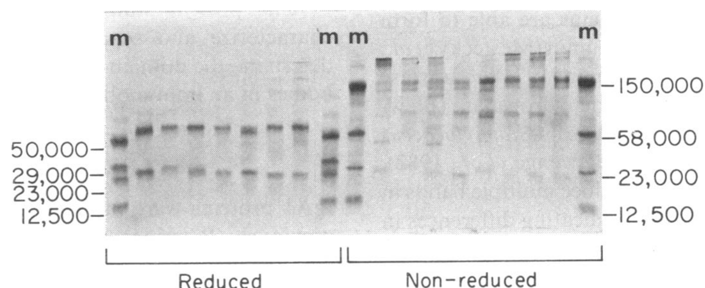

Figure 1. SDS–PAGE patterns of reduced (left) and non-reduced (right) IgA proteins in a 5% gel. m: marker proteins with a molecular weight of 50,000, 29,000, 23,000 and 12,500 (left) and 150,000, 58,000, 23,000 and 12,500 (right).

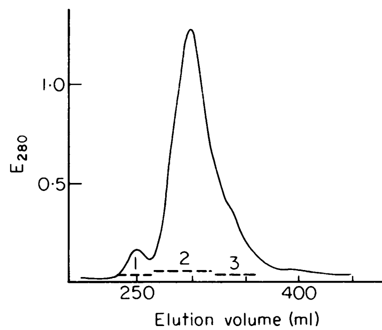

Figure 2. Typical elution profile of the pepsin digestion products of an IgA1 protein on an Ultrogel AcA 34 column (96 × 2·6 cm). The digestion time was 4 hr.

protein, which hardly migrated into 7–17% gradient gels. Its molecular weight was higher than that of the original IgA1 proteins. Based on its elution volume (Ve/Vt) in gel filtration experiments, its molecular weight was about 300,000. Further analysis of this protein by SDS–PAGE after reduction revealed a composition of α_{abc} and light chains, thus indicating that it consisted of dimers of F(abc)$_2$ fragments. Non-reduced samples of the main peak produced seven bands in SDS–PAGE gels (Fig. 3). The molecular weights of the various proteins were

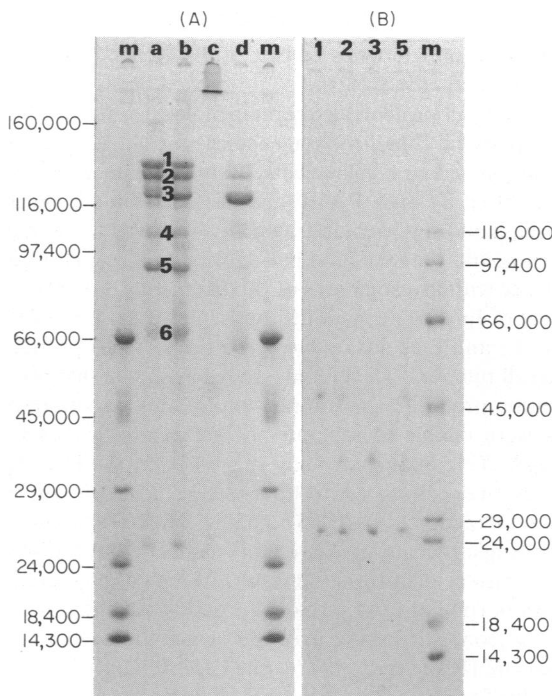

Figure 3. (A) SDS–PAGE gradient gel of (a) Pool 2 and (b) Pool 3 from gel filtration of a pepsin digest of IgA, (c) secretory IgA and (d) purified F(ab')$_2$ fragments. The molecular weight of the F(ab')$_2$ fragments is 120,000. m: marker proteins with a molecular weight of 66,000 45,000, 29,000, 24,000, 18,400 and 14,300. The positions of intact IgA and marker proteins of 116,000 and 97,400 (run in another lane) are also indicated. For gel filtration pattern, see Fig. 2. (B) SDS–PAGE gradient gel of reduced re-electrophoresed bands 1, 2, 3 and 5. m: marker proteins with a molecular weight of 116,000, 97,400, 45,000, 29,000, 24,000, 18,400 and 14,300.

Table 1. Pepsin digestion products of IgA1

Component	Composition*	Estimated MW†	Fragment	Calculated MW‡
Band 1	α_{abc}+L	140,000	F(abc)$_2$	140,000
Band 2	α_{abc}+$\alpha_{ab'}$+L	135,000	F(abc)(ab')	130,000
Band 3	$\alpha_{ab'}$+L	126,000	F(ab')$_2$	120,000
Band 4	α_{abc}+($\alpha_{ab'}$+L)	109,000	NI§	—
Band 5	αabc+L	94,000	Fabc	70,000
Band 6	$\alpha_{ab'}$+L	68,000	Fab'	60,000
Band 7	ND¶	25,000	L	25,000

* Composition as determined by SDS–PAGE after reduction and re-electrophoresis.

† Molecular weight estimated from electrophoretic mobility in 7–17% SDS–PAGE gels.

‡ Molecular weight calculated from the molecular weights of the heavy and light chains detected in reduced SDS–PAGE gels.

§NI, Non-identified. Band 4 contained α_{abc} with small amounts of $\alpha_{ab'}$ and light chains.

¶ ND, not determined.

estimated from their migration distances. SDS–PAGE of reduced samples showed the presence of intact α, α_{abc}, $\alpha_{ab'}$, and light chains. The composition of the proteins was determined by re-electrophoresis of single protein bands after reduction and by immunoblotting as previously described. Thus, intact IgA, disulphide-linked four-chain F(abc)$_2$, F(ab')$_2$ fragments, disulphide-linked two-chain Fabc and Fab' fragments and single light chains were identified (Table 1). These proteins could not be separated by ion exchange chromatography (Biewenga *et al.*, 1985). Repeated gel filtration on Ultrogel AcA 34 had previously produced a small amount of pure (F(abc)$_2$ fragments for antigenic analyses (Biewenga *et al.*, 1983). Gel filtration of the proteins by the FPLC technique on Superose 6 gave an elution similar to that previously obtained on Ultrogel AcA 34. Only the separation of Fab' fragments was improved. Again, the first peak contained dimers of F(abc)$_2$ fragments. The main peak contained the fragments F(abc)$_2$, F(abc)(ab'), F(ab')$_2$, Fabc and some Fab'. It should be noted that the amount of intermediate F(abc) (ab') fragments in the main gel filtration peak was proportional to the amount of F(abc)$_2$ and F(ab')$_2$ fragments. Intermediate fragments with an intact α chain were not found in SDS–PAGE gels of pepsin digests.

As non-reduced pepsin fragments were difficult to purify, the monoclonal antibodies were tested by immunoblotting on the pepsin fragments and on Fab and Fc fragments produced by IgA1 protease (Biewenga & Van Loghem, 1983). The reactions were read on bands of well-defined fragments which, in the case of four-chain molecules, should have two α chains of the same length. The reaction with F(abc)$_2$ and Fabc fragments was determined on samples obtained by short pepsin digestion (Fig. 3A, Lane a), whereas the reaction with F(ab')$_2$ and Fab' fragments was determined on samples from prolonged pepsin digestions (Fig. 3A, Lane d). The samples had been partially purified by gel filtration. The samples of short pepsin digestion contained F(abc)$_2$ and Fabc but also F(abc)(ab'), F(ab')$_2$ and little Fab'. Nevertheless, the reaction of the monoclonal antibodies with F(ab')$_2$ was determined on different nitrocellulose strips (Fig. 4) because antibodies reacting with F(abc)$_2$ some-

Table 2. Reactions of monoclonal anti-IgA antibodies with IgA1 and IgA1 fragments

Antibody	(Sub)class specificity*	Reactivity with: IgA1	IgA1 HM	$F(abc)_2$	Fabc	$F(ab')_2$	Fab′	Fab	Fc	Specificity†
H194-5.1	α	+	+	+	+	+	+	+	−	CH1 or framework VH
H69-6.32	α	+	+	+	+	−	−	−	+	CH2
H69-11.4	α_1	+	±‡	+	−	−	−	−	+	CH2
H69-7.1	α_1	+	±	+	−	−	−	−	+	CH2
H194-7.1	α	+	+	−	−	−	−	−	+	CH3
HISA 43	α	+	+	−	−	−	−	−	+	CH3

* Defined by haemagglutination-inhibition or immunoblotting.

† The isotope detected by CH2-specific antibodies may not be recognized on two-chain molecules (see text).

‡ ±, Negative or weakly positive.

times produced a diffuse and obviously non-specific staining around the $F(ab')_2$ band. Table 2 shows the results obtained with six anti-IgA antibodies. One of the antibodies reacted with all Fab-containing fragments but not with Fc, which shows its specificity for the Fd part of IgA. Three anti-IgA antibodies

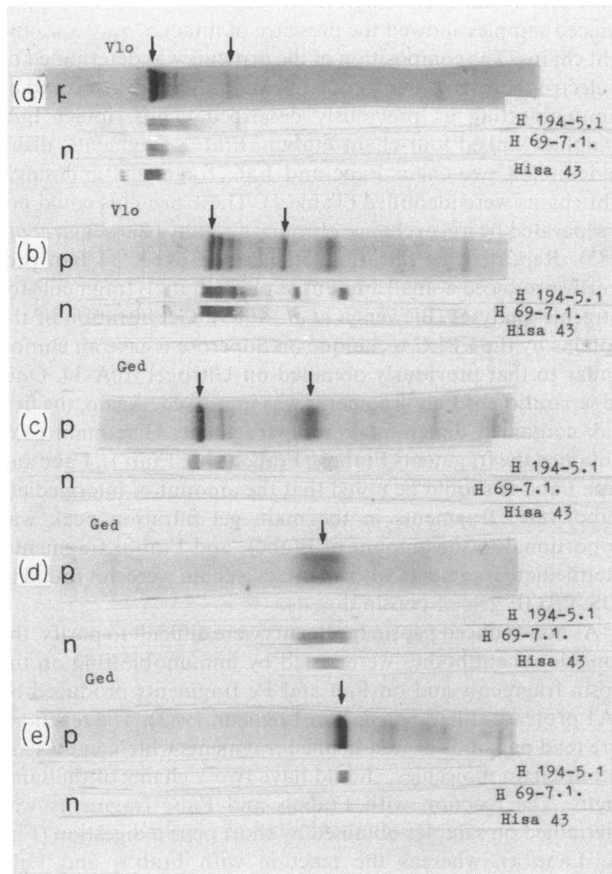

Figure 4. Typical SDS–PAGE and immunoblotting patterns. The protein bands used to determine antibody specificites are indicated by arrows. They represent (a) IgA1 and IgA1 half-molecules; (b) $F(abc)_2$ and Fabc; (c) $F(ab')_2$ and Fab′; (d) Fc; (e) Fab. p = protein patterns and n = corresponding nitrocellulose strips incubated with the indicated monoclonal antibodies. Vlo and Ged represent two different IgA proteins used for this study.

were reactive with Fc and $F(abc)_2$ molecules, which demonstrates CH2 specificity. In two of these three cases, expression of the isotype was restricted to four-chain molecules. Two anti-IgA antibodies reacted with Fc fragments only, which demonstrates that they were CH3-specific. Three antibodies with IgA2 specificity, according to haemagglutination-inhibition analyses, neither reacted with intact IgA1 nor with IgA1 fragments in the immunoblotting experiments (not shown in Table 2).

DISCUSSION

Basically, immunoglobulins consist of disulphide-linked four-chain molecules. However, the data reported here clearly show that human IgA1 proteins also contain molecules that lack H–H or H–L bonds. IgA1 half-molecules consisting of a single α chain disulphide linked to a light chain, were detected in all IgA1 proteins tested by SDS–PAGE analysis and immunoblotting. Three-chain and single-chain fragments were also found but in smaller amounts than the IgA1 half-molecules. These data are in accordance with investigations of Buxbaum *et al.* (1974) on the assembly of IgA. They identified molecules consisting of α_2L_2, α_2L, αL, L_2 and L chains in *in vitro* cultures of IgA-producing plasma cell tumours. The present study has shown that protein purification procedures performed under non-denaturing conditions were unable to separate the smaller molecules from intact IgA. This finding strongly suggests that the IgA1 half-molecules had associated to four-chain IgA through non-covalent interaction of the CH3 domains. Similarly, three-chain α_2L and single L chains had probably associated to four-chain IgA. It is also evident from the results of the pepsin digestions that a proportion of IgA1 myeloma proteins lack inter-H chain disulphide bonds. Pepsin cleaves IgA sequentially from the C-terminal end (Rivat *et al.*, 1977). The CH3 domains, which are responsible for non-covalent inter-H chain linkage, are cleaved off first. Thus, the molecules that lack CH3 and inter-H chain disulphide bonds fall apart to yield Fabc fragments, and further digestion yields Fab′ fragments. With respect to the origin of the Fab′ fragments, Rivat *et al.* (1977) suggested that pepsin cleaves non-reduced IgA proteins in the hinge region on each side of the inter-H chain disulphide bond(s), thus yielding both $F(ab')_2$ and Fab′ fragments. We have no evidence to support this. Such Fab′ fragments should have a somewhat shorter $\alpha_{ab'}$ chain than $F(ab')_2$ fragments, which was never seen in SDS–PAGE gels.

As shown in Fig. 5, pepsin cleaves IgA at two different α

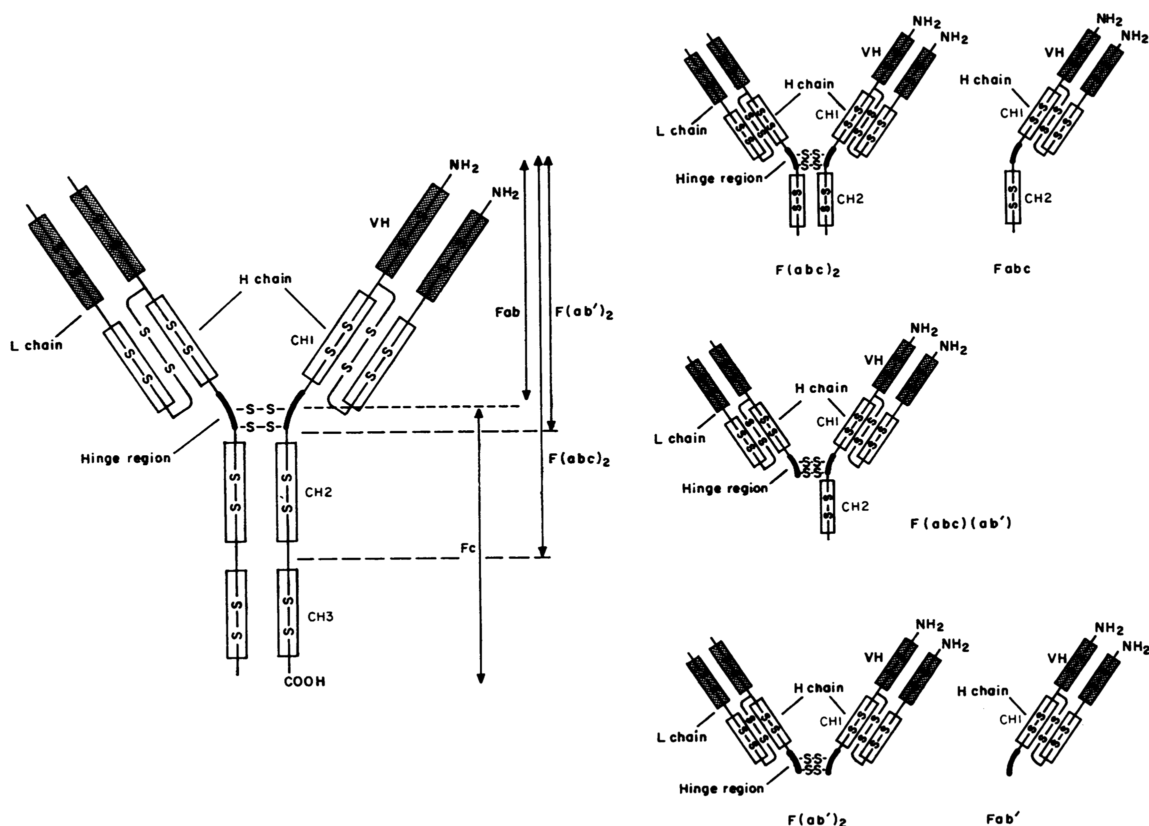

Figure 5. Schematic representation of an IgA1 molecule, its IgA1 protease (....) and pepsin (——) cleavage sites and peptic fragments. Pepsin cleavage of intact four-chain IgA molecules yields F(abc)$_2$, F(abc)(ab′) and F(ab′)$_2$. Molecules that lack the inter-heavy chain disulphide linkages (IgA half-molecules) yield Fabc and Fab′. Reduced Fabc (red Fabc) and reduced Fab′ (red Fab′) are peptic fragments from reduced and alkylated IgA. The latter lack the disulphide linkage between their H and L chain, which, however, are non-covalently linked.

chain sites. F(abc)$_2$ fragments are the products of cleavage between the CH2 and CH3 domains, whereas F(ab′)$_2$ fragments result from cleavage at the hinge region. The occurrence of the intermediate F(abc)(ab′) fragments shows that the IgA1 α chains may be cleaved independently at the hinge region site. Cleavage of the α chains between the CH2 and CH3 domains, however, seems to occur simultaneously because intermediate products between IgA and F(abc)$_2$ fragments were not found. These observations suggest that cleavage of one of the α chains between CH2 and CH3 increases the accessibility of the site on the other α chain. In contrast, cleavage of one α chain at the hinge region seems to have little effect on the other hinge region site.

The four-chain F(abc)$_2$, F(abc)(ab′) and F(ab′)$_2$ fragments could not be separated by ion exchange chromotography or by gel filtration. On gel filtration, however, disulphide-linked dimers of F(abc)$_2$ were obtained separately. Higher polymers were not found, indicating that distinct interactions rather than random aggregation had occurred. This could be due to non-covalent interaction of the CH2 domains, which lack structural stabilization through non-covalently linked CH3 domains (Dorrington & Klein, 1983). F(abc)$_2$ dimers do not result from dimeric IgA because the original IgA proteins were monomeric. The mechanism by which the dimers were disulphide-linked is not known. Our gel filtration experiments further demonstrated that, under non-denaturing conditions, Fabc fragments fail to separate from four-chain molecules. This observation suggests that the Fabc fragments also associated to larger, probably four-chain, molecules. Such association could again be explained by non-covalent interaction of the CH2 domains. In Fabc fragments, these lack stabilization through CH3 and through disulphide-linked α chains (Dorrington & Klein, 1983). In seeming contradiction is the fact that Fabc fragments produced by pepsin digestion of mildly reduced and alkylated IgA (Biewenga & Van Loghem, 1983; Klein *et al.*, 1981) do not associate to four-chain molecules. It is unknown whether or not the alkylation inhibits the molecular interactions.

Studies on IgG have demonstrated that the CH2 domain plays a major role in mediating biological activities (Dorrington & Klein, 1983). Consequently, conformational changes in the structure of the CH2 domain strongly affect the biological functions (Dorrington & Klein, 1982; Klein *et al.*, 1981). This explains why most of the CH2-mediated functions of IgG are lost on reduction. Similarly, non-disulphide-linked IgA has probably lost many IgA-specific biological properties. With respect to protein A binding, reduction of IgA1 proteins does not destroy the binding that is mediated by F(ab′)$_2$ (Inganas, 1981). However, reduction of F(abc)$_2$ fragments but not of F(ab′)$_2$ fragments causes loss of protein A reactivity (Bruin, Faber & Biewenga, 1985). These data suggest that the structurally labile CH2 domain of Fabc can block the binding of protein A to a Fab′-dependent structure. In another study (Biewenga &

Van Loghem, 1983), differences in antigenicity between four-chain $F(abc)_2$ and the corresponding two-chain Fabc fragments were demonstrated. These differences were then erroneously (Biewenga & Van Loghem, 1983; Van Loghem & Biewenga, 1983) ascribed to the anti-nA2m(2) antibodies in the antisera Ta and Wo. Later experiments showed that the antisera also contained anti-α antibodies. These appeared to be responsible for the discrimination between $F(abc)_2$ and Fabc fragments. The results of our present study show that Fd-, CH2- and CH3-specific monoclonal antibodies against human IgA can be easily distinguished using IgA1 protease and pepsin-produced IgA fragments in an immunoblotting assay. The Fd-specific antibody probably recognizes an epitope located in the CH1 domain of IgA. However, reactivity to a framework VH epitope is also possible. This antibody does not recognize an idiotype because it reacted with different IgA proteins. Our study demonstrates that CH2-specific isotypes found on four-chain molecules may be present in their two-chain counterparts. The CH2-specific isotypes detected by the antibodies H69-11.4 and H69-7.1 (Table 2) were either blocked on Fabc and IgA1 half-molecules or they depend upon a structure with two disulphide-linked α chains. Blockage of CH2-specific isotypes could be due to non-covalent interaction of structurally labile CH2 domains.

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