Comparison of the Structure of the Immunoglobulins from Horse Serum

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A study of the chemical structure of the horse immunoglobulins IgG and IgA (T) has shown that the amino acid contents of the peptide chains are very similar. These globulins differ most markedly in the products of papain digestion. IgG gives 3.5s products, whereas IgA (T) gives a 5s fraction and smaller components. This difference appears to be associated with the presence of an additional easily reducible disulphide bond in the Fd fragment of the heavy chain. There is two to three times as much carbohydrate in IgA (T) as in IgG. In both, this is in the heavy chain and in IgA (T) more than half is covalently bound to the Fd fragment. The differences in antigenic specificity between horse IgG and IgA (T) appear to be due to structural differences in the Fc fragment.

It has been known for a long time that if horses are repeatedly injected with protein antigens, such as diphtheria and tetanus toxoids, the antibodies that are produced are contained almost entirely in a serum protein fraction with an electrophoretic mobility intermediate between those of the γ - and β -globulins (Kekwick & Record, 1941; Van der Scheer, Wyckoff & Clarke, 1941). This fraction was named the β_2 - or T-globulin and its chemical structure is of some interest, as it appears to be equivalent to the IgA fraction, the most difficult to isolate of the three main human immunoglobulin types. Identification with IgA is based on the four main features of this protein: a molecular weight of about 150 000, and electrophoretic mobility at pH 8.6 higher than IgG, a higher carbohydrate content than IgG, and an antigenic cross-reaction, but not identity, with IgG. Whether this horse T-globulin is exactly equivalent to human IgA remains to be established, but clearly it is a distinct class of immunoglobulin present in large amounts in a repeatedly immunized horse and it is the main source of commercial antitoxins.

Analytical studies of horse IgG and IgA (T) globulins have been carried out by Schultze and collaborators (Schultze, 1959) and recently this has been extended to the peptic-digest products (Schultze, Haupt, Heide, Heimburger & Schwick, 1965). We now report studies of the peptide chains and the sub-units of these proteins obtained by enzymic digestion. Some of the results described below have been included in a review by Cohen & Porter (1964a).

In the present paper we have used the nomenclature recommended for human immunoglobulins

by the World Health Organisation (1964). It is summarized in Fig. 1.

MATERIALS AND METHODS

Horse antisera to crystalline diphtheria toxin (Pope & Stevens, 1958), to purified tetanus toxoid and to lecithinase from *Clostridium welchii* and the corresponding antigens were given by Wellcome Foundation Ltd., Beckenham, Kent. Rabbit antisera to horse IgG and IgA (T) were prepared by immunization by using Freund's adjuvant (Porter, 1959).

Recrystallized mercury papain was prepared by the method of Kimmel & Smith (1954).

Fig. 1. Nomenclature recommended for human immunoglobulins by the World Health Organisation (1964).

Horse IgG was prepared from normal horse serum by chromatography on Sephadex A-50 in 17.5 mM-sodium phosphate buffer, pH 6.3. IgG was not retained on the column and was obtained in a yield of 1.3–1.6 g./100 ml. of serum.

If immune horse serum was treated similarly, the protein eluted at the solvent front was a mixture of IgG and IgA(T) in a yield of about 1.6–1.9 g./100 ml. of serum. When rechromatographed on Sephadex A-50, equilibrated with 15 mM-potassium phosphate buffer, pH 8.4, most of the IgG was not adsorbed and the IgA(T) was subsequently eluted with a gradient to 175 mM-sodium phosphate buffer, pH 6.3. A 1 l. mixing vessel was used with a column (70 cm. x 3 cm.) of Sephadex A-50. The T-globulin was eluted as a single peak, but only the peak tubes were collected as the leading edge contained an appreciable amount of IgG.

Electrophoresis in starch gel was carried out in 8 M-urea-formate at pH 3.5 (Edelman & Poulik, 1961) or in 8 M-urea-glycine-NaOH at pH 7.8 (Cohen & Porter, 1964b). Electrophoresis on cellulose acetate strips was carried out in the buffer described by Aronsson & Grönwall (1958) and the strips were stained with Procion Brilliant Blue RS or Coomassie Brilliant Blue R250 (Fazekas de St Groth, Webster & Datyner, 1963). The micro-immunoelectrophoresis technique used was that of Scheidegger (1955). Peptide chains of the immunoglobulin were prepared as described by Fleischman, Pain & Porter (1962), except that 0.5 N-acetic acid, rather than N-propionic acid, was used for the separation of the chains, as the yield of light chain was 25% of the total protein, as judged by the extinction at 280 m μ , in both solvents. Papain digestion of immunoglobulin was carried out as described by Porter (1959). Amino acid analysis was kindly made by Mr J. M. Wilkinson (Crumpton & Wilkinson, 1963) and carbohydrate analysis as described by Fleischman, Porter & Press (1963). Labelling of proteins with ^{125}I was by the method of McFarlane (1958); 1 mol. of globulin contained approx. 1 atom of ^{125}I .

Molecular weights were estimated by Dr R. H. Pain by sedimentation equilibrium, by using the methods of Van Holde & Baldwin (1958) and Yphantis (1960).

RESULTS

Purity of preparation of IgG and IgA(T) globulin from anti-(diphtheria toxin) sera. Electrophoresis on cellulose acetate strips showed the IgG to be free of IgA(T), but the IgA to contain some IgG. These results were confirmed by immunoelectrophoresis, where the IgG appeared to be a single component but the IgA(T) preparation showed a spur due to contamination with IgG.

The contamination was estimated quantitatively by precipitation of ^{125}I -labelled IgA(T) globulin by anti-IgA(T) serum that had been made specific for IgA(T) by absorption with IgG. The radioactivity in the precipitate was 87.3% and examination of the supernatant and washings on Sephadex G-200 showed 0.9% of the radioactivity to be present as soluble complex. Hence this preparation of IgA(T) contained 12% of IgG and all analytical values were corrected accordingly.

In the anti-(diphtheria toxin) serum that was

Fig. 2. Antigenic relationship between horse IgG and IgA(T) and their chains. (a) Reaction of IgG (1), IgA(T) (2), IgG light chain (3) and IgA(T) light chain (4) with rabbit anti-IgG serum (5). (b) Reaction of IgG (1), IgA(T) (2), IgG light chain (3) and IgA(T) light chain (4) with rabbit anti-IgG serum absorbed with IgG light chains (6).

Fig. 3. Antigenic cross-reactions of horse IgG and IgA(T) and their Fab fragments. (a) Reaction of IgG (1), IgA(T) (2), IgG Fab fragments (3) and IgA(T) Fab fragments (4) with rabbit anti-IgG serum (5). (b) Reaction of IgG (1), IgA(T) (2), IgG Fab fragments (3) and IgA(T) Fab fragments (4) with rabbit anti-IgG serum absorbed with IgG Fab fragments (6).

used all the antitoxin was in the T-globulin fraction and this fraction was 60% precipitable by toxoid prepared from crystalline diphtheria toxin.

Antigenic relation of IgG and IgA(T). It is believed that the common antigenic determinants of the human immunoglobulins are contained in the light chains (Cohen, 1963). This is not true of these two horse immunoglobulins. There was extensive cross-reaction of each with antiserum prepared against the other: IgA(T) would precipitate 50% of the antibody in anti-IgG serum and IgG would precipitate 41% of the antibody in anti-IgA(T) serum. The light chains of both showed complete fusion in an Ouchterlony plate and were apparently antigenically identical (Fig. 2). However, absorption of anti-IgG or anti-IgA serum with light chains did not make them specific and hence the heavy chains must also carry common antigenic sites (Fig. 2). When the Fab fragments were allowed to react with anti-IgG serum they gave a reaction of identity (Fig. 3), suggesting that the distinct antigenic sites were on the Fc fragment half of the heavy chain. However, if anti-IgG serum is absorbed with IgG Fab fragment, the antiserum, which is now specific for IgG Fc fragment, still shows a cross-reaction with IgA(T). Hence it would appear that there are common antigenic sites on the light chain and both sections of the heavy

chain and that the distinct sites are localized in the Fc fragment of both.

Analysis of anti-(diphtheria toxin) IgG and IgA (T). The amino acid and carbohydrate analyses of the two immunoglobulins are given in Tables 1 and 2. The amino acid analyses show relatively small differences, which only exceed 10% for threonine, methionine, isoleucine and tyrosine. The carbohydrate content shows much bigger differences, the IgA(T) containing higher amounts of all components, particularly sialic acid. The relative contents of the neutral sugars, fucose, mannose and galactose, as judged by paper chromatography, were the same in both preparations and were about 1:2:1 respectively.

Peptide chains. The relative yields of heavy and

light chains, as judged by the extinctions at 280mμ, were 75 and 25% from both IgG and IgA(T). The amino acid compositions of the corresponding chains were similar (Table 3), as would be expected from the analysis of the whole protein. The only exception is in the S-carboxymethylcysteine, where there appears to be an additional residue in the heavy chain from the IgA(T) over the IgG.

The carbohydrate was, in both cases, almost entirely confined to the heavy chain (Table 2), as reported previously for rabbit IgG (Fleischman *et al.* 1963), human IgG (Cohen, 1963) and human IgM (Chaplin, Cohen & Press, 1965). It was observed

Table 1. *Amino acid composition of horse IgG and IgA (T) immunoglobulins*

The values were calculated by assuming the molecular weights of IgG and IgA (T) to be 150 000 (Pain, 1963).

Amino acid	Amino acid content (moles/mole)	
	IgG	IgA (T)
Lys	79	76
His	28	28
Arg	32	29
Asp	94	95
Thr	123	104
Ser	167	159
Glu	119	108
Pro	93	102
Gly	96	93
Ala	76	69
Val	123	116
Met	8.9	6.4
Ile	42	34
Leu	88	87
Tyr	51	44
Phe	39	35
CyS	31	28
Trp	20	20

Table 3. *Amino acid composition of the polypeptide chains of horse IgG and IgA (T)*

The assumed molecular weights were: heavy chain, 50 000; light chain, 20 000.

Amino acid	Amino acid content (moles/mole)			
	IgG		IgA (T)	
	Heavy chain	Light chain	Heavy chain	Light chain
Lys	26	9.5	28	10
His	10	2.1	12	2.1
Arg	9.9	4.4	10	4.2
Asp	30	12	35	13
Thr	37	17	32	19
Ser	45	28	47	33
Glu	38	16	38	16
Pro	31	11	38	12
Gly	28	16	28	18
Ala	22	12	21	13
Val	43	14	45	15
Met	3.6	0.4	3.2	0.2
Ile	13	6.2	11	6.9
Leu	29	11	31	12
Tyr	16	6.4	16	6.4
Phe	13	4.4	13	4.4
CyS	7.1	3.2	6.1	3.1
CyS·CH ₂ ·CO ₂ H	3.4	0.8	4.5	1.0
Trp	7.6	2.7	6.8	2.7

Table 2. *Carbohydrate content of horse IgG and IgA (T) and of their constituent polypeptide chains*

The assumed molecular weights were: IgG and IgA(T), 140 000; heavy chains, 50 000; light chains, 20 000.

Carbohydrate	Carbohydrate content (moles/mole)					
	IgG			IgA (T)		
	Native	Heavy chain	Light chain	Native	Heavy chain	Light chain
Hexose	9.4	4.6	0.4	24.3	11.6	0.6
Hexosamine	7.1	3.5	0.07	14.3	7.2	0.07
Sialic acid	0.3			2.7		

that the yield of light chains from horse IgA(T) could be raised by 2–3% if the dissociation of the peptide chains was carried out on a Sephadex column in 0.1% sodium dodecyl sulphate, pH 6.8, rather than 0.5N-acetic acid. However, the additional small yield of light chains showed a marked increase in hexosamine content from 0.05 to 0.40% by weight. Heavy chains contained 2.38% of hexosamine by weight. This suggested that some splitting of the heavy chains had occurred in this solvent or, perhaps more likely, that a few per cent of the heavy chain were already split in the original preparation and the halves dissociated in this solvent to be eluted with the light chains on gel filtration.

Starch-gel electrophoresis in 8M-urea at pH 3.5 showed no difference in the behaviour of the corresponding light and heavy chains of IgG and IgA(T). However, at pH 7–8 in 8M-urea there were obvious differences (Fig. 4). The heavy chain of IgG was aggregated and gave a spread band from the origin, whereas that from IgA(T) moved cleanly from the origin and gave a pattern of six to eight close, but clearly distinguished, bands. The light chain gave the bands expected (Cohen & Porter, 1964*b*) but the average mobility towards the anode of that from IgA(T) was greater than that from IgG (Fig. 5).

Papain-digest pieces. The difference between these two proteins was shown most sharply by this technique. When a papain digest of IgG was examined in the ultracentrifuge, it gave a single peak of 3.5s, as has been observed with a similar digest of IgG prepared from a variety of other species (Cohen & Porter, 1964*a*). However, digests of IgA(T) examined under the same conditions showed a major component with sedimentation coefficient 5.6s, together with other more slowly sedimenting components. The different fractions could be separated on a column of Sephadex G-100, as shown in Fig. 6. Dr R. H. Pain estimated the molecular weight of the main component by sedimentation-equilibrium studies and obtained a value of 97 000, assuming the same partial specific volume as that of whole horse IgG, i.e. 0.739 (Pain, 1963). When tested with diphtheria toxoid it gave a precipitate, suggesting that it contained two antibody-combining sites and was thus comparable to the fragment of molecular weight 100 000 obtained by peptic digestion of rabbit IgG (Nisonoff, Wissler, Lipman & Woernley, 1960). Of this 5.6s component 67% was precipitable by toxoid, compared with 60% of the undigested globulin, suggesting that the 12% contamination by inactive IgG had been eliminated,

Fig. 4. Electrophoresis of heavy chains of horse IgG and IgA(T) in alkaline 8M-urea-glycine starch gel. (1) Anti-lecithinase IgA(T) heavy chain; (2) anti-(diphtheria toxin) IgA(T) heavy chain; (3) normal IgG heavy chain.

Fig. 5. Electrophoresis of light chains of horse IgG and IgA(T) in alkaline 8M-urea-glycine starch gel. (1) Normal IgG light chain; (2) anti-(diphtheria toxin) IgA(T) light chain; (3) anti-lecithinase IgA(T) light chain.

Fig. 6. Fractionation of a papain digest of horse IgA(T) (63 mg.) by gel filtration through a column (1 cm. $\times$ 150 cm.) of Sephadex G-100 in 0.15M-NaCl-25 mM-potassium phosphate buffer, pH 7.4.

as would be expected, as 3.5s components on the Sephadex G-100 column.

It therefore seemed likely that the 5.6s component consisted, as in the peptic digest, of two 3.5s Fab fragments held together by a disulphide bond or bonds that were stable to the 0.01M-cysteine present in the digestion medium. The effect of higher concentration of reducing agent was investigated and it was found that 0.05M-mercaptoethanol at pH 8.2 for 1 hr. gave 70% of 3.5s fragments, and 0.1M-mercaptoethanol gave over 90% split to the smaller molecules. Such fragments could no longer be precipitated with toxoid, but could inhibit the precipitation of diphtheria toxoid and antibody. Hence it appeared that the interchain disulphide bond was much more resistant to reduction than that believed to hold together the two Fab fragments of IgG.

This was investigated further by isolation of the fragment (Fd) of the heavy chain contained in the reduced 5.6s pieces. Separation of Fd fragment and light chain is effected by chromatography of the reduced 5.6s piece on Sephadex G-75 in *n*-propionic acid. As in the Fab fragment from rabbit IgG, the Fd fragment dimerizes in *n*-propionic acid and is separable from the light chain, which behaves as a monomer (Fleischman *et al.* 1963). Amino acid analysis of Fd fragment (Table 4) showed that there were 2.7 *S*-carboxymethylcysteine residues/mol., rather than 1.9, in the Fd fragment from IgG. This additional residue is also apparent in the

Table 4. Amino acid composition of Fd fragment of horse IgA(T)

The molecular weight of Fd fragment was assumed to be 22 000.

Amino acid	Amino acid content (moles/mole)
Lys	11
His	3.7
Arg	4.7
Asp	15
Thr	14
Ser	25
Glu	11
Pro	15
Gly	15
Ala	11
Val	19
Met	1.8
Ile	6.1
Leu	16
Tyr	7.5
Phe	3.4
CyS	2.9
CyS $\cdot$ CH ₂ $\cdot$ CO ₂ H	2.7
Trp	2.9

Table 5. Carbohydrate content of Fab fragments of horse IgG and IgA(T)

The values were calculated by assuming molecular weights of 50 000 for the fragments from IgG and IgA(T).

Carbohydrate	Carbohydrate content (moles/mole)	
	IgG	IgA(T)
Hexose	0.59	7.8
Hexosamine	0.49	4.2

analysis of the whole heavy chain of IgA(T), which also shows an additional *S*-carboxymethylcysteine residue/mol. compared with the heavy chain of IgG.

Carbohydrate analysis (Table 5) shows that two-thirds of the carbohydrate remains covalently bound to the Fab fragment from IgA(T), in contrast with the behaviour of the Fab fragment from rabbit IgG (Fleischman *et al.* 1963), where the much smaller amount present was shown to be dissociated from the protein in *n*-propionic acid. As the light chain is known to be almost free of carbohydrate it is clear that two-thirds must be bound to the part of the heavy chain associated with the Fab fragment.

DISCUSSION

A point that has emerged in this work is that, when heavy chain prepared by dissociation of reduced IgA(T) in 0.5N-acetic acid is re-run on

Sephadex G-100 in 0.1% sodium dodecyl sulphate, there is a further release of material that behaves on gel filtration as light chains. This suggested that the true content of light chains should be 27–29% as judged by the extinction at 280 m μ , but estimation of the hexosamine content of this material showed it to be much higher than in the bulk of the light chain and it seems likely that the solvent has either dissociated heavy chains already split into approximately half molecules or has itself caused the splitting. The latter is improbable, as prolonged pretreatment with higher concentrations of the detergent solution increased only very slightly the yield of this lower-molecular-weight material, and it seems likely that in the original preparation of IgA (T) or IgG there has been some splitting of the heavy chain (perhaps by serum enzymes) in a small percentage of the molecules. This, in turn, suggests that the low carbohydrate content of the light chain, when prepared from the immunoglobulin of several species, may be due to slight contamination with split heavy chains and is not an intrinsic feature of light chains.

Comparison of the structure of the two immunoglobulins has shown them to be similar in amino acid composition and antigenic specificity. They differ markedly in carbohydrate content and also in the electrophoretic behaviour of the whole molecules and of the peptide chains at slightly alkaline pH. In each case, IgA (T) has a higher mobility and the heavy chain shows multiple components, whereas the heavy chain of IgG, as in other species, is partly aggregated under the same conditions. Another striking difference is the stability of the interchain linkage between the two antibody-site-containing 3.5S pieces from papain digestion. This is apparently due to an additional disulphide bond between the parts of the heavy chain contained in these pieces and seems to be a unique structural feature among the immunoglobulins of different species that have been examined (see Cohen & Porter, 1964a). The difference may arise from the papain hydrolysis occurring slightly more towards the carboxyl end of the heavy chain, but analysis shows that the IgA (T) heavy chains contain an additional disulphide bond that is split by reduction with mercaptoethanol in the absence of a denaturing agent.

Identification of the T-globulin with the IgA class of human immunoglobulin is doubtful, even though the four points of size, mobility at pH 8.6, carbohydrate content and antigenic behaviour are similar. There appears to be greater chemical similarity between the heavy chains of horse IgG

and T-globulin, with the exception of the extra interchain disulphide bond, although a sufficient study of human IgA has not yet been made. Also, the biological origin of the T-globulin is probably different as its presence in significant amounts appears to depend on prolonged immunization with protein antigens. However, there are already reports in several species of variants of the three main immunoglobulin groups. This variety of different types may be far greater than is at present recognized and may not fall into neat classes.

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