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CHROMATOGRAPHIC STUDY OF THE INTERRELATIONSHIPS OF IMMUNOGLOBULIN A AND α_1-MICROGLOBULIN IN MYELOMATOSIS

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SUMMARY

The binding of α_1-microglobulin (α_1-m) to serum immunoglobulin A (IgA) myeloma proteins have been examined by analytical and preparative Superose high-performance gel chromatography. Enzyme immunoassays showed that in the serum α_1-m was bound to monomeric IgA, but not to the polymeric IgA, and was also present in a free form. The IgA–α_1-m complexes involved covalent and non-covalent bonds. Considerable variation in the ratio of bound to unbound forms of α_1-m was observed that appears to be a result of variation of the IgA α heavy chains. Reduction of monomeric IgA produced α_1-m–heavy chain complexes, free α_1-m, light and α heavy chains, and traces of α_1-m attached to IgA that was resistant to reduction.

INTRODUCTION

Low-molecular-weight plasma proteins are cleared from the blood by the kidney. This process involves ultrafiltration through the glomeruli followed by a very efficient reabsorption by the tubular cells where the low-molecular-weight proteins are catabolized. When tubular function is impaired the excretion of low-molecular-weight proteins in the urine is increased. When glomerular function is impaired the levels of low-molecular-weight proteins in the blood rise. Several low-molecular-weight plasma proteins are important in clinical medicine as indicators of renal disease as well as diseases affecting their syntheses. The plasma and urine levels of β_2-microglobulin, retinol binding protein and α_1-microglobulin (α_1-m) have been widely studied in disease[1,2].

α_1-Microglobulin, a brown-coloured glycoprotein, has been isolated from urine by multistep gel chromatography and DEAE-cellulose chromatography[3–7]. The molecular weight is 31 kilodaltons (kdalton), as demonstrated by sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE), but its weight appears to be only 24.8 kdalton by gel chromatography in 6 *M* guanidinium chloride[8]. The protein has a p*I* between 4.3 and 4.9[8] and is well recognised as having a heterogenous charge. One of its alternative names is human complex-forming glycoprotein (protein HC)[3].

Surveys of the serum levels of α_1-m in disease have shown that apart from impaired renal function, very few disorders are associated with alterations of α_1-m

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concentrations[2]. A notable exception is immunoglobulin A (IgA) myeloma, a disease characterized by high levels of monoclonal IgA, in which very high levels of α_1-m may occur in patients with normal renal function[9], apparently due to α_1-m–IgA complexes[10].

Previous studies had suggested that α_1-m might bind to IgA and albumin[8], and it was known that purified α_1-m, isolated from urine, shows a tendency to polymerise in aqueous solution[8]. Crossed immunoelectrophoresis indicated the ratio of α_1-m bound to IgA to free α_1-m in the serum was variable. Evidence for covalent binding between α_1-m and IgA heavy chain that was resistant to reduction has been reported in a patient with IgA myeloma[11]. In our present study we have used a combination of gel chromatography and immunochemical procedures to investigate the distribution and nature of the bound and free α_1-m in the serum of patients with IgA myeloma and to isolate the main forms of α_1-m.

MATERIALS AND METHODS

Sera were obtained from patients in the Medical Research Council's Vth Myeloma Trial. The identification and measurement of the IgA myeloma protein concentration was made by the Department of Immunology, University of Birmingham, U.K.

Gel chromatography

High-performance analytical gel chromatography was performed on Superose 6 and Superose 12 gels, pre-packed in glass columns HR 10/30 (30 cm × 10 mm I.D.) obtained from Pharmacia, Uppsala, Sweden. The Superose 6 columns were used to separate proteins with a molecular weight higher than 100 kdalton and Superose 12 for proteins with molecular weights lower than 100 kdalton. A Superose 6B gel column (50 cm × 15 mm I.D.) was used for preparative high-performance chromatography to separate IgA monomers from serum. The columns were operated with a back pressure of less than 1.5 MPa for Superose 6 and Superose 6B and less than 3.0 MPa for Superose 12 at flow-rates of 0.2, 0.5 and 0.5 ml/min, respectively. The columns were cleaned with 30 ml 0.1 *M* sodium hydroxide after every 10–15 runs.

Buffers

The buffer for the gel chromatography under non-reducing conditions was 0.05 *M* sodium phosphate, containing 0.15 *M* potassium chloride and 0.05% sodium azide (pH 7.2). Under reducing conditions, the eluent was 6 *M* guanidinium chloride in 0.05 *M* phosphate buffer (pH 7.2).

The application and washing buffer for the immunosorbent columns was 0.02 *M* phosphate, containing 0.05% sodium azide (pH 8.0). The proteins were desorbed with 4 *M* magnesium chloride (pH 5.0).

Reduction

Proteins were reduced by incubating them in 0.1 *M* Tris–HCl (pH 8.0) containing 10 m*M* dithiothreitol (DTT) for 1 h at room temperature and subsequently alkylated by the addition of iodoacetamide to a concentration of 20 m*M*. Alternatively, proteins were boiled for 10 min in the presence of 0.7 *M* 2-mercaptoethanol.

Immunoassays

PAGE was performed on linear gradient gels (5–15%), containing 1% SDS. Proteins were identified by immunofixation after transfer to nitrocellulose membranes by the Western blotting technique[12]. The primary antibody was rabbit anti-human α_1-microglobulin antiserum, provided by Behringwerke (Marburg, F.R.G.). Goat anti-rabbit IgA was then bound to the rabbit anti-human α_1-microglobulin and reacted with rabbit peroxidase anti-peroxidase (PAP) (Miles Laboratories, Slough, U.K.). Protein bands were stained with 3-amino-9-ethylcarbazole as the substrate for peroxidase.

Enzyme linked immunoassay (EIA) for α_1-m was performed with kits supplied by Fuji Rebio (Tokyo, Japan)[13]. A rapid latex agglutination test for α_1-m was carried out using latex-particles coated with anti-α_1-m antisera prepared by T. Kawai, Jichi Medical College, Japan[14]. Crossed immunoelectrophoresis was performed with rabbit anti-human α_1-m antisera in the second dimension gel[15].

Instrumentation

The chromatography was performed using an automated fast protein liquid chromatography system supplied by Pharmacia.

Immunosorbent chromatography

Polyvalent rabbit anti-human α_1-microglobulin (Dako, Copenhagen, Denmark) and polyvalent sheep anti-human α chain antisera (University of Birmingham) were linked to CNBr-activated Sepharose 4B by the coupling method described by Pharmacia[16], and packed into columns (10 cm × 10 mm I.D.).

All buffers were made up with HPLC-grade water, then degassed and filtered through a 0.22-μm cellulose nitrate membrane (Uniscience, London, U.K.). The samples were also filtered through a 0.22-μm membrane filter before being injected into the columns.

RESULTS

Serum gel profiles

Analytical gel chromatography of IgA myeloma sera on Superose 6 columns, resolves the monomer, dimer and high polymers of IgA (Fig. 1). For routine analysis 200 μl of serum diluted 1:20, a flow-rate of 0.2 ml/min and an a.u.f.s. of 0.5 was considered to be optimal. The concentrations of the various forms of IgA were measured from the peak areas. The analytical determinations were found to be reproducible. An analysis of ten consecutive analyses showed that the albumin, IgA monomer, and IgA dimer peaks had retention times of 84.4 ± 0.13, 74.1 ± 0.43 and 69.9 ± 0.44 min, respectively. For the resolution of the higher polymers reduction of the flow-rate to 0.1 ml/min was advantageous. The flow-rate could be increased to 0.4 ml/min for rapid screening of the extent of polymerization of IgA paraproteins. The relatively serum viscosity of IgA myeloma sera compared to normal was increased. There was a significant correlation between the concentration of IgA dimer and higher polymers as measured by the areas under the peaks and the relative serum viscosity, the correlation coefficient in 53 samples was 0.73 ($p = 0.0001$). Normal serum, analyzed under the same conditions produced no detectable absorption at the

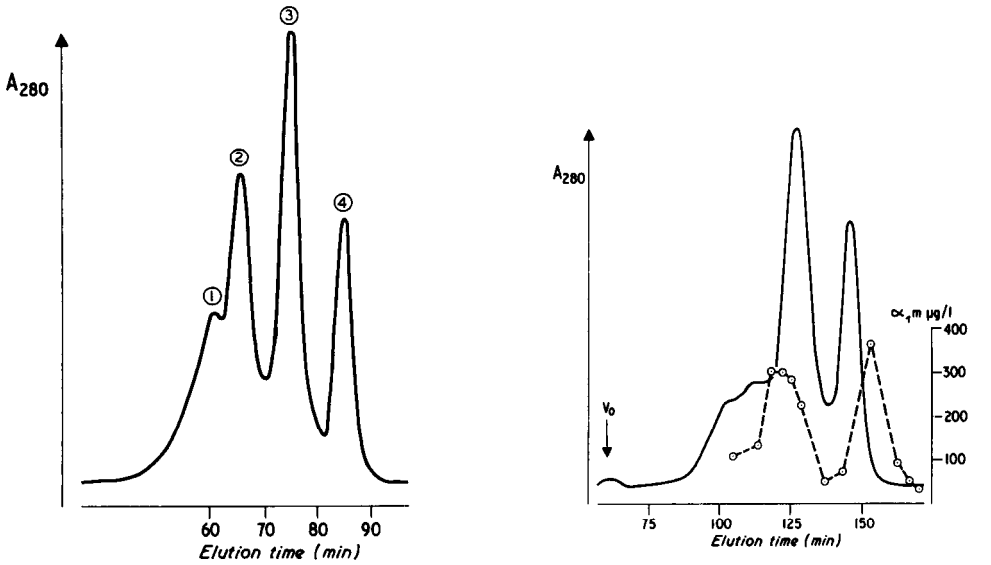

Fig. 1. Separation of IgA myeloma serum on a Superose 6 gel chromatography column. Elution was with phosphate–KCl buffer, at a flow-rate of 0.2 ml/min. The load was 200 µl of serum, diluted 1:20. Absorbance setting at 280 nm was 0.5 a.u.f.s. Peaks: 1 = IgA trimer (molecular weight 500 000); 2 = IgA dimer (340 000); 3 = IgA monomer (160 000); 4 = albumin (66 300).

Fig. 2. Separation of a monomeric IgA myeloma serum on Sepharose 6B with phosphate–KCl buffer, at a flow-rate of 0.5 ml/min. The fractions were assayed for α_1-m concentration using an enzyme immunoassay. The concentration of α_1-m is indicated by the open circles. V_0 is the void volume of the column, demonstrated with Blue Dextran-2000.

times corresponding to the elution of monomeric and polymeric IgA. This confirms earlier studies in which gel chromatography on Sepharose 6B[17], thin-layer gel chromatography followed by crossed immunoelectrophoresis[18], or analytical ultracentrifugation[19] were used, which indicated that the polymerization of IgA myeloma protein is a key factor in producing an elevated serum viscosity.

Semi-quantitative assays of the concentration of α_1-microglobulin by latex agglutination of the fractions eluted from the Superose 6 column indicated a strong α_1-m reaction in fractions eluted after the albumin peak and variable agglutination by the IgA monomer fractions. Quantitative analysis of the α_1-m by EIA confirmed that α_1-m bound to IgA was eluted shortly before the main IgA monomer peak and a peak of free α_1-m eluted after albumin (Fig. 2). α_1-Microglobulin was not associated with dimeric IgA or its higher polymers. In 45 IgA myeloma sera from untreated patients the range of α_1-m concentration was 60–271 mg/l, and the corresponding IgA monomer concentration was 17.4–74.4 g/l. The α_1-m and IgA monomer concentrations were significantly correlated ($r = 0.573$, $p = 0.0001$). However, there was no significant correlation between the dimeric IgA in these sera and the α_1-m levels ($r = -0.227$, $p = 0.146$). Fractions eluted after albumin containing free α_1-m from the sera of IgA myeloma patients with and without renal failure were examined by SDS–PAGE. Immunofixation for α_1-m after Western blotting confirmed that the free α_1-m was present mainly as monomer with very small amounts of dimer (Fig. 3).

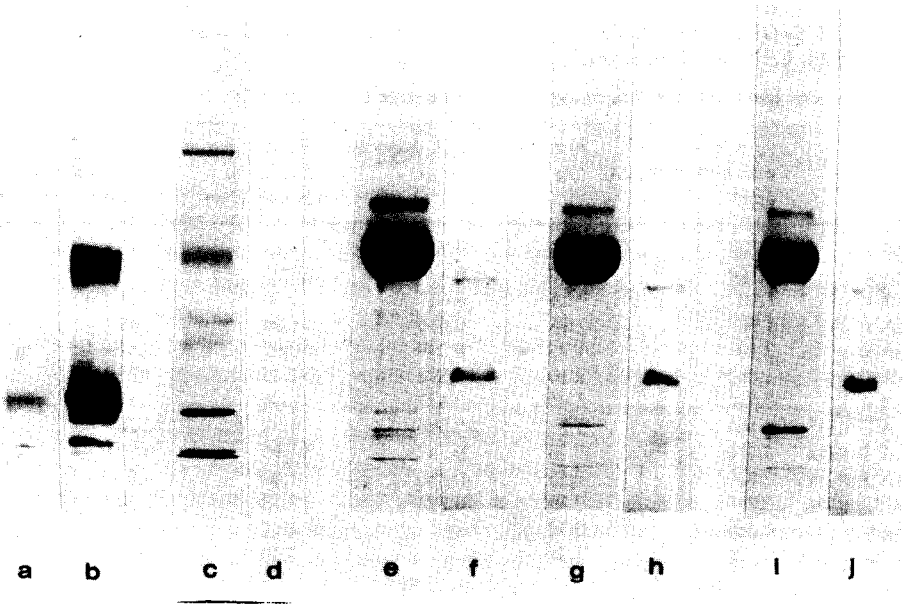

Fig. 3. SDS–PAGE and Western blotting of free α_1-m, separated from IgA myeloma sera by chromatography on Sepharose 6B. Track a is α_1-m, purified from urine and b its corresponding blot. In track c are low-molecular-weight markers (94, 67, 43, 30 and 20 kilodalton) and d their blot. Immunofixation of all blots was for α_1-m. Tracks e, g and i are the free α_1-m from 3 individual sera and f, h and j their corresponding blots.

A series of IgA myeloma sera was chromatographed on a Superose 6B column at a flow-rate of 1 ml/min and the IgA monomer fraction and the fraction containing free α_1-m were assayed by EIA for α_1-m concentration. Analysis of monomeric IgA myeloma proteins and IgG (sub-classes 1, 2 and 4) myeloma proteins showed the average peak retention times for these two groups of proteins were 74.1 ± 0.43 and 78.81 ± 0.56 min under the conditions described above. In myelomatosis the level of polyclonal IgG is suppressed; it was considered that the combination of the resolution and the relatively low concentration of polyclonal IgG in IgA myelomatosis allowed the absorbance of the IgA monomer peak to be used as an estimation of the IgA concentration ($E^{1\%}_{280}$ for IgA = 13.4). Marked differences in the α_1-m binding capacity of individual monomeric IgA myeloma proteins are apparent. The variation in the ratio of bound to unbound α_1-m, and the binding capacity of IgA monomer is shown in Table I. IgA myeloma proteins of the IgA2m(1) type have light chains that are joined to each other by disulphide bridges but held to the α_2-heavy chains by non-covalent forces. This contrasts to all other classes of immunoglobulins in which the light and heavy chains are joined by inter-chain disulphide bridges.

Crossed immunoelectrophoresis of the IgA monomer fraction showed that part of the α_1-m could be displaced from the IgA by electrophoresis and ran with an α_1 mobility. The dissociated α_1-m had an electrophoretic mobility that is slightly slower than α_1-m isolated from urine.

TABLE I

DISTRIBUTION OF BOUND AND FREE α_1-m IN IgA PARAPROTEINAEMIA SEPARATED BY SUPEROSE 6B CHROMATOGRAPHY

All samples came from patients with normal serum creatinine (< 130 μmol/l)

Sample	*α_1-m complexed with monomer IgA (μg)*	*Free α_1-m (μg)*	*IgA monomer (mg)*	*Ratio free α_1-m: bound α_1-m*	*Ratio μg bound α_1-m: mg IgA monomer*
1 IgA λ	0.768	1.40	0.280	1.82	2.74
2 IgA κ	2.160	4.80	0.396	2.22	5.46
3 IgA κ	1.70	4.25	0.617	2.50	2.76
4 IgA κ	1.90	1.98	0.636	1.04	2.99
5 IgA κ	2.10	1.95	0.599	0.93	3.51
6 IgA κ	1.680	2.10	0.392	1.25	4.29
7 IgA κ	0.510	1.05	0.437	2.06	1.17
8 IgA 2m(1)λ	1.560	3.0	0.366	1.92	4.26
9 IgA 2m(1)λ	0.516	7.20	0.742	13.95	0.70
10 IgA 2m(1)κ	2.880	5.55	0.360	1.93	8.0
11 IgA 2m(1)κ	0.984	7.80	0.394	7.93	2.50
12 Normal	0.668	0.78	–	1.17	–
13 Normal	0.420	1.34	–	3.20	–

α_1-m–IgA Complexes

IgA monomer fractions from the Superose 6B were purified on two alternative immunosorbent columns. One aliquot was adsorbed on an immobilized anti-α chain to separate IgA and its complexes, and a second aliquot was adsorbed on immobilized anti-α_1-m to separate the α_1-m–IgA complexes. After elution from the affinity columns, the eluates were dialysed against phosphate buffer and applied to an analytical

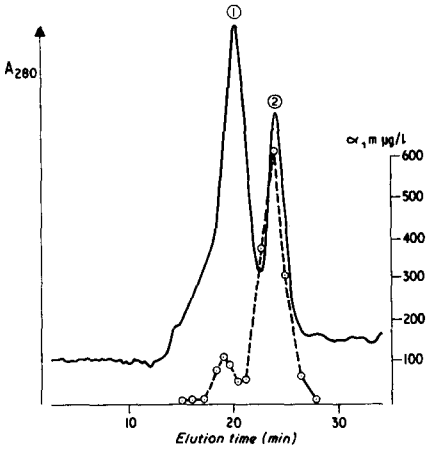

Fig. .4. Chromatography on Superose 12 of the reduction products of α_1-m–IgA complexes, isolated from IgA myeloma sera by immunosorbent chromatography. The desorbed eluate was incubated with 10 m*M* dithiothreitol for 1 h at room temperature and alkylated with 20 m*M* iodoacetamide. Running buffer, 6 *M* guanidinium chloride in 0.05 *M* phosphate (pH 7.2); flow-rate 0.5 ml/min. The two peaks of α_1-m concentration as assayed by enzyme immuno-assay (open circles), eluted at 18.0 and 24.0 min; the peaks of α-heavy chains and light chains were at 20.5 and 24.3 min, respectively.

Superose 6 column. The purified IgA monomer from the α-chain immunosorbent column gave a single peak with the same retention time (74.0 min) as the crude monomer fraction (73.8 min) before the immunosorbent chromatography. When the eluate from the α_1-m immunosorbent column was chromatographed on the Superose 6 column and the fractions assayed for α_1-m using EIA, α_1-m was found as two peaks. The first peak, eluted at 71.6 min, contained α_1-m and IgA. The second peak of α_1-m was eluted with its peak at 86.2 min, which was later than the elution time of albumin (84.3 min). The distribution of α_1-m containing fractions was consistent with being IgA–α_1-m complex and free α_1-m. When the eluates from both the immunosorbent columns were chromatographed on Superose 12 in the presence of 6 *M* guanidinium chloride, the purified IgA monomer and the bound fraction eluted

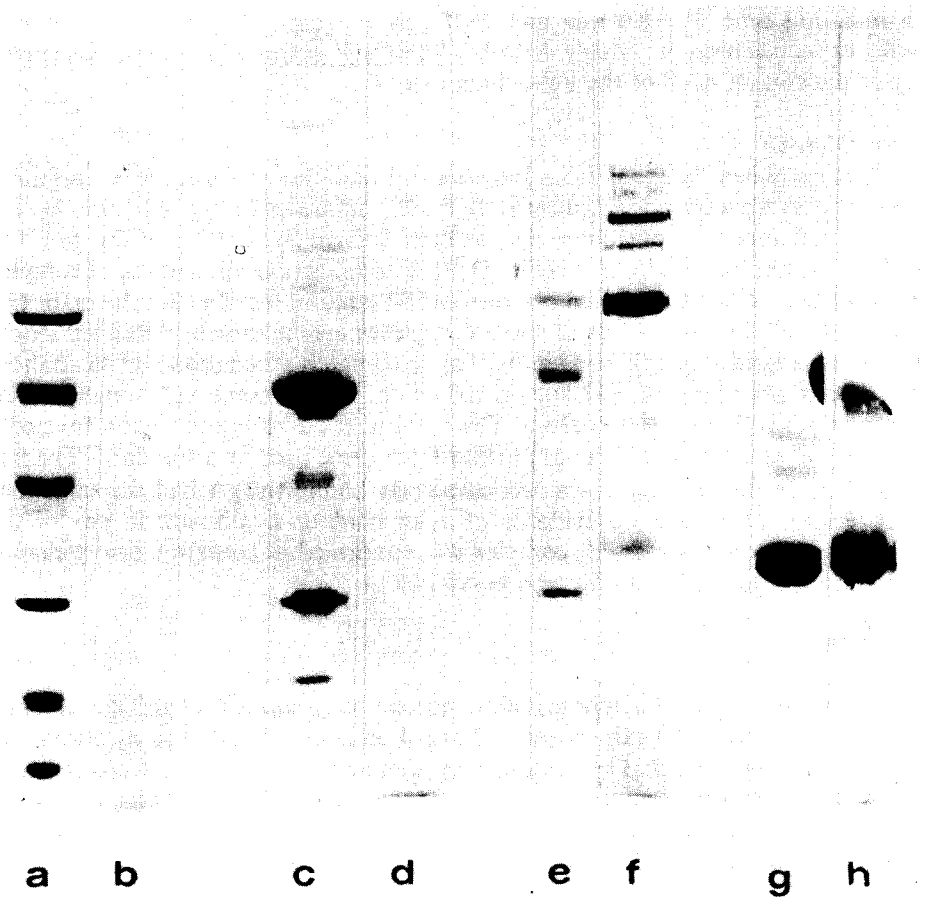

Fig. 5. SDS–PAGE and Western blotting. Pooled IgA monomer fractions separated on Superose 6B column were applied to an α_1-m immunosorbent column, the bound material desorbed with $MgCl_2$. All samples were reduced in 1% 2-mercaptoethanol before SDS–PAGE. In track a are low-molecular-weight standards 94, 67, 43, 30, 20, and 14.4 kilodalton, b corresponding blot; track c is non-bound fraction from α_1-m immunosorption, d corresponding blot; track e is bound fraction, eluted from α_1-m immunosorption, f corresponding blot; track g is purified urinary α_1-m, h corresponding blot. Immunofixation of all blots was for α_1-m.

from the α_1-m immunosorbent column had the same retention time and appeared as a single peak when monitored at 280 nm. EIA demonstrated that the α_1-m was partially dissociated from the IgA under these conditions. α_1-Microglobulin could be detected by EIA in fractions eluting both in association with the monomeric IgA and as free α_1-m. The bound fractions from the two immunosorbent columns were then reduced and the reduction products were separated by chromatography on a Superose 12 column in the presence of 6 *M* guanidinium chloride at a flow-rate of 0.5 ml/min. The purified IgA monomer showed two peaks, corresponding to heavy and light chains. The enriched α_1-m–IgA complex extracted from the IgA monomer fraction by immunosorbent chromatography also showed two peaks. The heavy chain (mol.wt. 55 kdalton) eluted at 20.5 min and the light chains (mol.wt. 25 kdalton) at 24.3 min. Analysis of the fractions for α_1-m immunoreactivity by EIA showed peaks with retention times of 18.0 min and 24.0 min, corresponding to the 90-kdalton fragment that has been previously described[11], as well as free α_1-m in the close proximity of the elution peak of the light chain (Fig. 4).

Western blotting

IgA monomer fractions from Superose 6B and eluates from the α_1-m-immunosorbent column were examined by SDS–PAGE before and after reduction by 1% 2-mercaptoethanol. The positions of α_1-m were located by blotting of the gels, followed by immunofixation for α_1-m (Fig. 5). Prior to reduction, the IgA–α_1-m complex split into a least 4 bands, situated between the 94 kdalton marker and the main IgA monomer band. The structures of these complexes are unknown. After reduction, the α_1-m was found in mainly two bands of approximately 90 kdalton and 30 kdalton. Minor bands of complexes, resistant to reduction, were present with weights intermediate between 90 and 160 kdalton. The α_1-m in the 30-kdalton region formed a band of equal mobility to free α_1-m purified from urine used as a standard. The free α_1-m present in the sera and α_1-m non-covalently bound to IgA had the same molecular weight. There was no evidence of α_1-m binding to albumin in any of the electrophoretic experiments. The complex was confirmed to contain α heavy chains, by immunofixation with antisera to α heavy chains.

DISCUSSION

Monomeric IgA monomer myeloma protein has a mol.wt. of 160 kdalton and can be resolved from IgG sub-classes 1, 2 and 4, mol.wt. 150 kdalton by chromatography on Superose 6 gel. The reasons why these proteins of comparable mol.wt. separate are uncertain. It is known that the hinge region of IgA is twice the size of IgG, and the α heavy chains are 55 kdalton compared to the γ heavy chains of 50 kdalton. By contrast, IgG3 cannot be separated from IgA monomer by gel chromatography[20]. The IgG3 has a hinge region which contains up to 13 disulphide bridges[21]. This suggests it is the difference in shape and hydrodynamic volume that enables IgA1 to be separated from IgG1, 2 and 4.

IgA has been established to bind several proteins: lactic dehydrogenase and α_1-antitrypsin are bound to its kappa light chains[22,23], and IgA–albumin complexes have been detected, but the binding of other proteins seems to be relatively small in amount[18,24]. The present studies have indicated α_1-microglobulin is bound to IgA

by two mechanisms, covalent binding to an α heavy chain and non-covalent binding. It is unlikely that the non-covalent binding involves light chains as patients with IgA myeloma who are excreting kappa or lambda light chains and α_1-m in their urine show no evidence of complex formation between these two proteins[10]. Furthermore, the type of light chain and its link to the α chains does not appear to influence the α_1-m binding to IgA. There is considerable variation in the α_1-m binding to IgA that is probably a reflection of differences in the α-heavy chains and possibly in the α_1-m. It is well recognised that the ratios of bi- tri- and tetra-antennary glycans of plasma glycoproteins such as α_1-m may be modified in disease[25]; this could affect the non-covalent binding of α_1-m to IgA. The non-covalent binding dissociates under several conditions, during elution with 4 *M* magnesium chloride, exposure to 6 *M* guanidinium chloride or electrophoresis. The non-covalently bound α_1-m appears to vary in its affinity for IgA as it becomes progressively displaced with harsher conditions. A similar dissociation of non-covalent binding is observed when monomeric IgA2m(1) is exposed to 6 *M* guanidinium chloride which causes the light chain dimers and heavy chain dimers to separate without disrupting the inter chain bridges.

The high levels of free α_1-m in the serum in IgA myelomatosis in the absence of renal impairment are likely to be the result of the attraction of the IgA monomer for the α_1-m, holding it in the blood by opposing the ultrafiltration of free α_1-m in the glomerulus.

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