A SUBCLASS OF HUMAN γA-GLOBULINS (γA2) WHICH LACKS THE DISULFIDE BONDS LINKING HEAVY AND LIGHT CHAINS*

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It has been shown that γA-immunoglobulin is the major immunoglobulin present in a variety of external secretions, and it has been postulated that the capacity of this protein to enter these secretions is perhaps the most important biologic function that this class of immunoglobulins performs (1–3). Immunochemical analysis of human γA-myeloma proteins has led to the subdivision of γA-proteins into two subclasses, γA1 and γA2. This differentiation was based on the capacity of certain antisera made against γA1-proteins to demonstrate an antigenic deficiency of the γA2-proteins which was shown to be due to antigenic differences in the heavy chain (4–6). In the previous studies, it was not possible to obtain a specific antiserum for the γA2-proteins and this prevented quantitation of this fraction.

The present studies were undertaken to learn more about the chemistry of the γA2-proteins which distinguished them from other proteins of the γA-class and to obtain specific antisera which permitted quantitation in serum and external secretions. Such antisera were obtained and studies utilizing them indicated higher relative concentrations of γA2-proteins in external secretions than in serum. The chemical studies on γA2-proteins led to the surprising finding that the heavy and light chains were not linked by disulfide bonds as for other γA-proteins and other immunoglobulins.

Materials and Methods

Protein Isolation.—Human γA-proteins were isolated by block electrophoresis (7) followed by G-200 Sephadex gel filtration (8) in phosphate-buffered saline, pH 7.2. Polymeric and monomeric forms of the same protein were isolated separately. Colostrum was obtained

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from lactating mothers 1–4 days following delivery. Prior to antigenic analysis, it was clarified by ultracentrifugation at 35,000 rpm for 1 hr in a Spinco model L ultracentrifuge.

Starch Gel Electrophoresis.—Vertical starch gel electrophoresis was performed utilizing an 8 M urea-formate buffer, pH 3.0 (9). Electrophoresis was carried out at room temperature for 16 hr at 170 v.

Antigenic Analysis.—The methods utilized for quantitation of γA- and γA2-levels were essentially those described previously (10). Hemagglutination inhibition reactions were performed after coating human red cells with myeloma proteins of the γA1- and γA2-subclasses as described for other systems (11).

Preparation of Heavy and Light Chains.—Heavy and light chains were isolated as previously described (12), using a Sephadex G-100 column equilibrated in 1 M acetic acid to separate the reduced and alkylated chains.

Sulfhydryl Determination.—To determine the number of sulfhydryl (SH) groups present following partial reduction, γA–myeloma proteins were reduced with 0.02 M dithiothreitol (DTT) and alkylated with a 50% molar excess of ^{14}C-labeled iodoacetic acid over SH groups present. Heavy and light chains were separated as described above, and the specific activity of the separated chains was determined in a Packard Tri-Carb liquid scintillation counter using Bray's solution as solvent (13). The number of SH groups present per mole of heavy chain was calculated by taking the average specific activity present on all light chain preparations to represent 1 mole of SH group per mole of peptide chain.

RESULTS

Chemical Studies

Separation of Light and Heavy Chains.—In the course of isolating γA-proteins of the two heavy chain subclasses, it was observed that proteins of the γA2-subclass, when examined by starch gel electrophoresis in 8 M urea-formate buffer, demonstrated a protein band which was more cathodically migrating than any band seen with proteins of the γA1-subclass (Fig. 1). This band migrated more rapidly than monomer γA-proteins and was accompanied in some instances by a fainter, even more rapidly migrating component (arrows, Fig. 1).

These rapidly migrating components were observed in proteins isolated by starch block electrophoresis and G-200 gel filtration. The G-200 gel filtration pattern of one γA2-protein is shown in Fig. 2. The major protein peak was eluted just following the void volume of the column in the region where polymer γA-proteins would be expected to be eluted. This peak was asymmetric in the descending portion due to the presence of a small amount of γA-monomer. A small second peak, composed mainly of transferrin as well as other non-immunoglobulin β-globulins, was also present. The rapidly migrating bands shown in Fig. 1 were seen in the material pooled from the major polymer peaks. The elution volume of dimer and monomer human Bence Jones proteins is indicated by the arrows.

Since under the acid conditions used for electrophoresis the size of the proteins being separated is a major factor in determining the mobility in the starch gel, and since the cathodic bands observed with the γA2-proteins migrated

more rapidly than monomeric γA-proteins, the possibility arose that under the dissociating conditions of 8 M urea some low molecular weight fragment which was noncovalently bound to the γA2-proteins was split off from the parent molecule. To test this hypothesis further, the polymer peak from the γA2-proteins isolated by starch block electrophoresis and G-200 gel filtration, was applied to a Sephadex G-100 column equilibrated in 1 M acetic acid. The elution curve is shown in Fig. 3. Two incompletely separated peaks were observed. The first peak was eluted with the void volume of the column and consisted predominantly of the more slowly migrating bands observed on starch gel electro-

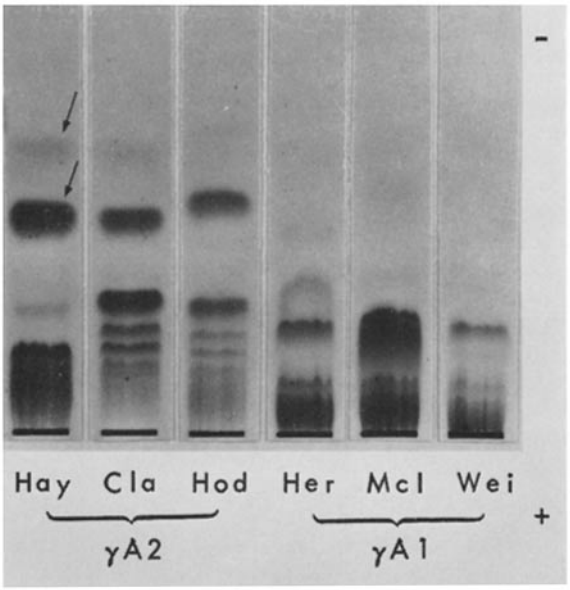

FIG. 1. Formate–8 M urea starch gel electrophoresis of unreduced human γA-myeloma proteins. Arrows point to cathodic bands present in γA2-proteins but not in γA1-proteins.

phoresis. The second, smaller peak consisted almost exclusively of the more rapidly migrating starch gel band. This second peak was eluted at the same position as a dimer Bence Jones protein examined on the same column. These two peaks were examined antigenically by immunodiffusion with antisera specific for γA, and for κ-light chains (Fig. 4). Almost all of the γA-specific protein was located in the first peak. κ–light chains were present in both peaks, but were predominant in the second peak. These data indicated that a large fraction of the light chains present in this protein were noncovalently linked to the heavy chains, and were dissociated from the heavy chains as L-L dimers. Nitrogen analysis of the two peaks indicated that 26% of the total protein was present in the light chain dimer peak.

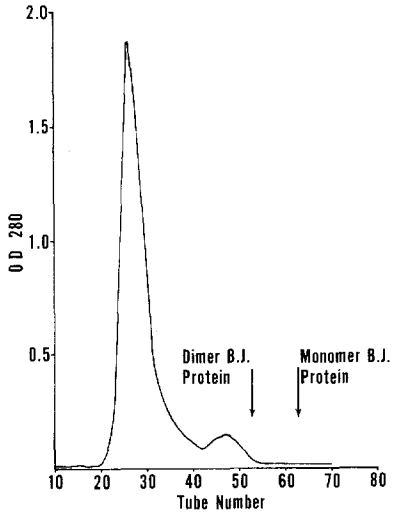

FIG. 2. G-200 gel filtration in phosphate-buffered saline, pH 7.2, of starch block isolated γA2-myeloma protein, Hay. The first peak consisted almost entirely of polymer-γA and emerged shortly after void volume of column. The second minor peak consisted of transferrin and other nonimmunoglobulin β-globulins. Arrows indicate elution volumes for monomer and dimer Bence Jones proteins on this column.

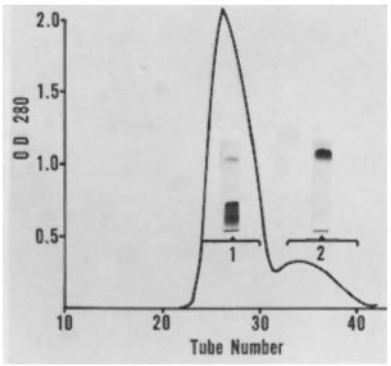

FIG. 3. Sephadex G-100 gel filtration in 1 M acetic acid of unreduced γA2-myeloma protein, Hay. The two peaks were pooled as indicated by the brackets and the acid-urea starch gel electrophoresis patterns of each peak are shown. Peak 1 contained the slow-migrating bands as well as a small amount of the electrophoretically more rapidly migrating band. Peak 2 consisted almost entirely of the rapidly migrating band. Dimer Bence Jones proteins were eluted in the same volume as peak 2.

To obtain more information regarding the extent to which the light chains were noncovalently bound to the heavy chains, the first peak obtained by gel filtration of the unreduced protein on Sephadex G-100 in 1 M acetic acid, was reduced with 0.2 M 2 mercaptoethanol, alkylated, and reapplied to a G-100

1 M acetic acid column. The elution pattern is shown in Fig. 5, and is compared with the pattern obtained with the reduced and alkylated γA-polymer of the same protein which was not previously gel filtered on Sephadex G-100 in 1 M acetic acid. The whole protein (dashed curve) gave a typical filtration pattern for heavy and light chains. The light chain peak made up 32% of the total protein eluted from the column. In contrast, the reduced and alkylated first peak obtained from the G-100 1 M acetic acid column gave a barely detectable

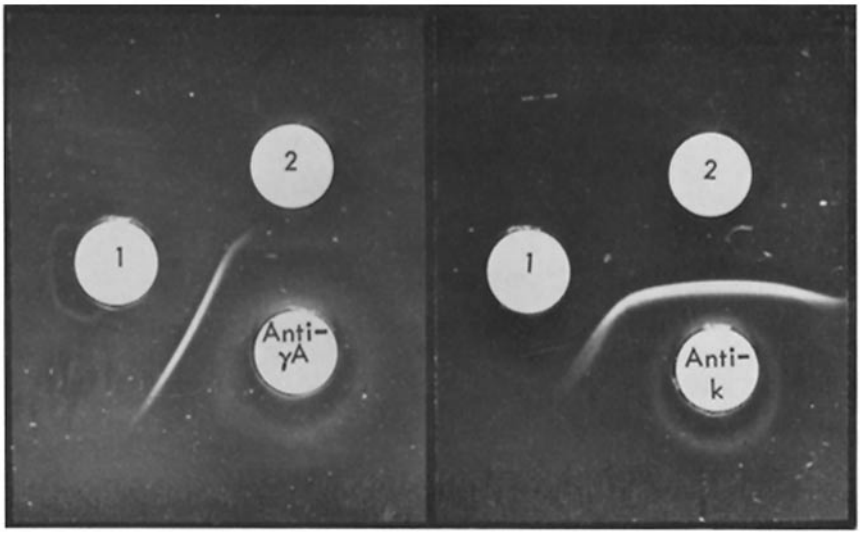

FIG. 4. Immunodiffusion analysis of the two peaks obtained by G-100 gel filtration in 1 M acetic acid of unreduced γA2-protein, Hay. The two peaks were pooled as shown in Fig. 3 and reacted against rabbit antisera specific for α-chains and κ-chains. Almost all α chain determinants were found in peak 1. κ-chain determinants were present in both peaks, but the predominant reaction occurred with peak 2.

peak in the light chain region (solid lines). Since 26% of the total protein was released as light chains without reduction and 32% with reduction, and since starch gel electrophoresis indicated that not all of the noncovalently bound light chains were separated from the heavy chains under the conditions used for gel filtration (Fig. 3), it seems probable that most, and perhaps all, the light chains present in this γA2-protein were not disulfide-bridged to the heavy chains.

The fact that the light chains were eluted in the same volume as Bence Jones protein dimers, suggested that the light chains were in the form of disulfide bonded L-L dimers. This suggestion was confirmed by reducing and alkylating the second peak obtained from the G-100 1 M acetic acid gel filtration of the unreduced γA2-proteins and examining it by starch gel electrophoresis

and G-100 gel filtration in 1 M acetic acid. In both starch gel electrophoresis and G-100 gel filtration, the reduced and alkylated second peak appeared in the same position as monomer light chains.

A total of 20 γA1- and 5 γA2-proteins were examined by urea-formate starch gel electrophoresis. All five γA2-proteins demonstrated a rapidly migrating L chain dimer band similar to those illustrated in Fig. 1. In all cases a faint monomer light chain band was also observed. None of the 20 γA1-proteins had either a monomer or dimer light chain band present on starch gel

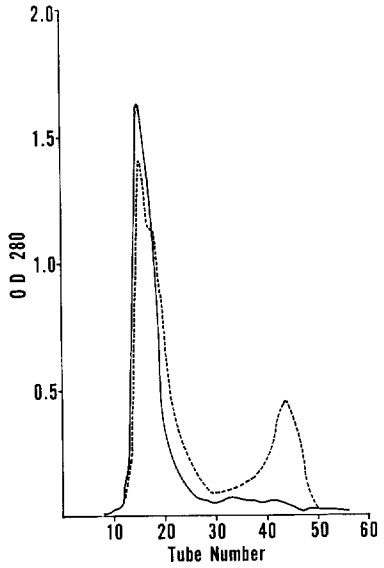

FIG. 5. Sephadex G-100 1 M acetic acid gel filtration of the partially reduced and alkylated first peak isolated as shown in Fig. 3 (solid line). No significant light chain peak was observed. Dashed line shows the elution of heavy and light chains from the same protein, which was not passed through a G-100 1 M acetic acid column prior to reduction.

electrophoresis. Sephadex G-100 gel filtration in 1 M acetic acid was performed in the unreduced state on all 5 γA2-proteins and 4 of the 20 γA1 proteins. None of the γA1-proteins studied showed any indication of a second peak similar to that shown in Fig. 3. Four of five γA2-proteins showed a distinct second peak similar to that shown in Fig. 3. In one instance, despite a clear L chain dimer band on starch gel electrophoresis (protein Cla, Fig. 1), the noncovalently bound light chains did not form a distinct second peak, but instead were present in the descending limb of the first peak, suggesting that residual noncovalent bonding between the dimer light chains and heavy chains was present even in the 1 M acetic acid used to dissociate the chains.

The presence of noncovalently bound light chains was not dependent on any particular polymeric form of the γA2-proteins. This is illustrated in Fig. 6. The light chain dimer band was present by starch gel electrophoresis in 8 M urea-formate buffer when the monomer and polymer of the same γA2-protein were examined (arrows, Fig. 6). The position of the reduced and alkylated heavy and light chains are shown for comparison. The reduced light chains occupied the same position as the faint monomer light chain band observed with the unreduced proteins.

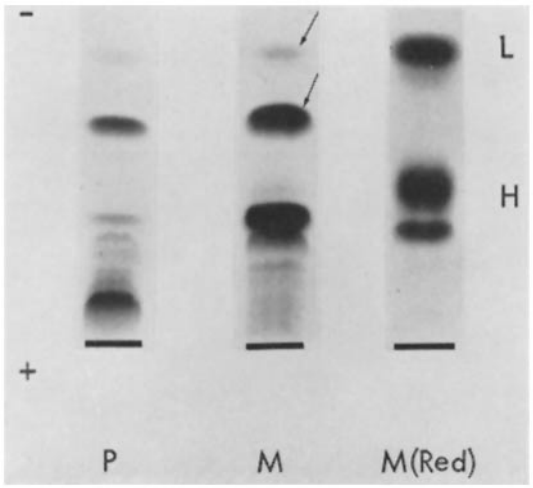

FIG. 6. Formate–urea starch gel electrophoresis of unreduced and reduced γA2-protein. P, unreduced polymer (Cou); M, unreduced monomer (Cou); M red, partially reduced and alkylated Cou monomer. Dimer light chain band as well as faint monomer light chain band was observed in both monomer and polymer-γA2 preparation (arrows). Position of light and heavy chains obtained from partially reduced and alkylated monomer is shown for comparison.

It was considered essential to evaluate the possibility that disulfide interchange reactions might have led to the observed results of the lack of L-H disulfide bridges in the γA2-proteins. To investigate this, the serum from one γA2-myeloma patient was dialyzed against 0.02 M iodoacetamide for 16 hr in the cold at pH 8 prior to the isolation of the myeloma protein by starch block electrophoresis and G-200 gel filtration. G-100 gel filtration in 1 M acetic acid of the unreduced protein and starch gel electrophoresis in 8 M urea-formate buffer gave results identical to those illustrated in Figs. 1 and 3. This is taken as evidence that the absence of the L-H disulfide bonds in the γA2-proteins was not a result of disulfide interchange since the alklylation of the myeloma protein prior to its isolation should inhibit this reaction from occurring.

Quantitation of SH Groups Released from γA1- and γA2-Heavy Chains

Following Partial Reduction.—Since the γA2-proteins lacked the L-H disulfide bridge and since it was apparent from their presence as L-L dimers that the light chains were capable of forming interchain disulfide bonds, it was concluded that the structural alteration which was responsible for the absence of the L-H disulfide bridge in the γA2-proteins was located on the heavy chains. It was considered of interest, therefore, to determine the number of SH groups which were involved in interchain disulfide bridges in the heavy chains of the two subclasses. To obtain this information, two myeloma proteins of each subclass were partially reduced with 0.02 M dithiothreitol for 1 hr at room temperature and then alkylated with a 50% molar excess over the thiol groups present using ^{14}C-labeled iodoacetic acid. Heavy and light chains were separated and the

TABLE I

Sulfhydryl Groups Released by Reduction with 0.02 M Dithiothreitol

Subclass	Protein	Peptide chain	cpm/μM × 10⁶*	MSH(H/L)
γA1	Van	H	2.80	7.6
		L	0.368	
γA1	Tri	H	2.60	7.1
		L	0.372	
γA2	Her	H	2.03	5.5
		L	0.357	
γA2	Cla	H	1.77	4.8
		L	0.374	

* Molecular weights used for this calculation: γA heavy chain, 64,000; light chain, 23,000.

molar specific activity calculated for both. The light chains from the four proteins all had specific activities within 10% of one another. The average value obtained with the four light chains was taken as representing 1 mole of SH group per mole of light chain. The figures for the heavy chains were then expressed as multiples of this figure. The data are shown in Table I and indicate that both γA2–heavy chains had a lower specific activity than the γA1-proteins and, based on the assumption of one mole of SH per mole of light chain, there were approximately seven SH groups alkylated per mole of γA1–heavy chain, and five per mole of γA2–heavy chain.

Antigenic Studies

In previous studies, the γA2-sublcass was only recognized as being deficient with γA1-antisera and no antisera were obtained which were specific for the γA2-subclass. As a result no direct quantitation was possible. In the present study an antiserum was obtained in a cynomolgus monkey which was specific

for the γA2-subclass and permitted quantitation of this component in normal serum and colostrum. Fig. 7 illustrates the results of agar plate analyses utilizing this antiserum after absorption with γA1-proteins. The reaction of the same proteins with a γA1-antiserum is also shown. The γA2-specificity was also brought out in a hemagglutination system which detected small amounts of γA2 by hemagglutination inhibition. All sera examined (200 from various population groups) showed the definite presence of γA2-protein by the inhibition technique. Many of these sera were also examined by quantitative immunodiffusion with very parallel results. Studies of colostrum and saliva also revealed the γA2-component in all specimens and at higher absolute and

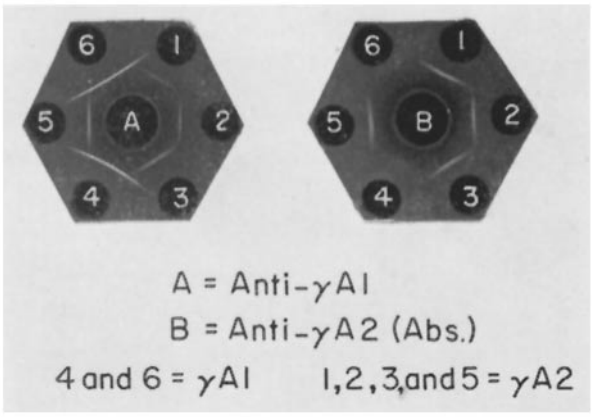

FIG. 7. Agar plate analyses showing the differentiation of γA1- from γA2-proteins with antisera specific for each type. The same six myeloma proteins were placed in the peripheral wells in the two rings.

relative concentrations than in serum. Table II shows some representative serum and colostrum levels of γA2-protein compared to the total γA-level utilizing the quantitative assay. A major problem in these quantitations concerned the question of the right standard that should be employed. These data were obtained using γA-monomer standards for the serum studies and γA-polymer standards for the colostrum. This was based on density gradient studies demonstrating that both γA1 and γA2 were primarily monomeric (7S) in serum and polymeric in colostrum. These analyses are continuing and will be published in detail separately.

In the course of these studies, two types of γA2-proteins were delineated based on antigenic differences in the heavy chains. Both types were found in all normal sera and in all colostrum and saliva specimens indicating that they did not represent genetic variants. In addition both γA2-types showed an absence of the L-H disulfide bonds.

DISCUSSION

The present studies indicate that the two subclasses of human γA-immunoglobulins differ from one another with regard to the manner in which their polypeptide chains are linked together. Myeloma proteins of the γA2-subclass lacked L-H disulfide bridges so that the light and heavy chains were held together only by noncovalent bonds. The light chains were present in the γA2-molecule almost exclusively in the form of L-L disulfide bridged dimers. The proteins of the γA1-subclass on the other hand possessed L-H disulfide bridges. The observation that the light chains in the γA2 proteins were in the form of disulfide linked dimers, has certain implications regarding the gross arrangement of the peptide chains within the γA2-molecule. At the point of disulfide linkage, the two light chains must be in close apposition to one another. This linkage almost certainly occurs at the C-terminal end of the light chains.

TABLE II

Quantitative Levels of Total γA and γA2 in Serum and Colostrum from Four Individuals

		γA	γA2
Wi.	Serum	0.80	0.29
	Colostrum	1.23	0.75
Ol.	Serum	1.11	0.22
	Colostrum	4.16	2.70
Le.	Serum	1.68	0.33
	Colostrum	8.4	3.1
Fu.	Colostrum	2.8	0.88

This arrangement is schematically represented in Fig. 8. The placing of both light chains inside of the two heavy chains was done only to allow formation of the L-L disulfide bond at the C-terminal end of the light chain, and is not meant to imply that the light chains must be in close apposition to one another along their entire length.

Other human immunoglobulins have been examined to determine whether the absence of the L-H disulfide bond was unique to γA2-proteins or whether other immunoglobulin classes possessed this property as well. For this purpose, 12 γG–myeloma proteins representative of the four heavy chain subclasses, and 4 γM-proteins were examined by starch gel electrophoresis in 8 M urea. No light chains dissociated from these proteins without prior reduction. Mouse γA–myeloma proteins were also examined by starch gel electrophoresis and G-100 gel filtration. Six of six mouse γA-proteins studied lacked L-H disulfide bonds. Quantitative results very similar to those described above for human γA2-proteins were obtained in all of the mouse γA-proteins studied and these will be published separately (14).

The structural variation in the γA2-proteins responsible for the lack of the L-H disulfide bridge is not known. The fact that the light chains are capable of forming interchain disulfide bonds as evidenced by the presence of L-L disulfide dimers, strongly suggests that a structural variation on the heavy chain is responsible. The finding that partial reduction releases seven SH groups per mole of γA1–heavy chain and only five SH groups per mole of γA2–heavy chain, indicates that structural differences within the heavy chains of the two subclasses exist with respect to the lability of their respective disulfide bonds. At present it is not known whether the cysteine residue on the γA2–heavy chain which is responsible for the L-H disulfide bridge is absent on that heavy chain, or whether it is present but involved in a stable intrachain disulfide bridge which was not susceptible to reduction under the conditions employed in the present study.

Recent work by Frangione et al. (15) has demonstrated that the γG–heavy

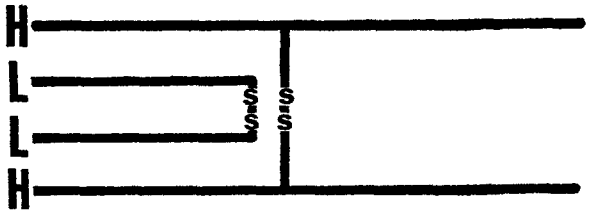

FIG. 8. Schematic structure of γA2-immunoglobulins.

chain subclasses differ from one another with regard to the position of the heavy chain cysteine which is involved in the L-H bridge. Also, these workers have been able to show considerable heterogeneity with regard to the number of H-H interchain bonds present from one γG–subclass to another. This heterogeneity of interchain disulfide bonds is in contrast to the relative constancy of the intrachain disulfides in γG–light and heavy chains (16–18). The importance of intrachain bonds in stabilizing the tertiary structure of the peptide chains may offer an explanation as to why the position of these bonds has been conserved throughout immunoglobulin evolution; the heterogeneity of the interchain bonds, on the other hand, suggests that these bonds may be of lesser importance to the antibody functions of mammalian immunoglobulins.

The antigenic studies described indicate that all normal sera contain significant amounts of γA2-protein. The concentration obtained varied in different sera but appeared somewhat higher than the approximately 10% incidence (4–6, 19) of γA2–myeloma proteins would suggest. However, further data are required to determine the exact mean concentration. A number of problems were encountered in this study which made this difficult. Among these was the finding of two distinct γA2-subgroups for myeloma proteins and all normal

sera. Of special interest were the high relative values found in colostrum and saliva. In some specimens close to one-half, the γA-concentration was composed of γA2-proteins. The latter consisted of both subgroups of γA2 which were found in serum. Preliminary evidence was obtained for antibody activity in the γA2-fraction. Isolated isoagglutinins showed the presence of some γA2 by antigenic analysis. This is of special interest because it would indicate that this immunoglobulin can function as an antibody with the light chains disulfide-bonded and without a covalent linkage to the heavy chains. It seems possible that the γA2-class may represent a more primitive form of immunoglobulin. The recent report (20) of a probable γM-protein in the lamprey which lacks L-H disulfide bonds would be in line with such a concept.

The finding of these relatively high concentrations of γA2-protein in secretory γA may help explain certain findings of other investigators working on secretory γA. It has been observed that easily detectable quantities of light chains are released when unreduced secretory γA is separated by gel filtration in dissociating solvents such as 1 M acetic acid, or 5 M guanidine (21–23). This can now be explained by the presence of γA2-molecules in secretory γA. Also, there is conflicting data regarding whether or not "secretory piece" (SP) is disulfide linked to the γA molecule. In man, some SP is released without reduction, but greater yields are obtained when the reduced and alkylated γA is separated (22, 23). In the rabbit it has also been observed that considerable light chains and secretory piece are released from the γA without reduction (24). It is possible that a situation exists with respect to SP-binding similar to that observed with light chain–binding and that SP is bound noncovalently to the γA2-molecules of secretory γA, and that it is disulfide linked to the γA-1 molecule. Experiments to test this hypothesis are in progress.

SUMMARY

The γA2-subgroup of γA-globulins, previously delineated by antigenic studies, was found to differ strikingly from other immunoglobulins in the manner in which the polypeptide chains are bound together. The heavy and light chains were not linked to each other by disulfide bonds. Instead the light chains were disulfide linked to one another, and were present in the γA2-molecule as disulfide bridged L-L dimers.

Antisera specific for γA2-proteins indicated the occurrence of two different antigenic types in all normal sera as well as saliva and colostrum. Both of these showed the unique interchain disulfide linkage. Quantitative analyses indicated higher levels of γA2-proteins in external secretions.

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