in which the sample is overlayed with mineral oil (Josephs and Harrington, 1967); pressure-sensitive equilibria show reversible pressure effects upon rapid shifting of rotor speed (Morimoto and Kegeles, 1971).

Irreversible reactions which are not pressure induced can be identified by analyzing the fraction rich in reactant after it has been aged for varying lengths of time.

Finally, these results also apply with only quantitative reservations to molecular sieve chromatography on Sephadex or other gel permeation supports.

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Structure of Immunoglobulin A. Amino Acid Sequence of Cysteine-Containing Peptides from the J Chain†

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ABSTRACT: A polymeric human IgA_1 myeloma protein (Oso) was partially reduced and alkylated with [^{14}C]iodoacetic acid. The J chain was isolated by several procedures. After partial reduction, at least eight cysteine-containing peptides were isolated and sequenced. Although the function and location of most of them remain unknown, one was shown to be the N-terminal peptide of the chain. The partial sequences of these carboxymethylcysteine peptides from the J chain indicated that they are different from those present in heavy and light chains. While this would suggest that the J chain has evolved independently, a possible relationship to the hinge region or other heavy-chain gene products whose structure is unknown deserves further study.

In 1970, Halpern and Koshland described the existence of a polypeptide chain in human and rabbit polymeric IgA[1] characterized by its rapid anodal mobility on alkaline urea gel electrophoresis. Shortly after, a similar chain was also observed in a Waldenstrom's macroglobulin (Mestecky *et al.*, 1971) and in proteins from several other species (Klaus *et al.*, 1971). In retrospect it would appear that this fast band had already been noted in secretory IgA in 1967 (Cebra and Small, 1967). Although its function remains unknown, its presence only in polymerized immunoglobulins suggests that it may serve to "join" the subunits and hence it was called the "J chain." Chemical studies of polymer IgA and IgM molecules and J chains have demonstrated that there is only one J chain per dimer (Morrison and Koshland, 1972) or pentamer (Mestecky *et al.*, 1971), that it is present only in polymeric IgA and IgM and not in monomeric IgA, and that it is rich in cysteine, containing approximately twice as much as light chains. Some question remains about the size of J chains since the molecular weight in sodium dodecyl sulfate polyacrylamide gel electrophoresis was 24,500, while

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[1] The nomenclature employed for the immunoglobulins follows that recommended by the World Health Organization, *Bull. W.H.O. 41*, 975 (1969).

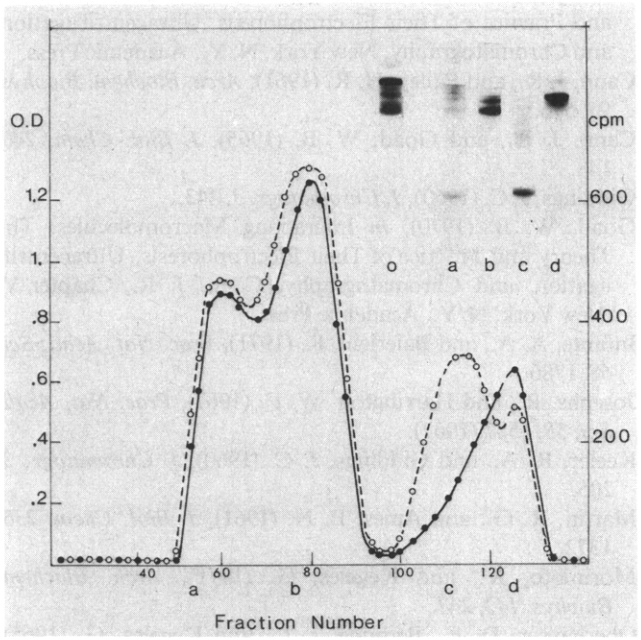

FIGURE 1: Fractionation on Sephadex G-100 of partially reduced and carboxymethylated myeloma protein Oso (IgA_1k): 250 mg of protein was dissoved in 12 ml of 1 M HAC and applied to a column (130 × 3 cm); 5-ml aliquots were collected. Polyacrylamide disc gels indicate that fraction a consists of polymers of heavy and J chains; fraction b of heavy chains; fraction c of J chain; and fraction d of light chains: (O) control of partially reduced and alkylated IgA_1 (Oso); (—) radioactivity; (⊖–⊖) absorbance 280 nm.

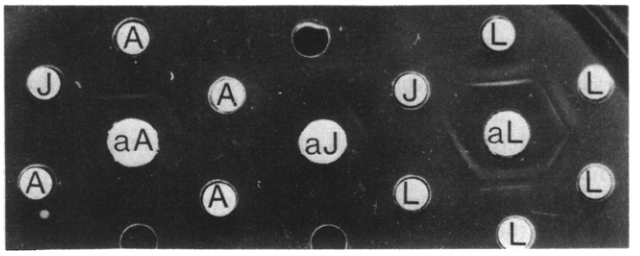

FIGURE 2: Immunodiffusion analysis of the J chain with antisera to α, J, and L chains: (A) IgA myeloma proteins; (J) pure J chain; (L) light chain; (a) antiserum to.

the result based in sedimentation equilibrium was 15,000 (O'Daly and Cebra, 1971). Peptide maps and antigenic studies of the J chain suggest that it has no homology to any immunoglobulin polypeptide chain (Mestecky *et al.*, 1971). In this paper we present sequences around cysteine residues isolated from the J chain of a human polymeric IgA_1 myeloma protein (Oso). In addition, we describe a new method for the purification of the J chain.

Materials and Methods

A human IgA_1 myeloma protein (Oso) was isolated from the serum by either starch zone or Pevikon electrophoresis at pH 8.6 followed by gel filtration on Sephadex G-200 in 0.3 M NaCl. Starch proved preferable since it yielded two peaks, one containing the monomer, the other rich in polymer.

Partial Reduction and Radioactive Alkylation. Solutions of the protein in 0.27 M Tris-HCl buffer, pH 8.2 (20 mg/ml), were treated with increasing concentrations of dithiothreitol from 0.1 to 10 mM. The reaction was allowed to proceed for one hour at room temperature under N_2. Radioalkylation was achieved by adding [^{14}C]iodoacetic acid (100% molar excess) and allowing the mixture to stand for one hour in absence of light. Excess reagents were removed by dialysis against water following which the samples were freeze-dried. Complete reduction and alkylation were carried out in the presence of 7 M guanidine hydrochloride using the same concentration of reducing and alkylating agents.

Polyacrylamide Gel Electrophoresis. Disc electrophoresis was performed in polyacrylamide gels as described by Reisfeld and Small (1966). The samples were dissolved in 10 M urea immediately before use and layered on top of the upper gel. The upper buffer (pH 8.91) was made with 5.1 g of Tris, 3.48 g of glycine, 700 ml of 10 M urea, and water to 1 l. The lower buffer consisted of 14.5 g of Tris, 60 ml of 1 N HCl, and water to 1 l. Electrophoresis was carried out at room temperature at a constant current of 2.5 mA/tube. Phenol Red was used as a marker and electrophoresis was stopped when the Phenol Red reached a position 0.5 cm from the bottom of the gel. After electrophoresis the gels were removed from the tubes, suspended in 5% CCl_3COOH–5% sulfosalicylic acid for 30 min at room temperature, and stained for 4 hr with 0.05% Coomassie Blue in 5% CCl_3COOH–5% sulfosalicylic acid. The gels were destained in a solution of 5% CCl_3COOH–5% sulfosalicylic acid.

Dodecyl Sulfate Polyacrylamide Gel Electrophoresis. It was carried out in 10 and 12% gels as described by Maizel (1969). The proteins were dissolved in 0.05 ml of 5 mM sodium phosphate (pH 7.1) containing 1% (w/v) sodium dodecyl sulfate, 1% (v/v) 2 mercaptoethanol, and 2 M urea and the solution was heated for 5 min in a bath of boiling water (Maizel *et al.*, 1968). Electrophoresis was carried out at a constant voltage of 4.5 V/cm (12 cm) for 15 hr. After electrophoresis, the gels were stained with 0.25% (w/v) solution of Coomassie Blue in methanol–acetic acid–water (5:1:1) for 2 hr and destained in 7.5% acetic acid.

Molecular Weight of J Chain. It was calculated in acrylamide gels containing sodium dodecyl sulfate (Shapiro *et al.*, 1967), by comparing the electrophoretic mobility with marker proteins of known molecular weight: bovine serum albumin, 75,000; ovalbumin, 45,000 (monomer) and 90,000 (dimer); pepsin, 35,000, and trypsin, 22,500.

Ion-Exchange Chromatography. A mixture of J and L chains containing 3.5 mg/ml was applied on DEAE-cellulose columns in a Tris–glycine buffer, pH 8.9 (5.16 g of Tris–3.4 g of glycine), containing 7 M urea and 0.1 M NaCl. After elution of the first L-chain peak with the starting buffer a linear gradient from 0.1 to 0.8 M NaCl in the same buffer was applied to the column. Finally the column was washed with the last buffer.

Enzyme Digestion. The labeled heavy, light, and J chains were dissolved in 0.2 M ammonium bicarbonate (pH 8.3) (20 mg/ml) and digested with trypsin (enzyme:substrate, 1:20, w/w) for 15 hr at 37°. The digest was freeze-dried and subjected to pepsin digestion (enzyme:substrate, 1:50, w/w) in 5% formic acid at a concentration of 20 mg/ml for 15 hr at 37°.

Characterization and Purification of Peptides. The digested material was subjected to electrophoresis on Whatman No. 3MM paper at pH 3.5. Radioactive peptides were identified by radioautography and were further purified at pH 6.5 and 2.1. The following buffers were used: pH 6.5 (pyridine–acetic acid–

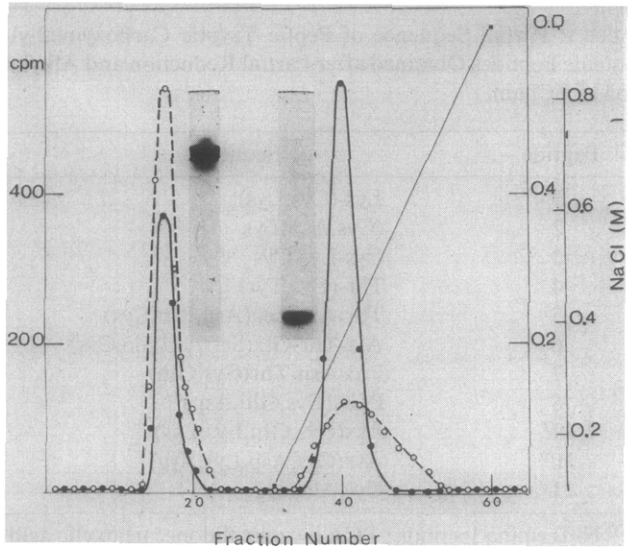

FIGURE 3: Purification of J chain. J chain contaminated with light chains (3.5 mg/ml) was passed through a column (1 × 23 cm) of diethylaminoethylcellulose equilibrated with Tris–glycine (pH 8.9) buffer containing 0.1 M NaCl and 8 M urea. Aliquots of 1 ml were collected. Fractions 1–30 were eluted with the starting buffer and fractions 15–19 contained the L chain. A linear gradient from 0.1 to 0.8 M NaCl was started at fraction 30. Fractions 37–45 contained the J chain. Gel electrophoresis was used to characterized the fractions: (⊖–⊖) absorbance 280 nm; (——) radioactivity.

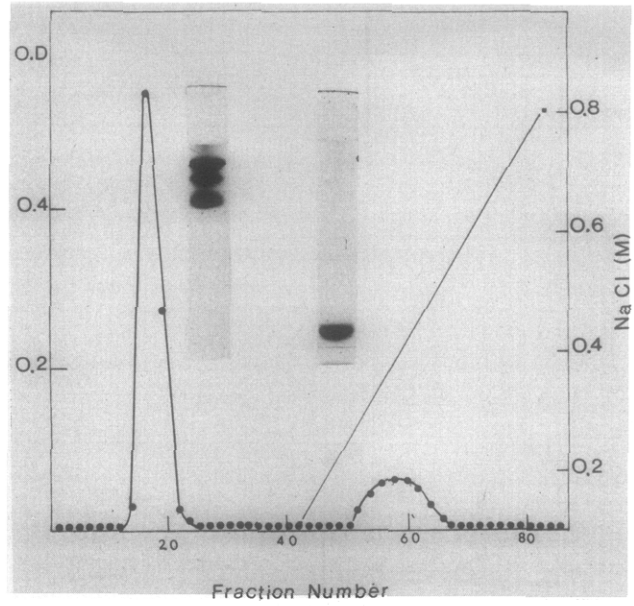

FIGURE 4: Chromatography of the (Oso) IgA_1 myeloma protein obtained by partial reduction and alkylation with cold iodoacetic acid on DEAE-cellulose. The protein, after dialysis against 0.1 M NaCl–Tris–glycine (pH 8.91), was chromatographed on a column (1 × 23 cm) of diethylaminoethylcellulose equilibrated with the same buffer. Fractions of 1 ml were collected with the same buffer till fraction 40. In fractions 14–23 heavy and light chains are present. A linear gradient was started in fraction 41–82. Fractions 52–62 contain J chain. The chains were detected by polyacrylamide gel electrophoresis in 8 M urea in the same conditions as Figure 1: (⊖–⊖) absorbance 280 nm; (——) radioactivity.

water, 33:1:300, v/v), pH 3.5 (pyridine–acetic acid–water, 1:10:190), and pH 2.1 (formic acid–acetic acid–water, 1:4:45, v/v). All mobilities are given relative to the distance between ε-DNP-lysine and aspartic acid at pH 6.5 (Offord, 1966).

Amino Acid Analysis. The peptides were hydrolyzed in 6 M HCl containing 0.1% phenol under vacuum in sealed ampoules at 110° for 20 hr. Amino acid analyses were carried out on a Beckman Model 121 automatic amino acid analyzer equipped with high-sensitivity cuvets and recorder.

Amino Acid Sequences. Edman degradation was done manually using dansylation to determine the new N-terminal residues (Frangione and Milstein, 1968). The N-terminal derivatives were identified by chromatography as described by Woods and Wang (1967).

Results

Dissociation of the J Chain. In order to establish the lowest concentration of dithiothreitol necessary to dissociate the J chain, the protein was partially reduced with increasing concentrations of dithiothreitol ranging from 0.1 to 10 mM followed by alkylation with [^{14}C]iodoacetic acid. The release of J chain was followed by polyacrylamide gel electrophoresis in the presence of urea. Since the J chain started to appear at concentrations greater than 1 mM dithiothreitol (Mendez *et al.*, 1972), we chose 5 mM dithiothreitol for the preparation of the J chain in all subsequent studies.

Preparation of the J Chain. After partial reduction and alkylation with [^{14}C]iodoacetic acid the chains were partially separated by chromatography on a column of Sephadex G-100 in 1 M acetic acid (Figure 1). Studies in polyacrylamide gel electrophoresis in urea of the different fractions obtained from each peak showed that the first peak (a) contained polymers of heavy and J chain, the second peak (b) consisted of heavy chain and the third peak (d) contained the L chain. The region corresponding to the J chain (c) immediately preceded the L chain and is characterized by a higher ratio between radioactivity and optical density as was shown recently in a canine polymeric IgA (Kehoe *et al.*, 1972). The estimated molecular weight by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate indicated a molecular weight of 61,000 for heavy, 22,000 for the light, and 24,500 for the J chain. The α and J chains had unreactive NH_2 terminals and the κ chain had aspartic acid. Figure 2 shows that the J-chain peak (fractions 100–110: 7.5 mg out of 250 mg of protein) from Figure 1 was relatively pure since at a concentration of 10 mg/ml it reacted with an antiserum to J chain, kindly provided by Dr. J. Mestecky, and failed to react with antisera to α and κ chains. Since these antisera detected α and κ chains at concentrations of 0.15 mg/ml, the J-chain preparation was at least 95% pure.

In order to purify the J chain further the peak containing a mixture of L and J chains obtained from the Sephadex column was chromatographed on DEAE-cellulose in 7 M urea (pH 8.91). Two peaks were obtained (Figure 3). The first, which eluted before the gradient started, contained L chains while the second peak eluted by the gradient contained the J chain. The ratio of counts per minute to optical density is high for the J chain due to its high content of cysteine. Purification of the J chain was subsequently simplified when it was noted that initial separation of L and J chain from the heavy chain was unnecessary. Figure 4 demonstrates that when partially reduced and alkylated protein (Oso) was chromatographed on DEAE-cellulose under the same conditions, two

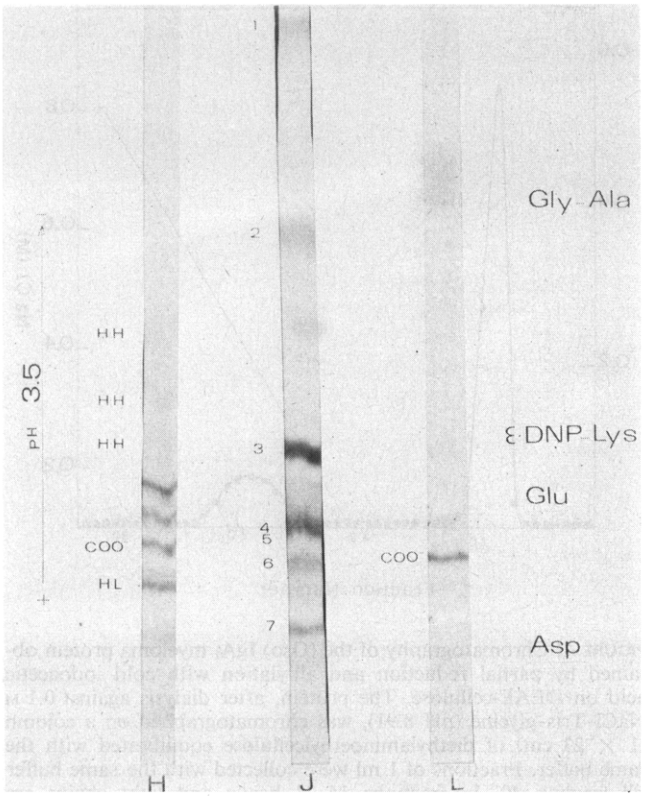

FIGURE 5: Diagram showing the radioactive bands obtained after electrophoresis at pH 3.5 of peptic–tryptic digests of partially reduced and radioactively alkylated heavy, light, and J chains. Abbreviations: H-H: inter-heavy; COO^-: carboxyl end: H-L: inter-heavy-light. Markers: aspartic acid, glutamic acid, ε-DNP-lysine and glycylalanine.

peaks were obtained (Figure 4). The first peak contained heavy and L chains while the second peak consisted of J chain. Thus J chain could be isolated from partially reduced IgA myeloma by one-step fractionation on DEAE-cellulose.

Characterization and Isolation of Peptic–Tryptic Peptides by High-Voltage Electrophoresis (Chemical Typing). Isolated partially reduced and radioactively labeled heavy, J, and light chains from protein Oso, were digested with pepsin and trypsin and then subjected to high-voltage paper electrophoresis at pH 3.5 (Frangione *et al.*, 1969). Figure 5 shows the main radioactive bands containing carboxymethylcysteine peptides obtained from partially reduced molecules. Among the peptides of the heavy chain there is the band containing the heavy–heavy disulfide bridges, the C-terminal peptide, and the peptide responsible for binding the H chain to the carboxyl end of the L chain as was shown for another IgA myeloma protein. The partial sequence of these peptides was previously reported (Wolfenstein *et al.*, 1971). In the light chain there is only one band which corresponds to the C-terminal peptide. The absence of any of these H- and L-chain peptides on the autoradiograph prepared from the J chain and failure to isolate them from the tryptic–peptic digest of the J chain provide additional evidence for the purity of the J chain used in these studies. Eight main radioactive bands were isolated from J chain and the partial sequence and mobilities of these carboxymethylcysteine peptides are shown in Table I and II. Peptide 8 (not shown in the figure) has a negative N terminal and a very rapid mobility of 1.25 at pH 6.5 (Table II). Since the NH_2 terminal of the J chain was unreactive there was a possibility that peptide 8 was coming from the amino terminal of the chain. To establish that this was indeed the case, the J chain was subjected to Pronase digestion followed by chromatography on Dowex 50. A similar peptide was isolated after purification on paper electrophoresis and its composition was identical with peptide 8 (Table I).

TABLE I: Partial Sequence of Peptic–Tryptic Carboxymethylcysteine Peptides Obtained after Partial Reduction and Alkylation of J Chain.

Peptide	Sequence
1	Lys-(Cys,Lys)
2	(Cys,Asp)Lys
3	Cys-Lys-Cys
4	Thr-(Cys,Tyr)
5	Thr-Ala-Leu(Asp,Pro,Cys)
6	Ala-Thr-Glx(Ser,Asp_2,Ile,Cys)
7	Pro-Asp-Thr(Cys,Glu)
8	PCA(Cys,Glu,Asp)[a]
9[b]	Asx(Cys,Glu,Lys,Lys)
10[b]	Asx(Cys,Asp,Lys,Arg)
11[b]	Cys,Ala,Arg

[a] N-Termina lpeptide; PCA = pyrrolidonecarboxylic acid. [b] Additional carboxymethylcysteine peptide isolated in low yield.

In another preparation of J chain three additional carboxymethylcysteine peptides were found although in very low yield. The amino acid compositions of these peptides are also listed in Table II.

Complete reduction of IgA Oso and alkylation with [^{14}C]-iodoacetic acid resulted in the labeling of four additional cysteine-containing peptides in the light chain and ten in the heavy chain (unpublished data). These probably represent the intrachain disulfide bridges. In contrast no additional peptides were radioactively labeled in the J chain.

Discussion

After partial reduction and alkylation of an IgA_1 myeloma protein, separation of the 3 constituent polypeptide chains and digestion of the chains with pepsin and trypsin, we isolated 18 cysteine-containing peptides. At least six were from the heavy chain, and probably are involved in interchain bonds. Their partial sequence and function were previously reported (Wolfenstein *et al.*, 1971). One peptide came from the C-terminal end of the L chain. At least eight CMCys peptides were isolated from the J chain (Table I). One, the very acidic peptide PCA(Cys,Glu,Asp), was shown to be the NH_2 terminal of the J chain, in support of the previous report of Meinke and Spiegelberg (1972). The function and location of the others remain uncertain. While the amino acid composition and partial sequence suggest that all of these peptides are different, the possibility that peptides 1–3 are overlapping peptides derived from a cysteine rich region of the molecule cannot be excluded. Among the three low yield peptides, two, 9 and 10 (Table I), show striking homologies in their amino acid composition, but so far insufficient

TABLE II: Amino Acid Composition of Peptic–Tryptic Carboxymethylcysteine Peptides Isolated after Partial or Complete Reduction and Alkylation of J Chain.[a]

Peptides[b]	PT-1	PT-2	PT-3	PT-4	PT-5	PT-6	PT-7	PT-8	PT-9	PT-10	PT-11
Lys	2.0	1.0	1.06						2.3	1.2	
Arg										1.0	0.9
$CMCysSO_2$[c]	0.6	0.5	1.7	0.6	1.0	0.3	0.3	1.0	0.8	0.8	0.4
Asp		1.0			1.1	1.8	0.9	1.0	0.5	2.0	
Thr				1.0	1.0	0.8	0.9				
Ser						1.1		1.0			
Glu							1.0	2.0	0.5		
Pro					1.0		1.1				
Gly											
Ala					1.0	1.3					1.1
Leu					0.57						
Tyr				1.06							
Ile						0.5					
N-Terminal	Lys	ND[e]	CMCys	Thr	Thr	Ala	Pro	PCA[f]	Asp	Asp	ND
Mobility, pH 6.5[d]	−0.60	N	0.55	0.57	0.67	0.8	1.2	1.25	N	N	N
Yield (μM)	0.01	0.01	0.01	0.01	0.02	0.03	0.02	0.01			

[a] Compositions are moles of amino acid per mole of peptide. [b] Hydrolysis for 20 hr; PT, pepsin–trypsin; $CMCysSO_2$, carboxymethylcysteine sulfone. [c] The low yield of $CMCysSO_2$ was due to destruction during hydrolysis (Frangione and Milstein, 1968). [d] Mobilities are given relative to the distance between ϵ-DNP-lysine and aspartic acid. [e] ND = not done. [f] PCA = pyrrolidonecarboxylic acid.

material is available for sequencing. Their origin and significance are still unclear.

Following complete reduction and alkylation of the molecule the expected number of peptides involved in intrachain disulfide bridges was isolated from the α and κ chains, but no additional carboxymethylcysteine-containing peptides were found in the J chain.

The partial amino acid sequences of the cysteine-containing peptides clearly indicate that the J chain differs from the known heavy and light chains of immunoglobulins and suggest a separate evolutionary origin for the J chain. The possibility that the J chain is related to an immunoglobulin polypeptide chain that has not yet been characterized in detail or possibly an unusual region of the molecule such as the hinge region deserves further study.

At the present time it is not possible on the basis of the available data to ascribe a definite function to the J chain or any of the cysteine-containing peptides. Their lability to reductive cleavage resembles that of the interchain disulfide bridges of the immunoglobulins rather than that of the intrachain bridges. However, it has been suggested that the J chain plays a role in joining the subunits of IgA and IgM polymers. If this were the case and if there is only a single J chain per polymer, one must postulate the existence of a series of asymmetric disulfide bridges between the J chain and individual subunits and the possibility of forming additional asymmetric intrachain bridges depending on the number of polymers to be joined. Thus disulfide interchange may play an important role in its function. Studies by others (Morrison and Koshland, 1972) have suggested that the J chain is linked to the α chain. While the position of the appropriate cysteine on the α chain has not been located, it has been possible to exclude a section consisting of 30 residues in the hinge region of the α chain as a site of linkage (unpublished observation). Additional studies of the structure of the J chain may provide clearer insights into its biological function and evolutionary origin.

Acknowledgment

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Antigenic Determinants in Lysine-Rich Histones†

Michael Bustin* and B. David Stollar‡

ABSTRACT: Antibodies to purified, unfractionated, calf thymus F1 histone were induced by immunizing rabbits with histone–RNA complexes. The reactions of these antibodies with chemically modified calf thymus F1 histone and with fragments of the F1 histone have been tested in order to approach the definition of serologically active regions of the molecule. Two fragments were obtained by treating the F1 histone with *N*-bromosuccinimide. The larger carboxyl-terminal portion (N1) reacted to give 40% complement fixation with a serum dilution (1:500) that gave over 85% complement fixation with intact F1 histone. The smaller amino-terminal portion (N2) failed to form a complement-binding complex with even higher serum concentrations (1:100). However, a 5-fold molar excess of the N2 fragment over intact F1 did completely inhibit the complement fixation of the F1 histone with a 1:1000 dilution of anti-F1 serum. The cleaved fragments have different antigenic determinants for the anti-F1 serum and do not recombine in dilute solution to re-form the native protein structure, since the complement fixation reaction of a mixture of peptides N1 and N2 was equal to that of N1 alone. Dinitrophenylated derivatives of N1 and F1 failed to fix complement upon reaction with anti-F1. The inhibitory capacity of N2, however, was not altered by dinitrophenylation. The results suggest that lysine residues in N1 but not in N2 are involved in immunogenic determinants. Compared to native F1 histone, F1 which was nitrated at the single tyrosine residue gave a weaker complement fixation reaction thereby pinpointing the tyrosine residue or its environment as an antigenic determinant. The tyrosine-containing peptide failed to inhibit the complement fixation activity of the F1–anti-f1 mixture at peptide concentrations which do not interfere with the complement system. However, an unfractionated tryptic digest of F1 did completely and specifically inhibit the system. When animals were immunized with RNA complexed with either N1 or N2, the animals immunized with N1 did not produce detectable antibodies, while those immunized with N2 did produce antibodies which reacted strongly with intact F1 and weakly with isolated N1 or N2 fragments. Both fragments N2 and N1 as well as poly(L-lysine) inhibited the reaction between anti-N2 and intact F1 suggesting that antibodies against lysine residues in N2 react with lysine containing determinants in N1 and F1. Indeed, when the reaction of this serum with dinitrophenylated derivatives of F1 and N1 was tested, it was found that the complement fixation reactivity of these derivatives was markedly reduced. On the other hand the N_2ph–N2 derivative fixed complement to somewhat a higher degree than N2. The latter results support the findings that N2 contained determinants other than lysine residues and suggest that lysine residues in fact inhibited effective complement-fixing aggregation of this fragment with antibody.

Complexes of histone and RNA, when injected into rabbits, elicit specific anti-histone antibodies (Stollar and Ward, 1970). Anti-F1 histone antibodies obtained in this way are not only specific for the F1 histone class in general, but in addition they can distinguish between subfractions of this class and between F1 molecules derived from various sources (Bustin and Stollar, 1972). Thus, the species and organ specificity of the F1 histone class, which has been detected by chromatographic (Bustin and Cole, 1968; Kinkade, 1969) and electrophoretic (Paynim *et al.*, 1971) techniques, is also immunologically demonstrable.

Structural studies on the F1 histone revealed that the molecule could be visualized as being composed of three regions, each of which displays characteristic amino acid composition and overall cationic charge (Bustin and Cole, 1970). Kinkade and Cole (1966b) mentioned the possibility that the structural differences between the various lysine-rich histones could be restricted to a particular portion of the primary structure of the molecule. Tryptic fingerprints of the two peptides isolated after *N*-bromosuccinimide cleavage of the molecule suggested that most of the differences between the various F1 molecules lie in the amino-terminal portion of the molecule (Bustin and Cole, 1969). Indeed, detailed sequence analysis (Rall and Cole, 1971) detected a variable region within the first 40 residues of the F1 molecule.

The presence of regions with distinct amino acid composition and overall cationic charge raises the possibility that these differences could be expressed conformationally and that the various parts of the histone molecule differ in their ability to interact with DNA (Boublick *et al.*, 1970; Bustin and Cole, 1970; Fasman *et al.*, 1971). Immunochemical methods may be useful in studying these conformational changes and interactions with DNA, especially if we can identify the immunologically reactive regions of the histone molecule. This article reports studies designed to gain further insight into the antigenic properties of the F1 histones.

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