Immunology, 1968, **15**, 93.

Chemical Differences between Individual Human Cold Agglutinins

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(*Received* 30*th December* 1967)

Summary. The μ- and κ-chains from four human cold agglutinins having anti-I specificity have been analysed:

The κ-chains have either Asp or Glu, but not both, at the N-terminus. This provides additional support for the view that cold agglutinins are of monoclonal origin.

Both heavy and light chains from cold agglutinins vary as much in amino acid composition as do monoclonal chains of a given type chosen at random. The chemical individuality of cold agglutinin μ-chains is shown also by differences in hexose, fucose and glucosamine content.

The apparent lack of correlation between overall antibody activity and chemical composition of constituent peptide chains indicates the need for close definition of combining specificity in structural studies of cold agglutinins.

INTRODUCTION

Studies on the chemical basis of antibody combining specificity would be facilitated if monoclonal immunoglobulins with defined antibody activities were available for chemical analysis. Several apparently homogeneous IgG and IgM proteins with some form of combining affinity have been described (reviewed by Cohen and Milstein, 1967). Among these are the antibodies associated with chronic cold agglutinin disease. These have affinity for the I antigen of the erythrocyte, have restricted electrophoretic mobilities and are usually IgM molecules with Type κ light chains (Harboe, van Furth, Schubothe, Lind and Evans, 1965); in addition, their isolated light chains are more homogeneous than those of normal or antibody Ig on electrophoresis (Cooper, 1968).

The present report provides further evidence for the monoclonal nature of cold agglutinins and demonstrates marked individual differences in the chemical composition of both μ- and κ-chains isolated from four such antibodies. The implications of these findings in regard to the chemical basis of antibody specificity are briefly discussed; part of this work has been the subject of a preliminary report (Cohen, 1967).

MATERIALS AND METHODS

Isolation of cold agglutinins

Sera were obtained from patients with chronic cold agglutinin disease. The cold

agglutinins were isolated by absorption and elution from red cell stroma and purified by gel filtration on columns of Sephadex G-200 as previously described (Cooper, 1968).

Isolation of peptide chains

Peptide chains of reduced and alkylated cold agglutinins were prepared using methods previously described for normal and pathological IgM (Chaplin, Cohen and Press, 1965).

Starch-gel electrophoresis

Starch gel electrophoresis of isolated peptide chains was carried out in vertical trays using 8 M urea–0·035 M glycine buffer, pH 8·8 (Cohen and Porter, 1964). Fractions were quantified by radioactive assay of 2-mm segments of starch gels after electrophoresis of ^{125}I-labelled light chains in urea–glycine buffer.

Amino acid analysis

This was carried out in a Spinco amino acid analyser on single samples hydrolysed for 24 hours under the conditions described by Crumpton and Wilkinson (1963).

N-terminal analysis

N-terminal amino acids were determined by the fluorodinitrobenzene method of Sanger (Porter, 1957).

Carbohydrate analyses

Hexose was determined quantitatively by the orcinol technique (Winzler, 1955) with mannose as standard. Hexosamine was determined by Cessi's modification of the Elson–Morgan method, as described by Johansen, Marshall and Neuberger (1960) with glucosamine as standard. Sialic acid was estimated by the thiobarbituric acid method described by Warren (1959) with *N*-acetylneuraminic acid as standard. Fucose was estimated by the method of Dische and Shettles (1948).

Gel diffusion

Double diffusion was carried out in agar and proteins were classified by reaction with appropriate specific antisera.

Iodination

Iodination of IgM—cold agglutinins with ^{125}I was performed according to the method of McFarlane (1958).

RESULTS

The cold agglutinins of four patients selected from the series studied by Cooper (1968), were investigated. Some general details concerning these patients and their cold agglutinins are shown in Table 1. All the antibodies studied were IgM molecules having light chains of Type κ and anti-I combining specificity. Heavy and light chains were separated from reduced, alkylated cold agglutinins by gel filtration on Sephadex G-100 in 0·1 M formic acid; the yield of light chain was 20–23 per cent of the total protein (Table 2).

TABLE 1

PROPERTIES OF COLD AGGLUTININS

	Sex	Age	Age at onset	Specificity	Heavy chain	Light chain	Titre		Clinical state*
							Agglutination†	Lysis‡	
Hyl	M	65	59	I	μ	κ	8,000	80	R
But	M	62	55	I	μ	κ	16,000	1,280	R, H, A
Cut	M	65	55	I	μ	κ	4,000	640	R, H, A
Rei	F	73	69	I	μ	κ	16,000	2,560	R, H, A

* R = Raynaud's phenomenon; H = haemoglobinuria; A = anaemia.
† Using normal adult erythrocytes in saline.
‡ Using papain-treated normal adult erythrocytes in normal fresh serum.

TABLE 2

PROPERTIES OF COLD AGGLUTININ κ-CHAINS COMPARED TO NORMAL κ-CHAIN

	Total (per cent) IgM	Electrophoretic fractions (per cent total)†										N-terminus (moles/mole)	
		1	2	3	4	5	6	7	8	9	10	Asp	Glu
Norm κ*		1·0	1·9	9·7	12·4	17·7	18·5	12·8	8·8	5·7	2·6	0·4‡	0·3‡
Hyl	20	0	2·0	80	11	3·4	2·6	0	0	0	0	0	0·6
But	23	0	0	2·7	20	38	17	9·2	0	0	0	<0·05	0·4
Cut	20	0	0	0	2·0	19	43	24	10	1·3	0	0	1·0
Rei	21	0	0	0	0	7·4	14	24	30	12	7	0·8	<0·05

* Cohen and Gordon (1965).
† Electrophoretic fractions were quantified by radioactive assay of 2-mm segments of starch gels after electrophoresis of ^{125}I-labelled samples in urea–glycine buffer, pH 7–8.
‡ Analysis of normal light chain assuming 70 per cent κ-chain.

ANALYSES OF LIGHT CHAINS

Isolated light chains were analysed by electrophoresis on urea–glycine starch gels. Normal light chain is resolved into about ten components under these conditions (Cohen and Porter, 1964). The cold agglutinin light chains as shown by Cooper (1968b) were relatively homogeneous and contained four to six bands of which one or two were predominant. The four chains showed considerable individual differences in electrophoretic patterns as shown by the quantitative distribution of ^{125}I in trace-labelled samples (Table 2). The restricted heterogeneity of these light chains is also indicated by the results of N-terminal analysis using the fluorodinitrobenzene method. Whereas both Asp and Glu are found on normal κ-chains, the N-terminus of three cold agglutinin κ-chains was Glu only and in the remaining case was Asp only (Table 2). These differences in electrophoretic mobility and N-terminal residue indicate that cold agglutinin κ-chains are chemically different from one another and this is borne out by amino acid analyses (Table 3). Of the fifteen amino acids for which reliable values are available, twelve differed by more than 15 per cent among the four κ-chains.

TABLE 3

AMINO ACID ANALYSES OF COLD AGGLUTININ κ-CHAINS

Residue	Residues per 205*				Difference†	No. in variable‡ $V\kappa$
	Hyl	But	Cut	Rei		
Lys	11·7	10·4	11·2	12·3	1·9	6
His	3·8	2·8	3·3	3·4	1·0	0
Arg	8·2	8·1	8·6	5·0	3·6	5
Asp	15·4	14·6	15·7	19·2	4·6	10
Thr	15·9	13·6	16·5	16·4	2·9	7
Ser	24·4	26·3	24·5	30·4	6·0	10
Glu	24·4	25·7	27·6	25·6	—	12
Pro	13·2	15·6	15·6	10·5	5·1	3
Gly	14·6	19·2	16·3	15·7	4·6	4
Ala	16·1	15·5	14·6	13·9	—	5
Val	13·5	13·7	12·8	15·3	2·5	9
Met	1·7	0·7	0·4	1·2	1·3	2
Iso-Leu	6·5	6·1	5·8	5·6	—	9
Leu	15·5	17·0	17·9	14·4	3·5	11
Tyr§	9·3	9·4	7·1	7·4		4
Phe	10·3	7·0	8·1	8·8	3·3	6

* Excluding 1/2 Cys and Tyr.

† Maximum–minimum where difference >15 per cent.

‡ Indicates the number of sites in the variable portion of $V\kappa$ at which given residues have been observed in human κ-chains (see text).

§ All values are for 24-hour hydrolysis and estimates of Tyr are unreliable.

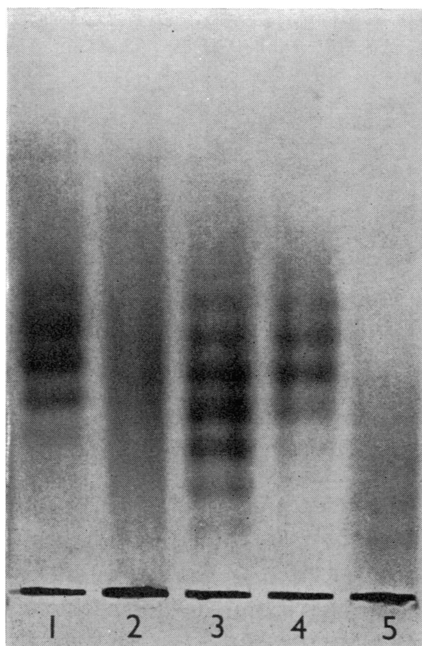

FIG. 1. Electrophoresis in urea–glycine starch gel, pH 8, of μ-chains from cold agglutinins: (1) Hyl, (2) Cut, (3) But, (4) Rei, and (5) μ-chain from monoclonal IgM.

ANALYSES OF HEAVY CHAINS

Electrophoretic patterns of isolated μ-chains from the four cold agglutinins and from one monoclonal Waldenström macroglobulin appear in Fig. 1. All showed considerable electrophoretic heterogeneity on urea–glycine starch gels. Three of the cold agglutinin μ-chains showed six to eight distinct and regularly spaced components; a similar pattern of banding occurred with the μ-chain of the monoclonal macroglobulin. As in the case of the light chain, amino acid analyses show considerable difference among the four cold agglutinin μ-chains. Eight out of fifteen of the amino acids for which reliable data are available differed by more than 15 per cent (Table 4). The chemical individuality of cold agglutinin μ-chains is further demonstrated by carbohydrate analyses which show differences in hexose, fucose and glucosamine content (Table 5).

TABLE 4

AMINO ACID ANALYSES OF COLD AGGLUTININ μ-CHAINS

Residue	Residues per 470 residues				
	Nor*	Cut	Hyl	But	Rei
Lys	23	24	25	25	25
His	8·7	8·7	8·6	11	12
Arg	20	21	18	23	19
Asp	38	37	43	41	40
Thr	44	48	48	47	45
Ser	48	47	50	46	53
Glu	46	45	49	48	45
Pro	33	28	34	31	36
Gly	32	33	34	35	31
Ala	30	28	33	31	27
Val	41	37	40	40	37
Met	6·1	4·2	6·4	4·6	2·7
Ile	15	14	15	16	16
Leu	34	34	37	34	40
Tyr	14	14	17	17	15
Phe	17	16	18	18	17
Cys (half)	8·5	—	—	—	—
Cys $CH_2.CO_2H$	3·7	3·5	3·9	2·5	5·2
Trp	8·7	—	—	—	—

* Normal heavy chain from IgM (U.K. blood donor)—Chaplin, Cohen and Press (1965).

TABLE 5

CARBOHYDRATE ANALYSIS OF COLD AGGLUTININ μ-CHAINS

	Carbohydrate content (g/100 g protein)				
	Nor*	Cut	Hyl	But	Rei
Hexose	7·0	4·0	5·7	9·8	6·6
Sialic acid	1·0	1·1	1·0	1·2	0·9
Fucose	Present	1·1	1·4	1·2	Trace
Glucosamine	4·4	3·1	3·6	4·7	4·2
Total (g/100 g)	12·4	9·3	11·7	16·9	11·7

* Normal heavy chain from IgM (U.K. blood donor)—Chaplin *et al.* (1965).

DISCUSSION

Several lines of evidence indicate that individual immunoglobulin producing cells synthesize single chemical variants of the heavy and light chain (reviewed by Cohen and Milstein, 1967). Most antigens must, therefore, activate many different cell clones since specific antibody preparations commonly contain multiple variants of both peptide chains. Other antigens appear regularly to induce a more restricted cellular response, since antibody preparations in any individual are relatively homogeneous. Among such antibodies are the cold agglutinins of anti-I specificity. These appear to have a monoclonal origin since they are almost invariably IgM molecules of restricted electrophoretic mobility and have light chains of Type κ only (Harboe and Lind, 1966) which are relatively homogeneous when analysed by urea–glycine starch gel electrophoresis (Cooper, 1968). In addition, as shown above, cold agglutinin light chains contain either Asp or Glu, but not both, at the N-terminus and in this respect resemble Bence Jones proteins and light chains separated from monoclonal immunoglobulins. The μ-chains of cold agglutinins show multiple components on urea–glycine starch gel electrophoresis, but similar heterogeneity was found with a μ-chain isolated from a monoclonal Waldenström macroglobulin (Fig. 1); such heterogeneity does not necessarily indicate differences in amino acid structure but may result from differences in carbohydrate content as demonstrated in a human myeloma IgA (Clamp, Dawson and Hough, 1966).

The individual differences in amino acid composition found for both κ- and μ-chains of cold agglutinins (Tables 3 and 4), are similar in extent to those reported for Type κ IgM proteins from six individuals with Waldenström's macroglobulinaemia (Putnam, Kozuru and Easley, 1967). Human κ-chains are now known to consist of a constant C-terminal half (Cκ) and a variable N-terminal half (Vκ) of approximately equal length. The differences in amino acid composition shown in Table 3 must, therefore, be confined to Vκ sections of chains and the extent of these differences is of course considerably enhanced if the residues of Cκ are substracted from the total values for each chain. In Table 3 the maximum difference between residues of cold agglutinin light chains is compared with the number of sites in human Vκ at which each may occur as an alternative residue (see Cohen and Milstein, 1967). This comparison shows that for some residues the variability of the four cold agglutinin κ-chains appears to exceed the known variability of human Bence Jones κ-chains chosen at random. For example, Pro is known to occur as an alternative amino acid at three sites in Vκ and yet cold agglutinin κ-chains differ by up to five Pro residues; similarly Gly is known to be an alternate residue at four sites in Vκ and the cold agglutinin κ-chains differ by up to four or five Gly. In the case of μ-chains from cold agglutinins, chemical individuality is shown also by the differences in hexose, fucose and glucosamine content (Table 5).

It seems reasonable to assume that the remarkable degree of structural variation observed in immunoglobulin chains of a single type is related to combining specificity. This is so because variation occurs in sections of chains known to carry antibody activity and the successful refolding of antibody molecules in the absence of antigen has provided strong evidence that such specificity is dependent upon covalent structure. The present study has shown, however, that in monoclonal antibodies there is a lack of correlation between combining specificity and the chemical structure of constituent μ- and κ-chains. This apparent discrepancy can be attributed to many factors: (i) The erythrocyte I antigen may be complex and the antibody in different subjects may have distinct com-

bining specificities. Such complexity is indicated by the finding that anti-I sera vary in the extent to which they are inhibited by sheep liver hydatid cyst fluid (Tippett, Noades, Sanger, Race, Sausais, Holman and Buttimer, 1960; Marsh, 1961) and the extent to which they interact with erythrocytes after treatment with β-galactosidase and β-glucosaminidase (Marcus, Kabat and Rosenfield, 1963). (ii) Antibodies directed against the same determinant may have variable affinities reflecting structural diversity of combining sites. (iii) V-sections of chains must include variable sequences determining the specific association between homologous pairs of heavy and light chains (Grey and Mannik, 1965; Gordon and Cohen, 1966). (iv) V-sections of chains must include variable sequences responsible for the remarkable antigenic individuality which has been demonstrated on antibody molecules (including cold agglutinins) and myeloma proteins (see Gell and Kelus, 1967). Such idiotypic specificity appears to be associated in some way with combining specificity, but does not seem to depend directly upon the configuration of the combining site. (v) Different sequences of variable regions of heavy and light chains may generate identical combining sites by complementary interaction of different pairs of residues.

These observations indicate the considerable difficulties that are likely to arise when attempts are made to relate the variable primary structure of monoclonal heavy and light chains to the combining specificities of the parent molecules. The possibility of finding a straightforward correlation between primary structure of chains and overall antibody specificity seems remote. If cold agglutinins are to be used in such work, it is evident that a detailed analysis of the I antigen is required in order that sequence studies can be carried out on heavy and light chains from antibody molecules of closely defined specificity and affinity.

ACKNOWLEDGMENTS

We wish to thank Dr C. Milstein, who performed some amino acid analyses of κ-chains, Dr M. Wilkinson for advice on analyses of μ-chains and Miss Marjolijn Krauss and Miss Rebecca Schein for valuable technical assistance. This work was made possible by grants from the Medical Research Council, The Nuffield Foundation and The Wellcome Trust.

One of us (A.C.) was supported in part by a United States Public Health Service Fellowship No. 1/F2/AI/8963/01 from the National Institute of Allergy and Infectious Diseases.

REFERENCES

Chaplin, H., Cohen, S. and Press, E. M. (1965). 'Preparation and properties of the peptide chains of normal 19S γ-globulin (IgM).' *Biochem. J.*, **95**, 256.

Clamp, J. R., Dawson, G. and Hough, L. (1966). 'Heterogeneity of glycopeptides from a homogeneous immunoglobulin.' *Biochem. J.*, **100**, 35C.

Cohen, S. (1967). 'Heterogeneity of immunoglobulin light chains.' *Third Nobel Symposium*, p. 21. Almqvst & Wiskell, Stockholm.

Cohen, S. and Gordon, S. (1965). 'Dissociation of κ- and λ-chains from reduced human immunoglobulins.' *Biochem. J.*, **97**, 460.

Cohen, S. and Porter, R. R. (1964). 'Heterogeneity of the peptide chains of γ-globulin.' *Biochem. J.*, **90**, 278.

Cohen, S. and Milstein, C. (1967). 'Structure and biological properties of immunoglobulins.' *Advanc. Immunol.*, **7**, 1.

Cooper, A. G. (1968). 'Purification of cold agglutinins from patients with chronic cold haemagglutinin disease. Evidence of their homogeneity from starch gel electrophoresis of isolated light chains.' *Clin. exp. Immunol.*, **3**, 691

Crumpton, M. J. and Wilkinson, J. M. (1963). 'Amino acid compositions of human and rabbit γ-globulins and of the fragments produced by reduction.' *Biochem. J.*, **88**, 228.

Dische, Z. and Shettles, L. B. (1948). 'A specific color reaction of methylpentoses and a spectrophotometric micro method for their determination.' *J. biol. Chem.*, **175**, 595.

Gell, P. G. H. and Kelus, A. (1967). 'Anti-antibodies.' *Advanc. Immunol.*, **6**, 461.

Gordon, S. and Cohen, S. (1966). 'Recombination of heavy and light chains from human immunoglobulins.' *Immunology*, **10**, 459.

Grey, H. M. and Mannik, M. (1965). 'Specificity of recombination of H and L chains from human γG-myeloma proteins.' *J. exp. Med.*, **122**, 619.

Harboe, M. and Lind, K. (1966). 'Light chain types of transiently occurring cold haemagglutinins.' *Scand. J. Haemat.*, **3**, 269.

Harboe, M., van Furth, R., Schubothe, H., Lind, K. and Evans, R. S. (1965). 'Exclusive occurrence of κ-chains in isolated cold haemagglutinins.' *Scand. J. Haemat.*, **2**, 259.

Johansen, P. G., Marshall, R. D. and Neuberger, A. (1960). 'II. The hexose, hexosamine, acetyl and amide-nitrogen content of hen's-egg albumin.' *Biochem. J.*, **77**, 239.

Marcus, D. M., Kabat, E. A. and Rosenfield, R. E. (1963). 'The action of enzymes from *Clostridium tertium* on the I antigenic determinant of human erythrocytes.' *J. exp. Med.*, **118**, 175.

Marsh, W. L. (1961). 'Anti-i: a cold antibody defining the Ii relationship in human red cells.' *Brit. J. Haemat.*, **7**, 200.

McFarlane, A. S. (1958). 'Efficient trace-labelling of proteins with iodine.' *Nature* (*Lond.*), **182**, 53.

Porter, R. R. (1957). 'Determination of amino acid sequence in proteins by the fluorodinitrobenzene method.' *Meth. Enzymol.*, **4**, 221.

Putnam, F. W., Kozuri, M. and Easley, C. W. (1967). 'Structural studies of the immunoglobulins. IV. Heavy and light chains of the γM pathological macroglobulins.' *J. biol. Chem.*, **242**, 2435.

Tippett, P., Noades, J., Sanger, R., Race, R. R., Sausais, L., Holman, C. A. and Buttimer, R. J. (1960). 'Further studies of the I antigen and antibody.' *Vox Sang.* (*Basle*), **5**, 107.

Warren, L. (1959). 'The thiobarbituric acid assay of sialic acids.' *J. biol. Chem.* **234**, 1971.

Winzler, R. J. (1955). 'Determination of serum glycoproteins.' *Meth. biochem. Anal.*, **2**, 279.