Clq metabolism in ulcerative colitis and Crohn's disease

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SUMMARY The metabolism of pure radioiodine labelled Clq has been observed in five patients with ulcerative colitis, five patients with Crohn's disease, and in five control subjects. Both the fractional catabolic rate and the synthesis rate of Clq were increased in the five patients with Crohn's disease and in four of the five patients with ulcerative colitis. The fifth patient was in remission and had a normal synthesis rate. These results support the hypothesis that complement activation plays a role in the pathogenesis of these disease states and that the increased complement activation is primarily *via* the classical pathway.

Circulating immune complexes have been found in patients with active inflammatory bowel disease (Jewell and MacLennan, 1973; Doe *et al.*, 1973; Mowbray *et al.*, 1973; Ezer and Hayward, 1974). They are frequently present in high titre in patients with extraintestinal manifestations (Hodgson *et al.*, 1977a) and may be involved in their pathogenesis. Although the role of immune complexes in the pathogenesis of mucosal lesions is not clearly defined, an experimental immune complex mediated colitis can be produced in rabbits which is histologically similar to human ulcerative colitis (Hodgson *et al.*, 1978).

Hypercatabolism of the third component of complement, C3, has been demonstrated in patients with inflammatory bowel disease (Hodgson *et al.*, 1977b) even though the serum concentrations are normal or raised (Ward and Eastwood, 1974; Hodgson *et al.*, 1977c). This component can, however, be activated either through the classical pathway by immune complexes, or the alternative pathway by substances such as bacterial endotoxins (Mergenhagen *et al.*, 1973). The aim of this study was therefore to investigate the catabolism of Clq, which is the subcomponent of the complement system that binds to immune complexes to activate the classical complement pathway.

Methods

PATIENTS

The subjects studied comprised five patients with ulcerative colitis and five patients with Crohn's disease. Details of these patients are given in Table 1. Diagnosis of ulcerative colitis and Crohn's disease was on the basis of clinical, radiological, and histological criteria. The severity of their disease was assessed according to the criteria of Truelove and Witts (1955) for ulcerative colitis and of de Dombal *et al.* (1974) for Crohn's disease. One patient with ulcerative colitis (E. McF) was in complete remission; five patients were on no treatment at the time of study and in the other five treatment was not altered immediately before or during the study. Five control subjects were also studied, four of whom were patients with non-inflammatory disorders (irritable colon syndrome, nutritional osteomalacia) and one healthy volunteer. The ages of the patients ranged from 24 to 70 years and that of the control subjects from 24 to 53 years. The Ethics Committee approved of the project and before each study began the informed consent and co-operation of the subject was obtained.

ISOLATION OF Clq

Pure, metabolically functional Clq was isolated from fresh human serum by the method of Reid *et al.* (1972) with minor modifications. The fresh human serum was obtained from a volunteer panel of donors at the Royal Free Hospital. The euglobulin fraction from 4 litres of serum was precipitated by dialysis against 0.02 M sodium acetate buffer, pH 5.5

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Received for publication 25 May 1979.

Table 1 Clinical details on patients studied

	Sex	Age (yr)	Extent/site	Severity	Treatment (daily)
<i>Ulcerative colitis</i>					
E.H.	F	26	Distal	Moderate	None
A.K.	M	70	Total	Moderate	None
P.K.	F	38	Total	Moderate	Sulphasalazine 2g
E.McF.	F	48	Distal	Remission	Sulphasalazine 2g
D.W.	F	55	Distal	Moderate	Sulphasalazine 2g
<i>Crohn's disease</i>					
J.C.	F	24	Ileocolitis	Moderate	Sulphasalazine 2g
L.E.	M	54	Ileal	Moderate	None
B.F.	F	27	Ileocolitis	Moderate	Prednisolone 20 mg
					Sulphasalazine 2 g
D.H.	F	41	Ileal	Mild	None
G.S.	M	50	Ileocolitis	Severe	None

and, after re-solution and high speed centrifugation to flotate contaminating lipid, it was subjected to ion-exchange chromatography on a 60 cm × 64 cm² column of DEAE-cellulose (Whatman DE52) equilibrated with 55 mmol sodium phosphate buffer containing 1 mmol EDTA, pH 7.4. The leading peak was concentrated by pressure ultrafiltration to 30 ml, dialysed against 56 mmol sodium phosphate buffer containing 200 mmol sodium chloride, pH 5.2 and eluted from a 90 cm × 32 cm² column of Sephadex G200 by upward flow. Again the first peak was concentrated to 30 ml and dialysed against 230 mmol sodium acetate buffer, pH 5.2. The sample was loaded on to a 30 cm × 4 cm² column of CM-cellulose (Whatman CM52) equilibrated with this buffer. After elution of the first peak, Clq was eluted using a linear salt gradient from the starting buffer to 230 mmol sodium acetate, pH 5.2, containing 270 mmol sodium chloride. Clq activity was assessed by Ouchterlony analysis against anti-Clq antiserum (Hoechst) and the positive fractions pooled and concentrated by pressure ultrafiltration to 6–8 ml.

Protein concentration was estimated using $E_{1\text{cm}, 280\text{nm}}^{1\%} = 6.82$ (Reid *et al.*, 1972).

All buffers were prepared in 'water for injection' (Travenol Laboratories) and contained either streptomycin sulphate (100 mg/l) and benzyl penicillin (1×10^6 units/l) or gentamycin sulphate (80 mg/l) and the sodium salt of ampicillin (250 mg/l). All apparatus and buffers were either autoclaved or sterilised using commercial sterilising agents.

Protein purity was assessed by polyacrylamide gel electrophoresis, isoelectric focusing, analytical ultracentrifugation and its reaction to multispecific antisera (Hoechst). Biological activity was assessed by its incorporation into the Clq, r, s complex. Aliquots of plasma samples, obtained from subjects injected with ¹²⁵I-Clq, were subjected to either gel filtration on a 90 cm × 8 cm² Sepharose 6B column

or sucrose density gradient ultracentrifugation on a 10–40% wt/wt phosphate buffered sucrose gradient. Gradients were prepared by the method of Britten and Roberts (1960) and centrifuged at 27 000 rev/min in a 3 × 25 ml swing-out rotor (MSE). In both cases fractions were tested for radioactivity and Cls activity against specific anti-Clis antiserum (Hoechst).

RADIOIODINE LABELLING OF Clq

Clq was labelled with ¹²⁵I by the method of McConahey and Dixon (1966). Five to six milligrams of Clq in 3 ml 100 mM sodium phosphate buffer containing 1 mM EDTA, pH 7.0, was stirred in a beaker on an ice bath. To this was added 1 mCi of carrier free Na ¹²⁵I (Radiochemical Centre, Amersham) followed by 50 µg chloramine-T in 0.5 ml pyrogen-free distilled water. After five minutes the reaction was stopped by the addition of 60 µg sodium metabisulphite in 0.6 ml. The mixture was allowed to stand for an additional 10 minutes before being exhaustively dialysed, first against 100 mmol sodium phosphate buffer containing 1 mmol EDTA, pH 7.0 and then against 0.85% sodium chloride containing 1 mmol EDTA (tetrasodium salt), in order to remove unbound ¹²⁵I. The ¹²⁵I-Clq was then centrifuged at 85 000 *g* (*r*_{av}) and 2°C for 1½ hours to remove aggregates and any bacterial contamination.

One aliquot of each labelled batch was tested for pyrogens by injection into rabbits (Consultox Laboratories, Ealing). Batches were used only if they met the acceptable standards of negligible pyrogenicity and bacterial contamination as laid down in the *European Pharmacopoeia* (1971).

EXPERIMENTAL PROCEDURE

Potassium iodide (30 mg twice daily) was administered to subjects 24 hours before the start of the study and then for seven days. After centrifugation to remove aggregates, weighed quantities of ¹²⁵I-Clq

were injected intravenously together with a weighed quantity of a solution of ^{131}I human serum albumin (Radiochemical Centre, Amersham). The total dose of radioactivity was less than 25 μCi .

Plasma samples were taken at five, 15, 30, and 60 minutes after injection, at two hours, four hours, eight hours, and thereafter twice daily for six days. The total volume of urine passed during the study was collected in 24 hour aliquots and, for the patients only, stool collections were also made. Two millilitre aliquots of the plasma and 5 ml aliquots of the urine were counted in an LKB 1280 ultragamma counter. The ^{125}I counts were corrected for crossover from the ^{131}I channel. The faeces were weighed, made up to a constant weight with water, and homogenised with a blender: 10 ml aliquots were counted. Two millilitre and 5 ml aliquots of standard solution of the injected proteins made up in 0.85% saline containing Tween 80 were also counted.

Serum Clq concentrations were determined by a single radial immunodiffusion (Mancini *et al.*, 1965) into 1% phosphate buffered agarose, pH 7.2, containing anti-human Clq antiserum (Hoechst). Diffusion was allowed to go to completion (approximately 60 hours). Under these conditions it was found unnecessary to incorporate EDTA into the buffered agarose. Standards were prepared from purified freeze-dried Clq. Serum albumin concentrations were determined colorimetrically using bromocresol green (Dumas *et al.*, 1971). At least five separate determinations of the serum protein concentrations were made throughout each study. In no case did the serum concentrations vary significantly.

ANALYSIS OF DATA

The plasma of radioactivity disappearance curves were analysed by the multiexponential analysis technique of Matthews (1957). Best fit curve stripping for two and three exponentials was achieved using a CDC 7600 computer (University College, London). The plasma volumes were estimated by extrapolating to zero time. Fractional catabolic

Table 3 Comparison of fractional catabolic rates obtained for control subjects by three different methods

	Multiexponential analysis (% IV pool/h)	Urine: plasma ratio (% IV pool/h)	Integral analysis (% IV pool/h)
1	2.85	2.48	2.67
2	1.92	1.86	1.79
3	2.08	2.31	2.73
4	2.96	3.39	2.03
5	1.88	2.92	1.59
Mean $\pm$ SEM	2.34 $\pm$ 0.23	2.59 $\pm$ 0.26	2.31 $\pm$ 0.21

rates (FCRs) were also determined using the integrated rate equations of Nosslin (1973) and the metabolic clearance of Berson and Yalow (1957). In the patient group, faecal counts were included in the calculation of metabolic clearance.

Results

The isolated Clq was pure as judged by polyacrylamide gel electrophoresis, immunoelectrophoresis, and analytical ultracentrifugation. Biological activity was indicated by density gradient ultracentrifugation and gel filtration studies which demonstrated that, within 15 minutes of injection, over 95% of the ^{125}I -Clq had been incorporated into the macromolecular Cl complex. The radioactivity was associated with the 19S fraction in which Cls activity was also demonstrated by immunodiffusion. When Clq was not incorporated into the Clqrs complex, as occurred on short-term *in vitro* incubation with normal human serum (30 minutes at 37°C), the radioactivity remained associated with the 11S fraction, corresponding to the sedimentation coefficient of the Clq subcomponent.

The fractional catabolic rates (FCRs) based on multiexponential analysis obtained for the control subjects are given in Table 2. The mean for the group was $2.34 \pm 0.23\%$ IV pool/hour. There was no significant difference in the values obtained by stripping two or three exponentials. Metabolic clearance data (U/P) from all of the control subjects did not vary significantly from the mean fractional catabolic

Table 2 Clq metabolism in control subjects

Subjects	Serum concn (mg/l)	Fractional catabolic rate* (% IV pool/h)	Plasma volume (ml/kg)	Synthesis rate (mg/kg/h)	IV pool (mg)	EV:IV ratio
1	113	2.85	46.5	0.153	287	0.82
2	105	1.92	47.5	0.100	321	0.28
3	73	2.08	44.7	0.068	201	0.98
4	115	2.96	47.4	0.161	470	1.35
5	152	1.88	37.2	0.106	347	0.51
Mean $\pm$ SEM	113 $\pm$ 12	2.34 $\pm$ 0.23	45.3 $\pm$ 0.22	0.118 $\pm$ 0.017	325 $\pm$ 44	0.79 $\pm$ 0.19

*Based on multiexponential analysis.

Fig. 1 Serial urine: plasma radioactivity ratios in a study of ^{125}I -Clq metabolism in a control subject

rates throughout the course of the study and were in good agreement with the values obtained from multiexponential analysis and the integrated rate equation. A comparison of the fractional catabolic rates obtained by these methods may be seen in Table 3 and a typical example of U/P values is shown in Fig. 1.

The ^{125}I -Clq plasma disappearance curves for the

Fig. 2 Plasma disappearance curves of ^{125}I -Clq after intravenous injection into five patients with ulcerative colitis. The mean for the control subjects (± 1 S.D.) is also given for comparison.

Fig. 3 Plasma disappearance curves of ^{125}I -Clq after intravenous injection into five patients with Crohn's disease. The mean for the control subjects (± 1 S.D.) is also given for comparison.

patients with ulcerative colitis are shown in Fig. 2 and those for patients with Crohn's disease in Fig. 3. In both the mean plasma disappearance curves for the control subjects (± 1 S.D.) is given for comparison. For the five patients with Crohn's disease there was an accelerated disappearance of the labelled protein from the plasma. This was also true of the four patients with active ulcerative colitis, but subject

Fig. 4 Retention curves of ^{125}I -Clq injected into a patient with ulcerative colitis (subject P.K.).

E. McF., who was in remission at the time of the study, showed a normal plasma disappearance rate. Figures 4 and 5 show the ^{125}I -Clq retention curves for a patient with active ulcerative colitis (P.K.) and for a patient with Crohn's disease (L.E.). It will be seen that, once equilibrium has occurred between

the plasma and the extravascular compartment, the rate of disappearance from the extravascular space is slower than that from the plasma. This therefore suggests that there is sequestration of Clq in an extravascular compartment.

The fractional catabolic rates for ^{125}I -Clq calcu-

Fig. 5 Retention curves of ^{125}I -Clq injected into a patient with Crohn's disease (subject L.E.).

Table 4 Clq metabolism in patients with ulcerative colitis and Crohn's disease

	Serum concentration (mg/l)	Fractional catabolic rate* (% IV pool/h)	Plasma volume (ml/kg)	Synthesis rate (mg/kg/h)	IV pool (mg)	EV : IV ratio
<i>Ulcerative colitis</i>						
E.H.	131	3.50	39.8	0.182	381	0.62
A.K.	90	7.81	30.4	0.214	144	0.40
P.K.	99	6.11	51.8	0.313	267	0.62
E.McF.	120	2.71	30.7	0.100	169	1.90
D.W.	81	4.66	36.9	0.139	210	0.88
Mean $\pm$ SEM	104 $\pm$ 9	4.96 $\pm$ 0.91	37.9 $\pm$ 3.9	0.190 $\pm$ 0.036	234 $\pm$ 42	0.88 $\pm$ 0.27
<i>Crohn's disease</i>						
J.C.	167	5.04	35.7	0.300	315	0.23
L.E.	148	3.81	75.7	0.427	543	1.29
B.F.	148	8.99	37.9	0.504	335	2.28
D.H.	82	8.44	48.1	0.333	187	3.97
G.S.	77	3.67	97.1	0.274	417	1.07
Mean $\pm$ SEM	124 $\pm$ 19	5.99 $\pm$ 1.14	58.9 $\pm$ 11.9	0.368 $\pm$ 0.043	359 $\pm$ 59	1.77 $\pm$ 0.64
Controls	113 $\pm$ 12	2.34 $\pm$ 0.23	45.3 $\pm$ 2.2	0.118 $\pm$ 0.017	325 $\pm$ 44	0.79 $\pm$ 0.19

*Based on Matthews' multiexponential analysis.

Table 5 Albumin metabolism in control subjects in patients with inflammatory bowel disease

	Serum concentration (g/l)	Fractional catabolic rate* (% IV pool/h)	Plasma volume (ml/kg)	Synthesis rate (mg/kg/h)	IV pool (g)	EV : IV ratio
<i>Control subjects</i>						
1	43	0.235	62.0	6.27	143	0.79
2	51	0.284	43.6	6.32	137	1.10
3	63	0.331	43.6	9.09	169	0.94
4	40	0.429	37.0	6.35	131	1.55
5	41	0.330	29.0	3.92	73	2.50
Mean $\pm$ SEM	47.6 $\pm$ 4.3	0.322 $\pm$ 0.032	43.0 $\pm$ 5.4	6.39 $\pm$ 0.82	131 $\pm$ 16	1.38 $\pm$ 0.31
<i>Ulcerative colitis</i>						
E.H.	44	0.313	33.1	4.56	106	1.07
A.K.	46	0.305	38.3	5.37	92	1.42
P.K.	44	0.390†	42.3	7.26	97	0.88
E. McF.	43	0.194	30.3	2.53	60	1.96
D.W.	42	0.668	43.4	11.89	128	0.33
Mean $\pm$ SEM	43.8 $\pm$ 0.66	0.374 $\pm$ 0.079	37.5 $\pm$ 2.5	6.32 $\pm$ 1.58	97 $\pm$ 11	1.13 $\pm$ 0.27
<i>Crohn's disease</i>						
J.C.	48	0.462	41.0	9.09	104	1.22
L.E.	35	0.893	40.6	12.69	69	0.76
B.F.	46	0.323	42.0	6.24	116	0.76
D.H.	46	0.456	42.5	8.91	92	1.11
G.S.	39	0.550†	72.9	15.64	159	1.22
Mean $\pm$ SEM	42.8 $\pm$ 2.5	0.537 $\pm$ 0.096	47.8 $\pm$ 6.3	10.50 $\pm$ 1.64	108 $\pm$ 15	1.01 $\pm$ 0.11

*Based on integral analysis.

†Based on U/P data.

lated from these curves using multiexponential analysis are shown in Table 4. Similar data for the catabolism of albumin are given in Table 5. Although there is no significant difference in the serum Clq concentrations, the fractional catabolic rates and synthesis rates in nearly every case are markedly above those for the control subjects: the exception being the patient in remission at the time of the study (E. McF.). The accelerated catabolism cannot be accounted for by loss into the gastrointestinal tract. Table 6 shows the percentage of the injected dose lost over the first five days of the patient studies. In only one case (B.F.) did this exceed 3%.

The associated albumin data for these patients show no significant difference in albumin metabolism for the patients with ulcerative colitis compared with the control subjects, but a marked rise in those with Crohn's disease.

Table 6 Percentage loss of injected dose into gastrointestinal tract over first five days

Ulcerative colitis	¹²⁵ I-Clq	¹³¹ I-HSA
E.H.	0.238	0.116
A.K.	0.829	2.081
P.K.	1.846	3.454
E. McF.	ND	ND
D.W.	—	—
<i>Crohn's disease</i>		
J.C.	0.528	1.060
L.E.	3.093	1.100
B.F.	24.793*	6.086
D.H.	0.338	1.237
G.S.	0.970	1.281

*Sixteen per cent lost on day 1.

Discussion

The present studies have shown that the metabolism of Clq is increased in inflammatory bowel disease. Although protein turnover studies are subject to many errors, it seems probable that this is a real finding. The data for Clq metabolism in control subjects are in good agreement with those of Kohler and Müller-Eberhard (1972). The ¹²⁵I-Clq injected appeared biologically active, as it was rapidly incorporated into the Clqrs complex. There was no evidence from the metabolic clearance data of a rapid excretion of ¹²⁵I within the first 36 hours, as would have occurred if denatured protein had been injected. The same batches of protein that showed normal catabolism in controls showed increased catabolism in patients with inflammatory bowel disease.

An interesting finding was the lack of *in vitro* incorporation of Clq into the Cl complex compared with the rapid incorporation seen *in vivo* and it seems likely that, once the subunits are synthesised and released, complex formation occurs instantaneously. Our *in vitro* findings suggest that the complex, once formed, is stable and little exchange takes place with exogenous Clq.

A possible explanation for the increased catabolism of Clq is faecal loss of the injected protein, as patients with active inflammatory bowel disease may lose protein by this route. The faecal excretion of ¹²⁵I-Clq in the patients studied (Table 6), however, cannot explain the increased catabolism, except in one patient, even though the losses may be an underestimate as the injected protein was labelled

with ^{125}I (Waldmann, 1970). It is also unlikely that we are merely recording a generalised increase in protein turnover, as in one group of patients (ulcerative colitis) raised Clq catabolism was seen with normal albumin turnover. In one patient, who was in remission, Clq metabolism was similar to the control subjects, which suggests that hypercatabolism occurs only in active disease. Obviously more patients in remission should be studied to confirm this, but it is difficult to admit well patients for metabolic studies when hospital admission is often a frequent event in the natural history of their disease.

The divergence between EV and IV distribution of Clq over the period of study in our patients is similar to that found by Kohler and Müller-Eberhard (1972) in patients with hypogammaglobulinaemia and in two patients with myeloma. Similar EV accumulation of C3 has been observed in patients with inflammatory bowel disease (Hodgson *et al.*, 1977b). These findings suggest retention of label within the EV compartment. This may occur as a result of increase in capillary permeability or of specific fixation of complement components at an EV site. In patients with inflammatory bowel disease it is possible that both mechanisms are operating within the inflamed mucosa.

Immunoglobulin production is greatly increased in the diseased intestine of these patients (McClelland *et al.*, 1976) and some of this immunoglobulin has antibody specificity for bacterial antigens (Monteiro *et al.*, 1971). It is highly probable, therefore, that the antigen-antibody reactions are occurring within the diseased mucosa with subsequent activation of complement *via* the classical pathway. The present studies do not exclude activation of the alternative pathway by such factors as endotoxin and IgA, nor do they rule out activation of the classical pathway by factors such as proteolytic enzymes and bacterial products. Nevertheless, the demonstration of Clq activation in patients with inflammatory bowel disease supports the hypothesis that antigen-antibody reactions are involved in the pathogenesis of mucosal inflammation.

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