Clin. exp. Immunol. (1989) **78**, 49–53

Differences in the metabolism of C4 isotypes in patients with complement activation

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(Accepted for publication 19 June 1989)

SUMMARY

The metabolism of the C4 allotypes C4A3,B1 and C4A3,BO was studied in five healthy control subjects and six patients with active immunological disease (five with systemic lupus erythematosus and one with rheumatoid arthritis). The specific aim was to identify any differences in the metabolism of C4A and C4B gene products that may be linked to their documented functional differences *in vitro*. The fractional catabolic rate of C4A3,B1 in patients was significantly greater than that of C4A3,BO ($3{\cdot}98 \pm 1{\cdot}37$ *versus* $3{\cdot}31 \pm 0{\cdot}85$%/h; mean ± s.d.; $P < 0{\cdot}05$) but there was no difference in control subjects (1·95 *versus* 1·99%/h). The extravascular: intravascular (EV:IV) distribution ratio of C4A3, B1 was also greater in both patients ($1{\cdot}19 \pm 0{\cdot}36$ *versus* $0{\cdot}97 \pm 0{\cdot}35$; $P < 0{\cdot}01$) and controls ($0{\cdot}43 \pm 0{\cdot}11$ *versus* $0{\cdot}31 \pm 0{\cdot}13$; $P = 0{\cdot}01$). We conclude that C4B1 was catabolized more rapidly than C4A3 in patients with pathological complement activation but not in control subjects. This difference could reflect the relatively greater extravascular distribution (i.e. EV:IV ratio) of C4B at sites of immune complex deposition or, alternatively, different rates of catabolism of inactive C4 isotypes (iC4b).

Keywords complement metabolism C4 allotypes activation immune complex

INTRODUCTION

Inherited and acquired abnormalities of the fourth component of complement (C4) occur commonly in several human diseases. An increase in the incidence of specific C4 allotypes, including those that contain heterozygous and homozygous null alleles, has been reported in disorders such as systemic lupus erythematosus (SLE) and type 1 (insulin-dependent) diabetes (Bertrams *et al.*, 1984; Hauptmann *et al.*, 1986). However, these substantial linkage data have not resolved the contribution of C4 genetic polymorphism to pathogenesis. In contrast, the biological consequences of acquired C4 abnormalities, which typically result from activation of the classical pathway by antibody-containing macromolecules, have been characterized in detail (Schur, 1985).

The protein products of the C4A and C4B genetic loci show several functional differences *in vitro*. These include variations in haemolytic activity (Awdeh & Alper, 1980), interaction with immune aggregates (Law, Dodds & Porter, 1984) and efficiency of inhibition of immune precipitation (Schifferli *et al.*, 1984). However, it is not known whether these differences are reflected in the behaviour of C4A and C4B proteins *in vivo*. We have compared the metabolic characteristics of human C4 prepared from healthy donors with C4A3,B1 and C4A3,BO allotypes. The purified proteins were studied simultaneously in both normal subjects and in patients with active immune complex disease. The specific aim was to identify differences in fractional catabolism and compartmental distribution that might be associated with functional differences between alleles *in vitro*.

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MATERIALS AND METHODS

Subjects

Five patients with active SLE, one with active rheumatoid arthritis (RA) and five healthy medical and scientific staff were studied. The SLE group satisfied the diagnostic criteria of the American Rheumatism Association (Tan *et al.*, 1982) and were in hospital at the time of investigation. Each patient in this group had active glomerular disease and involvement of at least one other system. At the time of study, no patient was taking more than 10 mg of prednisolone/day; two were also receiving cyclophosphamide, but there had been no change in this treatment in the preceding 4–6 weeks. The patients and control subjects were matched closely for age, but not for sex.

Complement measurements

The serum concentrations of C3 and C4 were measured by radial immunodiffusion using monospecific antisera (Dakopats, Glostrup, Denmark) (Mancini, Carbonara & Heremans, 1985). C4 allotypes were examined on fresh plasma (in EDTA) by the

use of methods previously described by Mauff and co-workers (1983). Briefly, neuraminidase-treated plasma was electrophoresed at 4°C (in agarose) before overlay with affinity-purified polyclonal anti-C4 (Silenus, Melbourne, Australia). The A and B genetic loci were defined where necessary by a haemolytic overlay technique, and heterozygous null alleles were detected by two-dimensional immunoelectrophoresis (Awdeh, Raum & Alper, 1979).

Immune complexes were detected in serum by a fluid phase C1q-binding assay (Zubler *et al.*, 1976). Samples were tested on the first day of the turnover study.

Purification of C4

C4 was prepared from fresh plasma (in EDTA) as previously described (Charlesworth *et al.*, 1987). Healthy members of the medical and scientific staff were used as donors to prepare the common C4 allotype, C4A3,B1 and the A locus isotype C4A3,BO. Donors were known to be negative for hepatitis B surface antigen (HBsAg) and HIV antibody. Proteins were tested for immunochemical purity by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and for functional activity by the use of a C4-deficient guinea pig serum (Gaither, Alling & Frank, 1974). These showed that preparations were essentially free of contaminants (i.e. $<5\%$) and that there was retention of haemolytic activity that was comparable with that observed in an equal concentration of donor plasma. Purified C4 was labelled with ^{125}I (C4A4,BO) or ^{131}I (C4A3,B1) by the lactoperoxidase method (Marchalonis, 1969). There was no significant change in haemolytic activity following radio-labelling and autoradiography of SDS–PAGE showed that there had been no significant production of radiolabelled contaminants or breakdown products. Proteins were sterilized by millipore filtration, cultured for aerobic and anaerobic organisms and pyrogen tested in rabbits prior to injection.

Turnover protocol

Turnover studies were performed in each of the five control subjects and six patients. A control subject was investigated simultaneously with one, two or three patients. Each received freshly labelled ^{125}I-C4A3,BO and ^{131}I-C4A3,B1 following pretreatment with oral potassium iodide (60 mg t.i.d. for three days) to block thyroidal uptake of isotope. Approximately 10 μCi ^{125}I-C4A3,BO and 2·5 μCi ^{131}I-C4A3,B1 were given by intravenous injection. Plasma and urine samples were collected for 140 h according to a previously described protocol (Charlesworth *et al.*, 1987). This protocol had been approved by the Hospital Ethics Committee, and informed consent was obtained from all patients. Purified C4AO,B1 was not used because of the possibility of sub-clinical autoimmunity in the donor.

Analysis of turnover data

Metabolic data were analysed by the integrated rate equations method (Nosslin, 1972). This permitted the day-to-day calculation of individual metabolic parameters in subjects in whom plasma disappearance of radioactivity was non-linear. The fractional catabolic rate (FCR) and extravascular : intravascular (EV : IV) distribution ratio were calculated by this method and further checked by the use of exponential analysis of the plasma disappearance curve (i.e. in those subjects in whom the final exponential reached linearity) (Matthews, 1957). No attempt was made to derive a value for plasma production because of the difference between injected and native C4 allotype in several subjects.

Differences between values for serum or metabolic parameters or both were examined by paired and unpaired Student's *t*-tests.

RESULTS

Immunological studies

Table 1 summarizes these data. There was significant elevation of antibodies to nuclear antigens in three of the five patients with SLE. The remaining two patients satisfied the 1982 revised criteria for diagnosis (Tan *et al.*, 1982); one of these patients had detectable lupus inhibitor. Immune complexes were detected in three patients and the sixth patient (with active RA) had an elevated titre of rheumatoid factor. C4 or C3 or both were reduced in three patients. Three patients also had detectable C4 null alleles (two had homozygous C4AO). Three control subjects had C4 null alleles (two had homozygous C4A3,BO).

C4 metabolic data

These data are shown in Fig. 1 and in Tables 2 and 3. The FCR of C4A3,B1 was significantly greater than that of C4A3,BO in patients ($3{\cdot}98 \pm 1{\cdot}37$ *versus* $3{\cdot}31 \pm 0{\cdot}85$%/h; $P<0{\cdot}05$) but there was no difference in control subjects (1·95 *versus* 1·99%/h). Three patients, with gross hypercatabolism of both proteins, showed a non-linear pattern of disappearance of plasma radioactivity (see Fig. 2). This was not observed in control subjects or patients with milder hypercatabolism where day-to-day iodide release was steady and plasma disappearance remained linear beyond 95% elimination. In two of the patients with a non-linear pattern, homozygous C4AO was detected with a low serum C4 concentration while no null alleles and a normal serum C4 were found in the other patient.

The mean value of EV : IV distribution for C4A3,B1 was also greater than that of C4A3,BO in both control subjects ($0{\cdot}43 \pm 0{\cdot}11$ *versus* $0{\cdot}31 \pm 0{\cdot}13$; $P=0{\cdot}01$) and patients ($1{\cdot}19 \pm 0{\cdot}36$ *versus* $0{\cdot}97 \pm 0{\cdot}35$; $P<0{\cdot}01$). The patient group also

Table 1. Immunological data of patients with active immunological disease and healthy controls studied for metabolism of C4 allotypes

Patient no.	Diagnosis	C4 allotype	Serum C4 (g/l)	Serum C3 (g/l)	Immune complexes (%)
1	SLE*	A3;B1	0·35	1·34	10
2	SLE	A3;B1,O	0·20	0·63	13
3	SLE†	AO;B1	0·11	0·40	25
4	SLE	AO;B1	0·11	0·56	16
5	SLE	A3;B2,1	0·22	0·79	63
6	RA	A3;B1	0·25	1·08	35
Control subjects ($n=5$)	—	—	$0{\cdot}32 \pm 0{\cdot}07$‡	$0{\cdot}96 \pm 0{\cdot}15$	9 ± 4

* Lupus inhibitor detected.

† Anti-nuclear antibody negative.

‡ Mean ± s.d.

SLE, systemic lupus erythematosus; RA, rheumatoid arthritis.

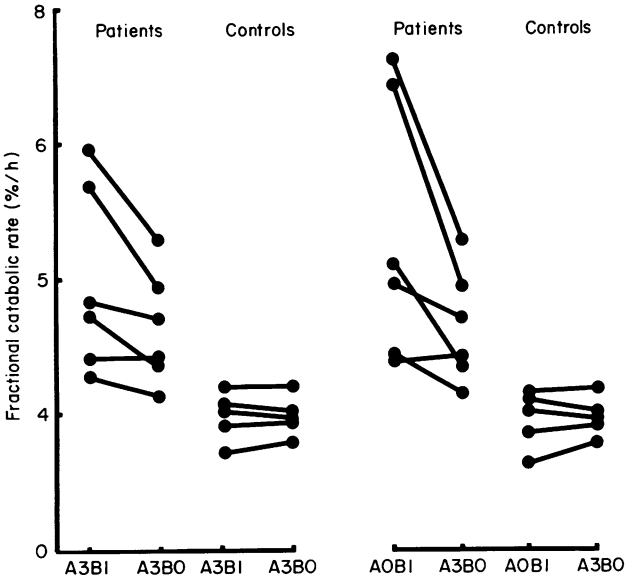

Fig. 1. The fractional catabolic rates of C4A3,B1 and C4A3,BO in patients and control subjects. Derived values for C4AO,B1 are also shown. There was a significant difference in the fractional catabolism of the two injected proteins for patients but not for control subjects.

Table 2. Metabolic data of I^{131}. C4A3,B1 in patients with active immunological disease and control subjects

Patient	$T_{\frac{1}{2}}$ (h)	FCR (%/h)	EV:IV distribution
1	44	2·58	1·46
2	43	2·83	0·69
3	NL	5·92	1·25
4	NL	3·67	1·15
5	NL	5·39	1·68
6	41	3·46	0·89
Mean ± s.d.		3·98 ± 1·37	1·19 ± 0·36
Control subjects ($n=5$)		1·95 ± 0·36	0·43 ± 0·11

$T_{\frac{1}{2}}$, half life; FCR, fractional catabolic rate; EV:IV, extravascular:intravascular ratio; NL, non-linear plasma radioactivity disappearance curve.

showed higher EV:IV ratios for both proteins compared with controls ($P<0·01$ for each protein).

Derived data for the B-locus isotype are shown in Fig. 1. The FCR for C4B1 was calculated by the formula:

$$\text{FCR} \cdot \text{C4B1} = 2 \times \text{FCR} \cdot \text{C4A3,B1} - \text{FCR} \cdot \text{C4A3,BO}$$

Two-dimensional electrophoresis of the injected C4A3,B1, with measurement of areas under precipitation peaks, showed that equal amounts of both alleles were present.

Table 3. Metabolic data for I^{125}. C4A3,BO in patients with active immunological disease and control subjects

Patients	$T_{\frac{1}{2}}$ (h)	FCR (%/h)	EV:IV distribution
1	44	2·30	1·42
2	48	2·89	0·58
3	NL	4·62	0·94
4	NL	3·44	0·97
5	NL	3·88	1·31
6	48	2·71	0·61
mean ± s.d.		3·31 ± 0·85	0·97 ± 0·35
Control subjects ($n=5$)		1·99 ± 0·31	0·31 ± 0·13

$T_{\frac{1}{2}}$, half life; FCR, fractional catabolic rate; EV:IV, extravascular:intravascular ratio; NL, Non-linear plasma radioactivity disappearance curve.

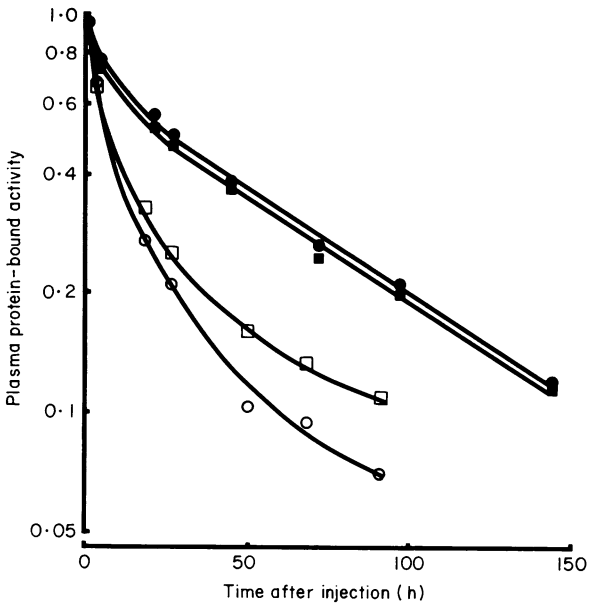

Fig. 2. Plasma radioactivity disappearance curves for ^{131}I-C4A3,B1 and ^{125}I-C4A3,BO in (a) a control subject (● and ■, respectively); and (b) a patient with C4 hypercatabolism (○ and □, respectively). The pattern in the patient is non-linear for both proteins with fractional catabolic rates of 5·92 and 4·62%/h (patient 3).

DISCUSSION

The fractional catabolism of purified C4A3,B1 was significantly greater than that of C4A3,BO in patients with pathological complement activation but not in control subjects. We could not demonstrate an influence of the allotype of the recipient (including the presence of null alleles), on the relative rate of turnover of the two purified proteins. Indeed, patients and

controls had a comparable frequency of null alleles at the A and B loci and thus received similar amounts of mismatched isotype. It could be argued that the metabolic differences between the two protein preparations resulted from variable loss of functional activity during purification (and radio-iodination). However, similarity of turnover in healthy subjects and the fact that the haemolytic activity of C4A3,B1 and C4A3,BO was comparable to that of aliquots of plasma from which they were purified argues against this possibility. Furthermore, the steady release of iodide from each protein throughout the period of sampling suggested that there was no significant percentage of denatured protein.

Three patients showed a non-linear pattern of disappearance of plasma radioactivity for both proteins; these three had the highest rates of fractional catabolism. Such a pattern may result from the injection of residual radiolabelled contaminants, with different rates of disappearance. However, no such contaminants were observed on autoradiography of SDS-PAGE, while *in vivo* plasma disappearance in healthy subjects remained linear beyond 95% clearance of injected radiolabelled material. (This was true of each of the four—two of each allotype—preparations of C4 made for this study.) A similar pattern of non-linear plasma disappearance of radioactivity has been observed with C3 turnover in patients with hypocomplementaemic membranoproliferative glomerulonephritis (Charlesworth *et al.*, 1974). The basis for this pattern of disappearance is not certain, although it may reflect generation of smaller C4 activation products with different rates of turnover and EV:IV distribution.

The data show that the turnover of C4B-derived protein was greater than that of C4A protein in patients with accelerated complement metabolism. Furthermore, the observation that the metabolism of both proteins was similar under normal conditions suggests that pathological complement activation *per se* was required before this unequal metabolic responsiveness became manifest. Activation of C4 through the complement cascade may not, therefore, be the major influence on the behaviour of C4 in normal subjects.

We know of no previous demonstration of biological differences between C4 isotypes *in vivo*. However, certain observations *in vitro* suggest selective roles for these products under pathological conditions. Law, Dodds & Porter (1984) have demonstrated variations in binding specificity of C4A and C4B products, despite their high degree of amino acid homology. For example, C4B-derived proteins show approximately two-fold efficiency for binding to antibody-coated erythrocytes. In contrast, proteins of the C4A locus interact more efficiently with pre-formed bovine serum albumin (BSA)–anti-BSA complexes. C4A3 is also known to be more efficient than C4B1 in the inhibition of immune precipitation (Schifferli *et al.*, 1986). One explanation for the difference in the metabolic characteristics between C4A and C4B would be more efficient cleavage of C4B by C1s *in vivo*. Miura *et al.* (1987) have used unpurified C4A and C4B from tissue culture to show preferential cleavage of C4B by C1s. However, two previous studies that used isolated C4 isotypes have found no difference in their interaction with C1s (Isenman and Young, 1984; Law *et al.*, 1984). Alternatively, the metabolic differences could reflect more rapid metabolism of inactivated C4B (iC4B) or the greater localization of this isotype at sites of classical pathway activation (we showed that C4B had a higher EV:IV ratio than C4A in both normal subjects and patients with immune complex disease).

No attempt was made to quantify the plasma production rate of either protein since we did not measure the plasma concentration of A and B locus isotypes. However, if it is assumed that the production of these products are comparable under both physiological and pathological conditions, then C4 hypercatabolism may increase the proportion of A locus to B locus proteins in the plasma (where both are present). Such metabolic sequelae may provide further evidence of the biological advantage of C4 gene duplication to the host.

ACKNOWLEDGMENTS

This work was supported by a grant from the National Health and Medical Research Council of Australia.

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