The metabolism of C3 and C4 in patients with immune complexes and normal complement levels

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THE METABOLISM OF C3 AND C4 IN PATIENTS WITH IMMUNE COMPLEXES AND NORMAL COMPLEMENT LEVELS

J. A. CHARLESWORTH
Associate Professor of Renal Medicine, Division of Medicine, Prince Henry Hospital and the University of New South Wales, Sydney, Australia

P. W. PEAKE
Senior Scientific Officer, Divisions of Medicine and Pathology, Prince Henry Hospital and the University of New South Wales, Sydney, Australia

J. GOLDING
Research Assistant, Division of Medicine, Prince Henry Hospital and the University of New South Wales, Sydney, Australia

B. A. PUSSELL
Renal Physician, Division of Medicine, Prince Henry Hospital and the University of New South Wales, Sydney, Australia

V. TIMMERMANS
Scientific Officer, Division of Medicine, Prince Henry Hospital and the University of New South Wales, Sydney, Australia

I. WICKS
Medical Registrar, Division of Medicine, Prince Henry Hospital and the University of New South Wales, Sydney, Australia

D. WAKEFIELD
Associate Professor of Pathology, Division of Pathology, Prince Henry Hospital and the University of New South Wales, Sydney, Australia

Abstract:
The metabolism of the complement proteins, C3 and C4 was examined in two groups of patients with a high incidence of detectable immune complexes but normal levels of complement components. The specific aim was to ascertain whether significant on-going complement activation occurred in these patients. Eleven patients with rheumatoid arthritis (RA), 11 with infection and 11 control subjects were studied. Each received approximately 10 uCi ^{125}I.C4 and 2.5 uCi ^{131}I.C3 by intravenous injection. Analysis of turnover data showed that there was significant hypercatabolism of both C3 and C4 in the two study groups compared to controls. Plasma production of C4 was normal for both groups (despite the presence of C4 null alleles in six out of 11 of the RA group), while C3 production was significantly elevated in both RA and infection ($p < 0.01$ and $p < 0.001$ respectively). Patients with infection showed a significant increase in extravascular/intravascular distribution of both proteins. The data show that immune complex formation is associated with accelerated turnover of complement proteins, irrespective of co-existing tissue damage or changes in the serum concentration of complement components. The findings suggest that both activation of complement and maintenance or enhancement of protein synthesis are important for the efficient processing of immune complexes *in vivo*. (Aust NZ J Med 1989; 19: 118-124.)

Key words: Immune complex, metabolism, complement, rheumatoid, infection.

Many diseases in humans are associated with the detection of complement-reactive macromolecules in the serum.[1] It is usually assumed that these reactants consist of antigen-antibody complexes even though the majority of assays used for their identification do not define the antigen component.

Reprint requests to: A/Professor J. A. Charlesworth, Division of Medicine, Clinical Sciences Building, Prince Henry Hospital, Anzac Parade, Little Bay 2036, Australia.

The many methods developed for this purpose reflect the relative inefficiency of detection of immune complexes (IC) in human serum. Furthermore, the wide range of diseases associated with assay positivity makes this finding of limited value in the understanding of pathogenesis or the clinical evaluation of the patient. Paradoxically, the majority of patients with (complement-dependent) IC show no change in the serum concentration of individual complement proteins. While activation by-products (such as C3a or C3d) may be found in fresh plasma samples,[2] this finding provides little insight into the metabolic basis for the maintained serum level.

An alternative to the investigation of plasma or serum samples is the direct study of metabolism using purified, functionally-active complement components. This quantitates the catabolism, plasma production and compartmental distribution of individual proteins and permits the more precise interpretation of complement behaviour in diseases where there is potential for immunological damage. In this study, we have examined simultaneously the turnover of purified C3 and C4 in normal subjects, 11 patients with infection and 11 with uncomplicated rheumatoid arthritis (RA). All patients had normal complement levels and 20 had high serum IC, as detected by a C1q-dependent assay. The specific aim of the project was to establish whether IC-associated diseases showed the metabolic features of on-going complement activation.

MATERIALS AND METHODS

Subjects

Eleven patients with RA, 11 with infection and 11 healthy members of the medical and technical staff were studied. All patients were in hospital for diagnosis and/or treatment. Each of the RA group had diffusely active joint disease and four had rheumatoid nodules; no other extra-articular manifestations were observed. None of this group were receiving corticosteroids or cytotoxic agents at the time of investigation. The patients with infection were studied as soon as possible after diagnosis. This group contained four subjects with subacute bacterial endocarditis (SBE), three with infectious mononucleosis, two with acute hepatitis B and one each with brucellosis and toxoplasmosis. One patient with infectious mononucleosis had acute Guillain-Barré syndrome. The other ten patients showed no clinical or laboratory evidence of immunological complications; specifically, there were no features of cutaneous or joint involvement and none had significant proteinuria or an active urine sediment. Bacteria cultured from the SBE group were: strept viridans (two); hemophilus influenzae (one) and strept fecalis (one). Both patients with hepatitis B were intravenous drug users but each was negative for human immunodeficiency virus (HIV)-antibody at the time of investigation.

Two further subjects with chronic disease (that is, one with ankylosing spondylitis and one with severe scleritis) were examined in order to observe the non-specific effect of chronic illness on complement turnover. A patient with essential cryoglobulinemia, a high level of IC and gross reduction in serum C3 and C4 was studied as a positive control.

Complement Studies

Serum samples were separated by centrifugation at 4 °C and stored in liquid nitrogen until analysed. The serum concentration of C4 and C3 was measured by radial immunodiffusion[3] using monospecific antisera (Dakopatts) and total hemolytic complement activity was tested by a standard tube assay.[4]

The C4 allotype of each patient was examined on fresh plasma (EDTA), using the method described by Mauff *et al.*[5] Briefly, neuraminidase-treated plasma was subjected to agarose electrophoresis at 4 °C before overlay with mono-specific polyclonal anti-C4 (Atlantic). A hemolytic overlay technique was used to further define the A and B genetic loci and two dimensional electrophoresis was performed to identify heterozygous null alleles at either locus.[6] Wherever possible, family studies were undertaken in those patients where the above techniques did not lead to satisfactory resolution of the protein pattern.

Preparation of C3 and C4

These proteins were prepared from fresh plasma (in EDTA) donated from healthy volunteers who were negative for hepatitis B surface antigen and HIV-antibody. All were in good health at the time of donation. Human C4 was prepared by the method of Bolitin *et al.*,[7] with modifications.[8] C3 was prepared by the method described by Harrison and Lachmann.[4] Both proteins were examined for purity by double diffusion in agarose against anti-whole human serum and by polyacrylamide gel electrophoresis in sodium dodecyl sulphate (PAGE-SDS). Functional activity was tested after purification by standard hemolytic techniques using C4 and C3 deficient sera.[9,4] These tests showed that both proteins were essentially free of contaminants and retained hemolytic activity comparable to that observed in an equal concentration of fresh normal serum.

C4 was labelled with I^{125} by the lactoperoxidase method[10] and C3, with I^{131}, by a modified

TABLE 1
C4 Metabolic Data in Patients and Control Subjects

Subjects	Se.C4 (g/l)	C4 Null* alleles (subjects)	IC (% binding)	t½ (h)	FCR (%/h)	Plasma production (mg/kg/h)	EV/IV distrib	EV† catabolism
RA (n = 11)	0.29 ± 0.06	6/11	51 ± 25‡	44 ± 6§	2.57 ± 0.45‡	0.34 ± 0.11	0.50 ± 0.18	0.64 ± 0.34
Infection (n = 11)	0.29 ± 0.09	2/11	37 ± 26§	39 ± 9‡	2.86 ± 0.52‡	0.38 ± 0.16	0.67 ± 0.19§	0.68 ± 0.10
Controls (n = 11)	0.38 ± 0.15	4/11	10 ± 5	54 ± 8	1.87 ± 0.17	0.30 ± 0.14	0.45 ± 0.13	0.73 ± 0.32

* = ≥ 1 null allele(s) detected.
† = Value < 1 indicates finite EV catabolism.
‡ = $p < 0.001$.
§ = $p < 0.01$.

chloramine T technique.[11] Radio-iodination caused no significant change in the hemolytic activity of either protein and autoradiography of PAGE-SDS confirmed that there had been no significant production of radio-labelled contaminants or breakdown products. Both proteins were sterilised by Millipore filtration prior to culture for erobic and anerobic organisms. Subsequent pyrogen tests in rabbits were negative for both proteins.

Turnover Studies

These were performed according to a previously described protocol.[12] This protocol had been approved by the hospital ethics committee and informed consent was obtained from each subject. Patients and controls (at least one per experiment) received approximately 10 uCi ^{125}I.C4 and 2.5 uCi ^{131}I.C3 by intravenous injection. Thyroidal uptake of radioactive iodide was blocked by the prior administration of oral potassium iodide (180 mg per day) for three days. Blood samples (in EDTA) were taken at 15 min, 4-6 h, 24 h and once or twice daily thereafter until there was less than 8% residual plasma radioactivity. Twenty-four h samples of urine were collected for the first 96 h of the study period. Serum C3 and C4 levels were measured at the beginning and end of each turnover to confirm a steady state.

Detection of IC

These were measured on the first day of the turnover study by a fluid-phase C1q binding assay.[13]

Analysis of Metabolic Data

Three methods of analysis were used: (a) metabolic clearance (that is, calculation of the fractional catabolic rate [FCR] from the ratio of urine to plasma radioactivity per unit time),[14] (b) exponential analysis,[15] and (c) the integrated rate equations method.[16] Specifically, the half-life (t½) was measured by regression analysis of the plasma disappearance curve, FCR was calculated by each method except where it was suspected that urine collections were incomplete or unsatisfactory (for example, patients with renal failure and iodide retention) and synthesis rate (mg/kg/h) was derived from the formula:

$$\frac{\text{serum concentration (mg/ml)} \times \text{FCR (\%/h)} \times \text{plasma volume (ml)}}{\text{weight (kg)}}$$

The extravascular (EV)/intravascular (IV) distribution ratio was calculated by exponential analysis and the integrated rate equations method. This latter technique was also used to determine the rate constant for EV catabolism. For most subjects, the final value for FCR was calculated as the mean of

TABLE 2
C3 Metabolic Data in Patients and Control Subjects

Subjects	Se.C3 (g/l)	t½ (h)	FCR (%/h)	Plasma production (mg/kg/h)	EV/IV distrib	EV* catabolism
RA (n = 11)	0.90 ± 0.16	48 ± 9†	2.00 ± 0.45†	0.79 ± 0.25‡	0.41 ± 0.21	0.45 ± 0.24
Infection (n = 11)	1.09 ± 0.22‡	45 ± 8†	2.33 ± 0.39§	1.14 ± 0.31§	0.54 ± 0.18	0.41 ± 0.12
Controls (n = 11)	0.84 ± 0.15	56 ± 8	1.61 ± 0.14	0.55 ± 0.12	0.39 ± 0.18	0.70 ± 0.44

* – Value < 1 indicates finite EV catabolism.
‡ = $p < 0.05$ (mean ± SD).
† = $p < 0.01$.
§ = $p < 0.001$.

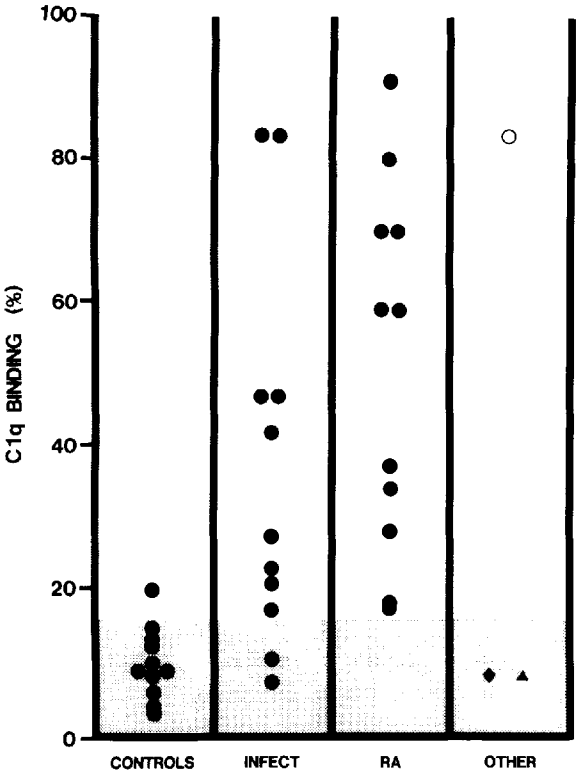

Figure 1: Detection of immune complexes by a fluid-phase C1q binding assay in controls and patients with RA and infection. Data for the patients with ankylosing spondylitis (◆), scleritis (▲) and essential cryoglobulinemia (○) are also shown. The normal range is shaded.

results obtained by the three methods of analysis. When there was disagreement of greater than 20%, the mean of the two more comparable values was used.

Differences between values for serum or metabolic parameters were examined by an unpaired t test and correlations between individual serum and metabolic parameters were examined by linear regression analysis with calculation of the coefficient 'r'.

RESULTS

Immune Complexes and Complement Studies

These data are shown in Figure 1 and Tables 1 and 2. IC were detected in 20 out of 22 patients with RA and infection, the patient with essential cryoglobulinemia and one of the control subjects. Both patients with other chronic diseases were negative. The mean serum C4 concentration was normal in both patient groups compared to controls and serum C3 was also normal in the RA group but significantly elevated in the patients with infection ($p < 0.01$). The total hemolytic complement activity (mean ± SD) was normal for controls and both patient groups (values were expressed as % pooled normal serum).

One or more C4 null alleles were detected in four control subjects, six patients with RA and two with infection (see Table 1). (The patient with ankylosing spondylitis was C4A3,BO.)

C4 Metabolic Data

These results are shown in Figures 2 and 3 and Table 1. The t½ in control subjects was 54 ± 7 h (mean ± SD) and the FCR was 1.80 ± 0.15 %/h. Day to day analysis of urine and plasma radioactivity showed a steady rate of fractional catabolism during the turnover period. Most subjects showed a finite constant for EV catabolism, when calculated by the integrated rate equations method. Serial measurement of the hemolytic function of purified C4 showed a decline after three to four weeks in storage and preparations were discarded once this activity fell below 75% of that observed in a similar concentration of fresh normal serum.

Both patient groups showed significant C4 hypercatabolism compared to controls ($p < 0.001$ for each

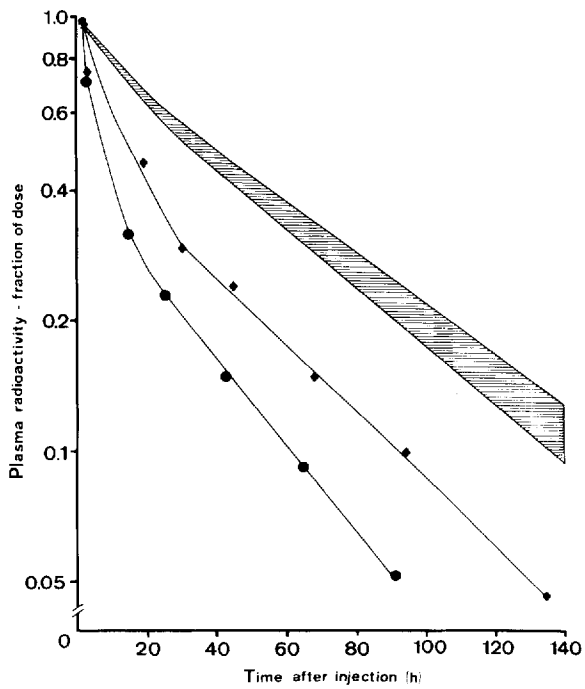

Figure 2: Plasma radioactivity disappearance curves for ^{125}I.C4 in control subjects (shaded) and a patient with infectious mononucleosis (●) and essential cryoglobulinemia (◆). Both patients showed an accelerated, linear pattern of disappearance which reflected fractional hypercatabolism (3.69 and 3.6%/h respectively) and an increase in EV/IV distribution of injected isotope.

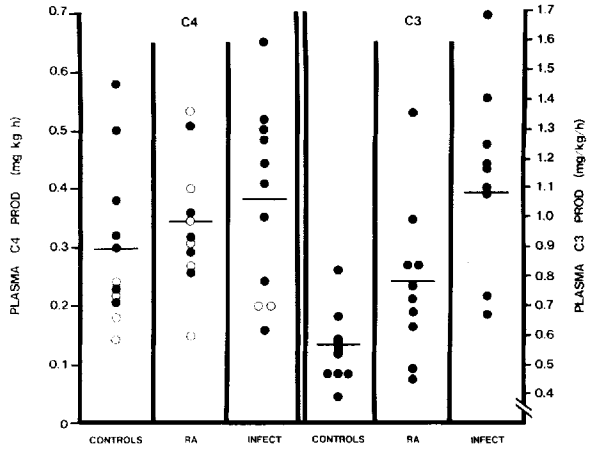

Figure 3: Plasma C4 and C3 production in control subjects and patients with RA and infection. Means are shown. (○) — C4 null alleles. There was significant elevation of C3 production in patients with RA ($p<0.01$) and infection ($p<0.001$).

group) (see Table 1) and the pattern of disappearance of plasma radioactivity was linear for both controls and patients (see Fig. 2). There was no significant difference in FCR between the groups with RA and infection. This parameter was normal in the patients with scleritis and ankylosing spondylitis (1.47 and 1.87 %/h respectively) but grossly elevated in the patient with essential cryoglobulinemia (3.6 %/h). The patients with infection showed significant elevation in the EV/IV distribution ratio ($p<0.01$), that is a greater fraction of injected radio-labelled protein resided outside the vascular compartment compared to normal subjects (see Table 1).

There was no significant difference in the rate of plasma production of C4 between either study group and controls, although mean levels were higher in patients with RA and infection. C4 production rates were comparable in RA patients, with (six patients) and without (five patients) detectable null alleles, that is 0.33 and 0.35 mg/kg/h respectively. However, there was a highly significant difference between the mean C4 levels in all subjects with null alleles (irrespective of group) and those without: 0.25 and 0.37 g/l ($p<0.001$).

C3 Metabolic Data

These results are shown in Table 2 and Figure 3. There was significant hypercatabolism in both study groups compared to controls and this was associated with a significant increase in plasma production of C3 (see Table 2). Patients with infection showed an increased EV/IV ratio. Both patients and controls showed evidence of EV catabolism.

Interrelationships Between C3 and C4 Metabolic Parameters

The data show that plasma production rate was the major determinant of the serum C4 concentration. Highly significant correlations were observed between these two parameters in controls, RA and infection: $p<0.001$; $p<0.01$ and $p<0.001$ respectively. There were also significant relationships between C3 production and serum C3: $p<0.01$; $p<0.05$ and $p<0.01$. No correlations were observed between serum concentration and FCR (that is, $p>0.05$). There was a significant correlation between C4-FCR and plasma production rate ($p<0.05$ for controls and each patient group) and between C3-FCR and production rate for the patients with RA ($p<0.01$) and infection ($p<0.05$).

DISCUSSION

This study shows that IC formation is associated with on-going complement activation, irrespective of co-existing tissue damage or detectable abnormalities in the serum concentration of complement proteins. Previous studies of complement turnover have concentrated particularly on diseases associated with hypocomplementemia and little is known of the behaviour of individual components where such abnormalities do not occur. Furthermore, other studies of C4 metabolism have been hindered by the presence of denatured material[17,18] which jeopardises the accurate analysis of turnover data. By contrast, our preparations showed no significant loss of hemolytic activity *in vitro* (other publications have reported up to 70% loss) and steady rates of fractional turnover *in vivo* (denatured preparations produce rapid elimination of isotope immediately following injection and excessive release of free iodide over the first 24 h). Harrison and Lachmann[4] have highlighted the technical advances which have led to the preparation of more functionally active human C4.

Assessment of the purified proteins in two patients with non-specific active chronic disease showed that catabolism was normal, while their responsiveness was confirmed by the rapid rate of turnover in a patient with essential cryoglobulinemia and gross hypocomplementemia. As previously,[8,12] we used three forms of turnover analysis to minimise errors inherent in each method. For example, exponential analysis may be inaccurate in the presence of small amounts of denatured protein while the integrated rate equations method permits the exclusion of these early (invalid) data. In contrast, exponential data provides a useful comparison when there is doubt about the validity of urine data which are essential for both other methods of calculation.

The results showed that patients with uncomplicated infection, that is where there was no immune damage detectable by clinical and simple laboratory methods, had rates of C3 and C4 catabolism comparable to those observed in RA. The values for C3-FCR were similar to those published previously in several IC-mediated diseases.[12,19] Both groups of patients had normal or elevated rates of plasma C3 and C4 production, even though six of the RA group had one or more detectable C4 null alleles. (Analysis of combined data, that is from all subjects, showed that C4 production was lower in patients with C4 null alleles.) Furthermore, regression analysis showed that production rate (rather than FCR or EV/IV distribution) was the major determinant of serum concentration. It is probable that acute phase reactivity influenced the production of C3 and C4. Several proteins of the complement system, including these two components behave as acute phase reactants,[20] and this phenomenon occurs in RA and various forms of infection.[21] This proposal is supported by the finding of elevated C3 production in both patient categories. The data do not explain the differences in phlogistic behaviour of IC between patients with RA and infection. However, it is recognised that immunological complications (such as cutaneous vasculitis or glomerulonephritis) in either group are associated with a higher incidence of hypocomplementemia. While there are no substantial data on the metabolic basis for low complement levels in either RA or infection, observations in other hypocomplementemic diseases (such as SLE or primary glomerulonephritis) suggest that this reduction results from changes in protein production, rather than higher rates of fractional catabolism.[12,22]

Although the derivation of rates of plasma production of C4 and C3 do not relate directly to the synthesis of individual proteins, it should be emphasised that extracellular complement behaviour is of relevance to those complement-dependent functions responsible for the processing of antigen-antibody complexes. Components of the alternative[23] and classical pathways[24] of complement are required for the effective dissolution of immune aggregates *in vitro* and it is considered likely that similar mechanisms apply *in vivo*. Our observations, particularly in patients with uncomplicated infection suggest that two aspects of complement kinetics are important to the physiological response to IC formation. Firstly, the accelerated turnover of complement proteins is required to generate those products (for example C3b) essential for the modification (or prevention) of antigen-antibody lattice formation. Secondly, that maintenance or increase in complement synthesis, in the presence of hypercatabolism is necessary to provide adequate plasma concentration of those components relevant to IC dissolution. Similarly, the increased EV distribution of both proteins in patients with infection may have implications for the prevention of IC-mediated tissue damage. ■

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BOOK REVIEW

TUMOUR MARKERS IN CLINICAL PRACTICE CONCEPTS AND APPLICATION. Edited by A. S. Daar. Blackwell Scientific, Oxford, 1987. 521 pp. Recommended retail price: $195 (hardback). ISBN: 0-632-0512-8.

The editor of this book has collated information on two aspects of the study of tumour markers (i) basic concepts, theoretical background and laboratory based experimental work and (ii) clinical applications. The book comprises 16 chapters which cover such topics as 'The malignant phenotype', Epstein-Barr virus transformed lymphoblastoid cell lines, various tumour markers in the management of human cancer and the application of monoclonal antibodies in diagnosis and therapy of tumours. The text in most chapters is lucid and should be easily understood by clinicians and scientists not familiar with the investigation of tumour markers. Although most of the references are no more recent than 1985 the background information was well covered and provides a good basis, for the critical reader, to understand more recent concepts derived from journal articles. The application of monoclonal antibodies to the study of tumour antigens in vitro and in vivo was well covered with a comprehensive chapter on immunohistology and chapters on radio immunoimaging, solid tumours, melanoma, Ca I antigen, toxin conjugates, Hodgkin's disease, anti-idiotypic antibodies for therapy of B cell tumours and bone marrow transplantation. A minor criticism concerns the lack of a more comprehensive review of the role of tumour markers in clinical practice. This is discussed in only one chapter although other chapters covered this aspect in part.

I believe that the editor sought to give a general overview of tumour markers and in terms of the basis concepts he has succeeded. However in the area of clinical applications I was left with the impression that the majority of studies had not progressed significantly from the experimental state.

In general this book is well written, with the contributions from a group of respected scientists and clinicians and succeeds in being informative and placing 'tumour markers in clinical practice' in perspective. ■

P. Thurlow,
Deputy Director of Hematology,
Hematology Dept,
Austin Hospital,
Heidelberg, VIC.