The complement system in Type 1 (insulin-dependent) diabetes

J. A. Charlesworth¹, V. Timmermans¹, J. Golding¹, L. V. Campbell², P. W. Peake¹, B. A. Pussell¹, D. Wakefield¹ and N. Howard¹

¹ Department of Medicine, Prince Henry Hospital and ² Diabetes Centre, St. Vincent's Hospital, Sydney, Australia

Summary. The complement proteins C1q, r, s, C2, C4, C3, factor B, C5, C6, and the inhibitors, C1 inhibitor, factors I and H were measured in 35 patients with recently diagnosed Type 1 (insulin-dependent) diabetes, 76 patients with longer-duration disease (30 with complications) and 43 first-degree healthy relatives. We found that C1q, C4 and C3 were reduced significantly in all groups of patients ($p < 0.001$ for each protein in recent onset and uncomplicated patients; $p < 0.01$, $p < 0.01$ and $p < 0.05$ respectively, for patients with complications) compared to 60 control subjects and that C4 was also reduced in healthy relatives ($p < 0.001$). C4 allotypes were examined in 63 subjects (selected from the patient groups) in order to clarify the role of null alleles in the production of the C4 abnormality. These showed serum C4 to be reduced significantly in 50 patients without null alleles (patient mean 0.24 g/l; control subject mean 0.34 g/l) ($p < 0.0001$), although levels were lowest in the 13 patients with

one or more null alleles (mean 0.19 g/l). Finally, to examine the metabolic basis for the low concentrations of C4 and C3, the turnover of highly-purified, radiolabelled C4 and C3 was measured in seven recently diagnosed patients; four of these had low levels of C4. The data showed that three out of four of these patients had reduced synthesis of C3 and C4 and normal values for fractional catabolic rate. Two patients showed features of C4 hypercatabolism. We conclude that several early complement proteins are reduced in Type 1 diabetes, irrespective of duration or complications. These abnormalities may result from hypercatabolism or, more commonly reduction in protein synthesis. Genetic factors may influence the low serum concentration of C4.

Key words: Type 1 (insulin-dependent) diabetes, complement proteins, C4 allotypes, protein synthesis, hypercatabolism.

Several groups have reported abnormalities of the complement system in patients with Type 1 (insulin-dependent) diabetes. These include a high incidence of uncommon alleles of the components coded for by genes within the major histocompatibility complex, i.e. C2, C4 and B [1, 2] and an increased frequency of null alleles at the C4A locus [3–5]. Vergani and co-workers [6] have shown that approximately 25% of Type 1 patients have low C4 levels and Barnett et al. [7] have reported a correlation between reduced C4 concentration and the presence of microvascular complications.

However, non-genetic factors could contribute to the complement abnormalities observed in Type 1 diabetes. These factors include pathologic activators such as immune complexes which have been detected by many groups, using complement-dependent assays. It has been suggested that immune complex formation could play a role in the pathogenesis of long-term diabetic complications [8, 9] although this proposal remains contentious. Sundsmo and co-workers [10] have detected activation products of C4, C3 and C5 in the

fresh plasma of a high percentage of Type 1 diabetic patients, but they did not attempt to relate this finding to the presence of immune complexes.

The established role of complement in both the enhancement and prevention of immunological inflammation makes it important to clarify the relative contribution of impaired synthesis and increased utilisation of complement proteins in Type 1 diabetes. To resolve this issue, we have examined components of each of the three major complement pathways, i.e., the classical and alternative pathways and the terminal membrane attack sequence. Four groups of subjects were examined: (a) patients with recently diagnosed Type 1 diabetes, (b) healthy first-degree relatives of this recent onset group, (c) patients with diabetes of more than 1-year duration without complications and (d) patients with documented retinopathy and/or nephropathy. The basis for the complement abnormalities was further clarified by C4 allotyping and by performing turnover studies with highly purified preparations of C3 and C4 in selected subjects.

Table 1. Clinical and laboratory data on Type 1 (insulin-dependent) diabetic patients and their relatives

Patients	Number	Sex (male:female)	Age (years)	Duration of diabetes (years)	Insulin dose (U·kg ⁻¹ ·day ⁻¹)	HbA1 (%)	Blood glucose (mmol/l)	Retinopathy background/ prolif.	Nephro- pathy
Recent onset diabetes	35	20:15	16 ± 9	< 0.25	0.75 ± 0.43	11.6 ± 6.4	11.0 ± 2.25	-	-
Diabetes without complications	46	25:21	22 ± 9	10 ± 7	0.75 ± 0.34	12.2 ± 2.5	13.9 ± 3.8	-	-
Diabetes with complications	30	14:16	29 ± 10	18 ± 8	0.80 ± 0.60	12.1 ± 2.2	14.8 ± 5.0	17/9	11
Relatives	43	23:20	21 ± 14	-	-	-	-	-	-

Values given as mean ± SD. Normal range for HbA1 = 6.0–8.5%

Subjects and methods

Patients

One hundred and eleven patients (59 males and 52 females) with Type 1 diabetes were selected for study. Their clinical data are shown in Table 1. Thirty-five recently diagnosed patients were investigated during their initial admission to hospital for stabilisation of blood glucose. Blood samples were taken after at least four days of insulin therapy. Onset of symptoms had occurred within the previous three months. Forty-three healthy first-degree relatives of these subjects were also studied at the time of the *propositus*' admission to hospital. These relatives represented one or more members of 19 families, each containing one patient with recently diagnosed Type 1 diabetes. They consisted of 28 parents and 15 siblings (mean age: 21 years; range: 7–47 years).

Two groups with longer-duration diabetes were also studied: (a) 46 patients with a mean duration of diabetes of 10 years and without clinical or laboratory evidence of complications and (b) 30 patients with a mean duration of 18 years and with confirmed retinopathy and/or nephropathy (see Table 1). The majority of these subjects were attending a diabetes outpatient clinic while the rest were in hospital for stabilisation of their disease. All patients had been examined recently by a consultant ophthalmologist for evidence of retinopathy and had had regular measurements of serum creatinine and 24-h urine protein. Diabetic nephropathy was diagnosed when there was persistent proteinuria of >300 mg/24 h, irrespective of the level of serum creatinine. HbA1 was determined by a chromatographic technique, using a commercial kit (Bio-Rad Laboratories, Richmond, Calif, USA). The normal range was 6–8.5%.

Patients and relatives with a history of recent infection, immunological disease or using pharmacological agents likely to affect immunological function were not considered for the study.

Measurement of complement proteins

The following complement components were measured by radial immuno-diffusion, using mono-specific antisera [11]: (1) classical pathway – C1q, r, s, C4 and C2 (2) alternative pathway – C3 and factor B (3) terminal membrane attack sequence – C5 and C6 (4) inhibitor proteins – C1 inhibitor, factors I and H. Serum for these assays was obtained from a fresh blood sample and stored in liquid nitrogen until tested. The concentration of C1q, C4 and C3 was measured in g/l, while other components and inhibitors were expressed as a percentage of pooled normal human serum (NHS). All values were compared to those quantitated in 60 healthy control subjects. The antisera used for these assays had been shown to produce a single precipitin line against normal serum on double diffusion in agarose.

C4 allotyping

This was performed by the method described by Mauff and colleagues [12]. Briefly, plasma samples (in EDTA) were treated with

Fig. 1. Autoradiograph of polyacrylamide gel electrophoresis in sodium dodecyl sulphate under reducing conditions. Molecular weight markers are shown. The α and β polypeptide chains of ¹³¹I-C3 are shown in column A and the α , β and γ chains of ¹²⁵I-C4 are shown in column B. There are no significant radio-labelled contaminants

neuraminidase prior to electrophoresis in agarose and fixation with polyclonal anti-C4 (Silenus). A haemolytic overlay technique was used to distinguish C4A and C4B loci. Heterozygous null alleles were defined, where necessary by two dimensional electrophoresis using the same anti-C4 for the second stage [13].

Detection of immune complexes

These were detected by the fluid phase C1q binding assay [14]. Samples were taken at the same time as those for complement assays and C4 allotyping.

Studies of the metabolism of purified C4 and C3

Turnover studies were performed with highly-purified functionally-active preparations of C4 and C3 in order to define the metabolic basis for the observed reduction in serum concentration of C4 and C3. Studies were performed in seven recently diagnosed Type 1 dia-

betic patients, four of whom had serum C4 > 1SD below the mean level in normal subjects. These patients were in hospital for stabilisation of blood glucose and the initiation of diabetes education. At least one healthy age-matched control subject was studied with each patient. One subject with active systemic lupus erythematosus (SLE), a high level of immune complexes and demonstrable hypocomplementaemia was used as a positive control for both purified proteins.

C3 and C4 were prepared from fresh plasma (in EDTA) donated by laboratory staff who had been screened for hepatitis B surface antigen and HTLV-III antibody. All donors had the common C4A3,B1 allotype. The methods have been described in detail elsewhere [15]. Briefly, fresh plasma was made to 0.5 mmol/l with phenylmethylsulphonyl fluoride and precipitated with 12% (w/v) polyethylene glycol 4000 at 4°C. The protein pellet was dissolved in buffer (100 mmol/l K₂HPO₄, 150 mmol/l NaCl, 5 mmol/l Na₂ EDTA and 5 mmol/l benzamidine HCL, pH 7.4) and run onto a 2.5 × 5 cm lysine-sepharose 4B column in order to deplete plasminogen. Fractions were pooled and loaded onto a diethylaminoethyl-sephadex-A50 column (5 × 10 cm) with elution of C4 by linear gradient of six column volumes from 0.1 mol/l-0.4 mol/l NaCl. Fractions were tested for C4 haemolytic activity using a standard assay with C4-deficient guinea pig serum [16]. Haemolytically active fractions were pooled and further precipitated with solid polyethylene glycol to a final concentration of 20% (w/v). The pellet was re-dissolved and applied to a sepharose CL 6B column (2.5 × 100 cm). The final C4 pool (average volume 100 ml) was concentrated by dialysis against Aquacide 1A to approximately 40 ml, thus yielding a C4 concentration of 0.5–0.8 mg/ml. Final preparations were tested for purity by Ouchterlony double diffusion against an anti-whole human serum and by polyacrylamide gel electrophoresis in sodium dodecyl sulphate (PAGE-SDS). Each protein showed a single line against anti-whole serum while PAGE-SDS, under reducing conditions showed the three polypeptide chains of C4 and the two chains of C3. Functional activity was tested by standard haemolytic techniques and showed both proteins to have comparable activity (i.e., at least 80%) to that observed in an equivalent concentration of NHS. C4 was labelled with ¹²⁵I, using the lactoperoxidase technique [17] and C3 was labelled with ¹³¹I using a chloramine T method [18]. Further testing of

functional activity showed that iodination had caused no significant loss of haemolytic activity and autoradiography of PAGE-SDS confirmed that there was no significant amount of radio-labelled contaminants or breakdown products (Fig. 1).

Both proteins were sterilised by Millipore filtration (0.22 µmol/l), cultured for aerobic and anaerobic organisms and pyrogen-tested in rabbits. Each patient received approximately 10 µCi of ¹²⁵I.C4 and 2.5 µCi of ¹³¹I.C3 intravenously after blockade of thyroidal uptake by the administration of oral potassium iodide (180 mg/day) for 3 days. The turnover protocol had been approved previously by the hospital Ethics Committee and written informed consent was obtained from each patient. Plasma samples (in EDTA) were taken at 15 min, 6 h, 24 h and twice daily until there was less than 8% residual plasma radioactivity. Twenty-four h samples of urine were collected throughout the study period. Metabolic parameters were calculated from plasma and urine data by (a) metabolic clearance [19], whereby the fractional catabolic rate (FCR) is calculated as the ratio of total urinary radioactivity/24 h to the mean protein-bound plasma radioactivity and then expressed as % plasma pool radioactivity/h and (b) integrated rate equations analysis [20]. This method uses measurements of plasma and urinary radioactivity to derive values for extravascular radioactivity and to solve equations where the slopes represent FCR and extravascular/intravascular distribution. Synthesis rate (mg · kg⁻¹ · h⁻¹) was derived from the formula:

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

Serum C3 and C4 levels were measured at the beginning and end of each turnover to confirm a steady state.

Statistical analysis

The normal range for each complement component was established by the analysis of sera from 60 age-matched healthy control subjects. Differences in serum concentration were analysed by a Student's t-test and by a Wilcoxon Rank Sum Test. This latter test was required for the analysis of C4 data, as C4 concentration in normal subjects may not have a normal distribution.

Fig. 2. Serum C4 concentration (g/l) in control subjects and patients in each study group. Means are shown by horizontal bars. There was significant reduction of C4 in (a) patients with recent onset Type 1 diabetes ($p < 0.001$), (b) patients with ($p < 0.01$) and without ($p < 0.001$) diabetic complications and (c) healthy first-degree relatives of the recent onset group ($p < 0.001$)

Fig. 3. Serum C3 concentration in control subjects and patients in each study group. Means are shown by horizontal bars. There was significant reduction of C3 in (a) patients with recent onset Type 1 diabetes ($p < 0.001$) and (b) patients with ($p < 0.05$) and without ($p < 0.001$) diabetic complications

Table 2. Serum concentration of classical and alternative pathway complement proteins in Type 1 (insulin-dependent) diabetic patients and their relatives

Group	Classical pathway					Alternative pathway	
	C1q (g/l)	C1r (%NHS)	C1s (%NHS)	C4 (g/l)	C2 (%NHS)	C3 (g/l)	B (%NHS)
Control subjects	0.19 ± 0.04	102 ± 17	100 ± 24	0.34 ± 0.08	110 ± 24	0.81 ± 0.14	100 ± 14
Patient with recent onset diabetes	0.14 ± 0.06 ^a	67 ± 20 ^a	86 ± 28	0.23 ± 0.08 ^a	100 ± 30	0.69 ± 0.11 ^a	104 ± 21
Patients with diabetes without complications	0.16 ± 0.04 ^a	65 ± 25 ^a	81 ± 41	0.24 ± 0.07 ^a	105 ± 32	0.72 ± 0.18 ^a	95 ± 29
Patients with diabetes with complications	0.16 ± 0.04 ^b	56 ± 22 ^a	59 ± 26 ^b	0.28 ± 0.09 ^b	111 ± 25	0.73 ± 0.17 ^c	99 ± 33
Relatives	0.18 ± 0.06	–	–	0.27 ± 0.08 ^a	94 ± 17	0.80 ± 0.14	103 ± 19

Values are given as mean ± SD; NHS = pooled normal human serum. ^a $p < 0.001$, ^b $p < 0.01$, ^c $p < 0.05$ when compared to control subjects

Table 3. Serum concentration of complement inhibitors and terminal attack sequence proteins in Type 1 (insulin-dependent) diabetic patients and their relatives

Group	Terminal attack sequence		Inhibitors		
	C5 (%NHS)	C6 (%NHS)	C1-Inhib. (%NHS)	I (%NHS)	H (%NHS)
Control subjects	101 ± 18	103 ± 19	91 ± 14	95 ± 15	93 ± 15
Patients with recent onset diabetes	103 ± 19	109 ± 25	115 ± 31	91 ± 25	83 ± 22
Patients with diabetes without complications	105 ± 38	95 ± 33	122 ± 34	94 ± 31	87 ± 25
Patients with diabetes with complications	120 ± 32	87 ± 38	120 ± 28	102 ± 23	96 ± 23
Relatives	103 ± 26	100 ± 21	106 ± 26	95 ± 20	96 ± 17

Values are given as mean ± SD; NHS = pooled normal human serum

C4 allotype data were divided into two groups: (a) those subjects showing homozygous or heterozygous null at either locus and (b) subjects without detectable null alleles. The difference in concentration of C4 between each group was analysed by a Student's *t*-test and the Wilcoxon test.

Linear regression analysis, with calculation of the coefficient 'r', was used to examine correlations between the level of immune complexes and the concentration of C1q, C4, C2 and C3 and between HbA1 and the serum level of C4 and C3. A *p* value of < 0.05 was considered statistically significant.

Results

Complement proteins

These data are shown in Figures 2 and 3 and Tables 2 and 3. There was a highly significant reduction in serum C1q, C4 and C3 in patients with recently diagnosed Type 1 diabetes compared to control subjects

($p < 0.001$ for each protein – unpaired *t*-test; $p < 0.001$ – Wilcoxon test for C4). These three components were also reduced in both groups of longer-duration patients: uncomplicated – $p < 0.001$ for each protein; complicated – $p < 0.01$; $p < 0.01$ and $p < 0.05$ respectively. The C4 concentration was also reduced in first-degree relatives ($p < 0.001$) but there was no difference between the level in parents and siblings (mean ± SD: 0.26 ± 0.08 and 0.28 ± 0.09 g/l respectively). There were no significant abnormalities of other complement components (i.e., C2, B, C5, C6 and inhibitors).

A comparison of complement levels in each patient group showed C4 to be significantly lower in the recent onset patients than in those with diabetic complications ($p < 0.05$). There was no difference in C4 levels in sub-groups of longer-standing patients (i.e., with and without complications) even when matched for duration of diabetes. In view of the significant reduction in C1q, other components of the C1 macromolecule were also measured; these data are shown in Table 2. C1r was significantly reduced in each patient group ($p < 0.001$) while C1s was low only in the patients with complications ($p < 0.01$). No correlation was observed between HbA1 and the serum concentration of C3 and C4.

Detection of immune complexes

Three of the recently diagnosed patients and eight of those with longer-duration disease had detectable immune complexes. However, there were no differences between the level in 60 control subjects and that observed in each study group. There was also no correlation between the concentration of C1q, C4, C2 or C3 and the level of immune complexes.

C4 allotypes

The analysis of C4A and C4B alleles in 63 patients (selected from each of the study groups) showed 13 (i.e., 21%) to have one or more null alleles. Ten involved the

A locus (five with a single null and five with homozygous C4A0) and three involved the B locus (one with homozygous C4B0). A comparison of C4 levels in con-

Fig. 4. Serum C4 concentration in (a) control subjects, (b) patients with Type 1 diabetes without null alleles and (c) patients with one or more null alleles. Means are shown by horizontal bars. There was a highly significant difference between control subjects (0.34 ± 0.08 g/l; mean $\pm$ SD) and each patient group (0.24 ± 0.09 and 0.19 ± 0.08 g/l, respectively), ($p < 0.0001$). There was also a significant difference between patients with and without null alleles ($p < 0.05$)

trol subjects, patients without detectable null alleles and those with one or more null alleles are shown in Figure 4. There was a highly significant difference between the concentration in controls (0.34 ± 0.08 g/l; mean $\pm$ SD) and that in patients without null alleles (0.24 ± 0.09 g/l; $t = 6.33$; $p < 0.0001$). There was also a difference between patients with (0.19 ± 0.08 g/l) and without null alleles ($p < 0.05$).

Studies of C4 and C3 metabolism

These data are summarised in Tables 4 and 5. Patients 1 and 7, with normal serum C4 and C3, had normal values for FCR and synthesis of both proteins. Three of the other five patients had normal FCR-C4, while patients 4 (Fig. 5) and 6 had reduced $T_{1/2}$ -C4 (38 and 44 h) and increased FCR-C4 (2.30 and 2.54%/h) respectively. C4 synthesis was normal in these two patients but reduced in the other three (i.e., patients 2, 3 and 5) with low/normal levels of serum C4; each of these latter three patients had a common allotype, without detectable null alleles. No significant abnormalities of C4 distribution were observed.

Values for FCR-C3 were normal in six out of seven patients; patient 6 had an FCR of 2.25%/h. Two other subjects had reduced C3 synthesis. Notably, the three patients with low C4 synthesis also had the lowest levels of C3 production. (There was close correlation between the two methods of calculation of metabolic parameters.)

Table 4. Half life, fractional catabolic rate and synthesis rate for 125 I-C4 in recent onset Type 1 diabetic patients and control subjects

Patient (sex)	Duration of diabetes (months)	HbA1c (%)	Blood ^a glucose (mmol/l)	C4 allotype	Serum C4 (g/l)	$T_{1/2}$ (h) ^b	FCR ^c (%/h)	Synthesis (mg $\cdot$ kg ⁻¹ $\cdot$ h ⁻¹)
1 (F)	< 1	10.3	5.9 ± 2.3	A4,B2	0.34	50	1.78	0.27
2 (M)	6	8.8	6.8 ± 2.0	A3,B1	0.18	47	1.54	0.11
3 (M)	2	9.7	6.9 ± 3.8	A3,B1	0.22	50	1.56	0.14
4 (F)	1	10.5	10.1 ± 4.6	A3,0,B1	0.24	38	2.30	0.23
5 (M)	3	10.5	7.9 ± 2.3	A3,B2	0.21	58	1.55	0.13
6 (F)	10	9.8	6.2 ± 1.7	A4,B2	0.34	44	2.54	0.37
7 (F)	2½	13.8	7.1 ± 2.6	A3,0,B1	0.32	60	1.75	0.24
Control subjects <i>n</i> = 11					0.34 ± 0.16^d	52 ± 8	1.86 ± 0.32	0.26 ± 0.12

^a mean $\pm$ SD of twice daily measurements performed during metabolic study; ^b half life; ^c fractional catabolic rate; ^d mean $\pm$ 2SD

Table 5. Half life, fractional catabolic rate and synthesis rate for 131 I-C3 in recent onset Type 1 diabetic patients and control subjects

Patient	Duration of diabetes (months)	Serum C3 (g/l)	$T_{1/2}$ (h) ^a	FCR ^b (%/h)	Synthesis (mg $\cdot$ kg ⁻¹ $\cdot$ h ⁻¹)
1	< 1	0.76	51	1.95	0.67
2	6	0.85	56	0.98	0.35
3	2	0.53	59	1.20	0.27
4	1	0.76	44	1.45	0.47
5	3	0.70	52	0.88	0.25
6	10	0.80	49	2.25	0.78
7	2.5	1.51	59	1.71	1.14
Control subjects		0.81 ± 0.28^c	57 ± 10	1.60 ± 0.30	0.53 ± 0.21

^a Half life; ^b fractional catabolic rate; ^c mean $\pm$ 2SD

Fig. 5. Plasma disappearance curves for ^{125}I -C4 in 11 control subjects (shaded), a patient with recent onset Type 1 diabetes (●) (patient 4, Table 4) and a patient with active systemic lupus erythematosus (○). The normal half-life was 52 ± 8 h (mean $\pm$ 2SD) with a fractional catabolic rate of $1.86 \pm 0.32\%/h$. The diabetic patient had a half-life of 38 h and a fractional catabolic rate of $2.30\%/h$ while the patient with systemic lupus erythematosus had a half-life of 30 h and a fractional catabolic rate of $3.20\%/h$

The patient with active SLE showed decreased $T_{1/2}$ for both C4 and C3 (30 and 36 h) with increased values for FCR (3.20 and $3.42\%/h$ respectively). The C4 plasma disappearance curve for this patient is shown in Figure 5.

Discussion

Our data demonstrate abnormalities of three early components of the complement system in patients with Type 1 diabetes. These changes occurred in both recently diagnosed patients and those with long-duration disease, irrespective of complications. Low levels of C4 in Type 1 diabetes have been reported previously [3, 6, 7] but we also found a highly significant reduction in serum C1q and C3. While there have been no previous reports of measurements of C1q in this disease, Vergani and co-workers [6] found that the frequency of low C3 levels in Type 1 diabetic patients was comparable to that in Type 2 (non-insulin-dependent) diabetic patients. However, the actual quantitation of C3 levels and their comparison to a control group was not reported.

This pattern of complement abnormality usually reflects activation of the classical pathway, either by immune complexes or IgG or IgM class of antibody. It is established that a high percentage of patients with diabetes, irrespective of type, have detectable immune complexes [8, 21] although our data show no correlation between their level – as measured by a C1q-de-

pendent assay – and the concentration of individual complement components. Nevertheless, several observations suggest that immune complexes may have contributed to the observed complement abnormalities: firstly, individual immune complex assays may fail to detect specific antigen/antibody systems; secondly, two of our (recently diagnosed) patients showed C4 hypercatabolism which is consistent with immune complex-mediated complement activation; and thirdly, we have observed a significant rise in C4 concentration in some patients when studied 3–6 months after diagnosis (unpublished data). The finding of normal concentrations of C1q, C4 and C3 in Type 2 diabetes [21] argues against the possibility that poor glucose control at the time of diagnosis contributed to the reduction in serum complement levels. Moreover, it is not surprising to find normal values for several complement components despite significant reduction in C1q, r, C4 and C3. This difference may reflect the variable metabolic response of individual complement proteins. For example, Sissons and co-workers [22] have shown that C5 exhibits significantly less hypercatabolism than C3 in patients with immune complex disease and hypocomplementaemia.

We found that C4 levels were also reduced in healthy relatives which supports the view that inherited factors contribute, at least in part, to the C4 abnormality. Our data confirm that C4 was lower in patients with detectable null alleles ($p < 0.05$) (see Fig. 4), and Uko and co-workers [23] have quantitated the influence of these defects on C4 concentration. However, we also found a significant difference between control subjects and those Type 1 patients where C4 null alleles were not found. This was despite the fact that our 60 control subjects were selected without regard for C4 allotype.

Our turnover studies suggest that reduction in protein synthesis was a major influence on the level of C3 and C4. This is in contrast to other immune complex-associated diseases, such as systemic lupus erythematosus (SLE) [24] and rheumatoid arthritis [25] where hypercatabolism and compartmental redistribution contribute significantly to the low complement levels. Previous reports of C4 metabolism show metabolic parameters similar to ours; however, those studies were hindered by reduction in functional activity of the purified protein and subsequent evidence of denaturation on turnover analysis [25, 26]. In our study, five out of seven patients had a normal half-life and fractional catabolic rate for C4 while these parameters were normal for C3 in six patients. Three out of the four subjects with a C4 level $> 1\text{SD}$ below the normal mean showed reduced synthesis of this component and these three patients also had the lowest values for C3 synthesis. Notably, each had a common C4 allotype (see Table 4). The two patients with C4 hypercatabolism (one with C4A3,0) showed linear disappearance of plasma radioactivity beyond 90% of the injected dose of radiolabelled protein. This argues against the possi-

bility that significant amounts of radiolabelled C4 breakdown products were generated during the course of the turnover study, and the simultaneous examination of a normal control emphasised the significance of the metabolic abnormalities observed in these two patients. Furthermore, there were no detectable radiolabelled breakdown proteins prior to injection (see Fig. 1). Previous studies (with C3 and B) demonstrate that patients with high levels of activation products show a non-linear plasma disappearance curve [24].

The basis for the defect in synthesis of C4 and C3 is uncertain, apart from the demonstrable influence of C4 null alleles on the concentration of the fourth component. Failure of insulin synthesis might influence hepatic protein production and therefore affect the level of those complement proteins produced by the liver. However, two observations make this unlikely: firstly, the selective involvement of C4 and C3, without reduction in other components such as B, C5 and C6 (all of which are synthesised by hepatocytes) and secondly, the persistence of the defect in long-standing disease.

The biosynthesis and intracellular processing of C3 and C4 are similar and the plasma products share several structural and functional properties. It is known that pro-C4 undergoes several modifications prior to secretion and that these include glycosylation, which has been shown to alter intracellular catabolism [27]. Several pieces of evidence suggest that genes other than those residing in the major histocompatibility complex may contribute to the control of C4 production. For example, it has been shown that additional genetic factors are responsible for the post-translational modification of pro-C4 [28]. Also, Muir and co-workers [29] have reported incomplete C4 deficiency in a patient with SLE and six healthy family members; the defect showed no linkage to HLA, Bf or C4 structural loci. (The proband's allotype was C4A3,0;B1,2 despite a C4 concentration of 2–5% normal). Finally, a high percentage of patients with juvenile chronic active hepatitis and their relatives have been reported to have low C4 levels [30]; these patients showed no evidence of complement activation nor defects in hepatic synthesis of albumin and transferrin. Despite a high incidence of C4 null alleles, it was suggested that the low C4 levels resulted partially from non-HLA genetic influences. Our data do not clarify the basis for the reduction in components of the C1 macromolecule. In contrast to C3 and C4, the site(s) of production of these three subunits are less well-understood. It seems probable that the bulk of plasma C1q, r, s, arises from gastrointestinal and genitourinary epithelial cells. It is notable that each of the subunits was reduced (to a variable extent) in patients with Type 1 diabetes. This is in contrast to patients with immune complex-induced complement activation where levels of C1s and C1r are usually normal despite reduction (through hypercatabolism) in C1q. The restriction of the abnormality to the patient groups suggests that the diabetic

state per se is responsible for the defect(s) in C1 (and C3). However, the finding of normal complement levels in Type 2 diabetes makes it unlikely that the metabolic disturbance is primarily responsible for the changes in complement proteins.

We did not establish whether the observed complement abnormalities caused alteration in complement function in vitro. Recent studies show that allotypic differences in C4 may affect protective complement mechanisms, such as the inhibition of immune precipitation. Schifferli and co-workers [31] showed that proteins of the C4A gene were 1.7 times more efficient in this regard than C4B products. (This is in contrast to studies of red cell lysis where C4B-derived products show greater activity.) They suggested that C4A proteins may be involved predominately in immune clearance reactions, and this proposal could be important in Type 1 diabetes where there is an increased incidence of C4A null. Several other publications show that relatively subtle defects of C4A and/or C4B (i.e., 1, 2 or 3 nulls) may be associated with an increased incidence of auto-immune disease [29, 30, 32]. This concomitant defect in C1 and C3 in our patients could further influence functions mediated by these early complement components. For example, the subunits of C1 are essential for binding of immune aggregates (i.e., C1q) and subsequent initiation of classical pathway activation (i.e., C1r, s), while C3 is required for assembly of the alternative pathway converting enzyme, C3b, Bb and its involvement in solubilisation of antigen/antibody complexes.

In conclusion, our data show significant reduction in the early complement components C1, C4 and C3 in patients with Type 1 (insulin-dependent) diabetes, irrespective of duration. The abnormalities of C1 and C3 were confined to Type 1 patients while low C4 levels were also seen in healthy first-degree relatives. However, significant C4 reduction occurred independently of null alleles at C4 genetic loci. Metabolic studies showed that the low levels of C4 and C3 resulted from reduction in synthesis in three patients, while two showed hypercatabolism. The data suggest that the diabetic state per se is responsible for reduced production of C1 subunits, C3 and possibly C4.

Acknowledgements. This study was supported by research grants from the National Health and Medical Research Council of Australia and the Rebecca L. Cooper Research Foundation. We wish to thank Professor G. Antony and Drs. W. Kidson and S. Colagiuri for allowing us to study their patients.

References

1. Raum D, Stein R, Alper CA, Gabbay KH (1979) Genetic marker for insulin-dependent diabetes mellitus. *Lancet* 1: 1208–1210
2. Wolf E, Cudworth AG, Marwick JR, Wells L, Bodansky HJ, Gorsuch AN, Spencer KM, Lister J (1981) Bf and C2 complement factors in Type 1 diabetes. *Diabetologia* 20: 667 (Abstract)

3. Dawkins RL, Uko G, Christiansen FT, Kay PH (1983) Low C4 concentrations in insulin dependent diabetes mellitus. *Br Med J* 287: 839
4. McCluskey J, McCann VJ, Kay PH, Zilko PJ, Christiansen FT, O'Neill GJ, Dawkins RL (1983) HLA and complement allotypes in Type 1 (insulin-dependent) diabetes. *Diabetologia* 24: 162-165
5. Mijovic C, Fletcher J, Bradwell AR, Harvey T, Barnett AH (1985) Relation of gene expression (allotype) of the fourth component of complement to insulin dependent diabetes and its microangiopathic complications. *Br Med J* 291: 9-10
6. Vergani D, Johnston C, B-Abdullah N, Barnett AH (1983) Low serum C4 concentrations: an inherited predisposition to insulin dependent diabetes? *Br Med J* 286: 926-928
7. Barnett AH, Mijovic C, Fletcher J, Chesner I, Kulkuska-Langlands BM, Holder R, Bradwell AR (1984) Low plasma C4 concentrations: association with microangiopathy in insulin dependent diabetes. *Br Med J* 289: 943-945
8. DiMario U, Iavicoli M, Andreani D (1980) Circulating immune complexes in diabetes. *Diabetologia* 19: 89-92
9. Brownlee M, Pongor S, Cerami A (1983) Covalent attachment of soluble proteins by nonenzymatically glycosylated collagen. Role in the in situ formation of immune complexes. *J Exp Med* 158: 1739-1744
10. Sundsmo JS, Papin RA, Wood L, Hirani S, Waldeck N, Buckingham B, Kershner A, Ascher M, Charles MA (1985) Complement activation in Type 1 human diabetes. *Clin Immunol Immunopathol* 35: 211-225
11. Mancini G, Carbonara AL, Heremans JF (1965) Immunochemical quantitation of antigens by single radial immunodiffusion. *Immunochemistry* 2: 235-254
12. Mauff G, Alper CA, Awdeh Z, Batchelor JR, Bertrams J, Bruun-Petersen G, Dawkins RL, Demant P, Edwards J, Grosse-Wilde H, Hauptmann G, Klouda P, Lamm L, Mollenhauer E, Nerl C, Olaisen B, O'Neill G, Rittner C, Roos MH, Skanes V, Teisberg P, Wells L (1983) Statement on the nomenclature of human C4 allotypes. *Immunobiology* 164: 184-191
13. Awdeh ZL, Raum D, Alper CA (1979) Genetic polymorphism of human complement C4 and detection of heterozygotes. *Nature* 282: 205-207
14. Zubler RH, Lange G, Lambert PH, Miescher PA (1976) Detection of immune complexes in unheated sera by a modified ¹²⁵I.C1q binding test. *J Immunol* 116: 232-235
15. Whaley K (1985) Purification of complement components and preparation of antisera. In: Whaley K (ed) *Methods in complement for clinical immunologists*. Churchill Livingstone, London, pp 21-76
16. Gaither TA, Alling DW, Frank MM (1974) A new one-step method for the functional assay of the fourth component (C4) of human and guinea pig complement. *J Immunol* 113: 574-583
17. Marchalonis JJ (1969) An enzymic method for trace iodination of immunoglobulins and other proteins. *Biochem J* 113: 299-305
18. McConeahy PJ, Dixon FJ (1966) A method of trace iodination of proteins for immunologic studies. *Int Arch Allergy Appl Immunol* 29: 185-189
19. Berson SA, Yalow RS (1957) Distribution and metabolism of ¹³¹I-labelled proteins in man. *Fed Proc* 16: 13s-18s
20. Nosslin B (1972) Analysis of disappearance time curves after single injection of labelled proteins. In: *Protein Turnover Ciba Foundation Symposium 9*. Elsevier/North Holland, Amsterdam, pp 113-120
21. Charlesworth JA, Campbell LV, Catanzaro R, Pussell BA, Pasterfield GV, Peake P (1982) Immune complexes in diabetes mellitus: studies of complement utilisation and tissue deposition. *J Clin Lab Immunol* 8: 163-168
22. Sissons JGP, Leibowitch J, Amos N, Peters DK (1977) Metabolism of the fifth component of complement, and its relation to metabolism of the third component, in patients with complement activation. *J Clin Invest* 59: 704-715
23. Uko G, Christiansen FT, Dawkins RL, McCann VJ (1986) Reference ranges for serum C4 concentrations in subjects with and without C4 null alleles. *J Clin Pathol* 39: 573-576
24. Charlesworth JA, Gwyn Williams D, Sherington E, Lachmann JP, Peters DK (1974) Metabolic studies of the third component of complement and the glycine-rich beta glycoprotein in patients with hypocomplementemia. *J Clin Invest* 53: 1578-1587
25. Kaplan RA, Curd JG, Deheer DH, Carson DA, Pangburn MK, Muller-Eberhard JH, Vaughan JH (1980) Metabolism of C4 and Factor B in rheumatoid arthritis - Relation to rheumatoid factor. *Arthritis Rheum* 23: 911-920
26. Ruddy S, Carpenter CB, Chin KW, Knostman JN, Soter NA, Gotze O, Muller-Eberhard HJ, Austen KF (1975) Human complement metabolism: An analysis of 144 studies. *Medicine (Baltimore)* 54: 165-178
27. Matthews Jr WJ, Goldberger G, Marino Jr JT, Einstein LP, Gash DJ, Colten HR (1982) Complement proteins C2, C4 and factor B - Effect of glycosylation on their secretion and catabolism. *Biochem J* 204: 839-846
28. Karp DR, Atkinson JP, Shreffler DC (1982) Genetic variation in glycosylation of the fourth component of murine complement. Association with haemolytic activity. *J Biol Chem* 257: 7330-7335
29. Muir WA, Hedrick S, Alper CA, Ratnoff OD, Schacter B, Wisniewski JJ (1984) Inherited incomplete deficiency of the fourth component of complement (C4) determined by a gene not linked to human histocompatibility leukocyte antigens. *J Clin Invest* 74: 1509-1514
30. Vergani D, Wells L, Larcher VF, Nasaruddin BA, Davies ET, Mieli-Vergani G, Mowat AP (1985) Genetically determined low C4: a predisposing factor to autoimmune chronic active hepatitis. *Lancet* 2: 294-298
31. Schifferli JA, Steiger G, Paccaud J-P, Sjöholm AG, Hauptmann G (1986) Difference in the biological properties of the two forms of the fourth component of human complement (C4). *Clin Exp Immunol* 63: 473-477
32. Rynes RI (1982) Inherited complement deficiency status and SLE. *Clin Rheum Dis* 8: 29-47

Received: 7 October 1986
and in revised form: 6 April 1987

Dr. J.A. Charlesworth
Renal Unit
Prince Henry Hospital
Little Bay, N.S.W. 2036
Australia