Serum C_3 and C_4 levels and complement-dependent antibody-mediated cytotoxic activity of islet cell surface antibody in Type 1 (insulin-dependent) diabetic children

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Summary. The role of complement in the pathogenesis of diabetes was studied in 31 Type 1 (insulin-dependent) diabetic children by assaying serum islet cell surface antibody, C_3, C_4 and serum complement-dependent antibody-mediated cytotoxicity. Nine of 21 islet cell surface antibody-positive children were within 5 months of disease onset and showed significantly lower serum C_3 and C_4 levels than either 1 year later or the remainder of the islet cell surface antibody-positive children at 6–12 months after disease onset. The overall trend of all islet cell surface antibody-positive diabetic children within 1 year of disease onset was toward increased serum C_3 and C_4 levels as the disease progressed. Serum C_4 concentration and complement-dependent antibody-mediated cytotoxicity which showed an initial negative correlation were uncorrelated 1 year later. Four children who were ini-

tially strongly islet cell surface antibody-positive but negative 1 year later also exhibited significantly higher ($p < 0.05$) mean serum C_4 levels after 1 year. There was a significant decrease in complement-dependent antibody-mediated cytotoxicity when sera from the diabetic children were treated with either ethylene glycol tetra-acetic acid or ethylene diamine tetra-acetic acid. These data strongly suggest that complement-dependent antibody-mediated cytotoxicity induced by the classical complement pathway involving an islet cell surface antibody may play an important role in the pathogenesis of Type 1 diabetes.

Key words: Human, insulin-dependent, diabetes mellitus, complement, antibody-dependent cell cytotoxicity, autoantibodies, antigens, surface, Islands of Langerhans.

Complement is required for the final phase of the humoral immune process that leads to pancreatic islet cell damage. Islet cell surface antibodies (ICSA) which are demonstrable in the circulating blood of Type 1 (insulin-dependent) diabetic patients shortly after disease onset have been shown to cause complement-dependent destruction of rat islet cells in vitro [1]. The experimental evidence that complement-dependent antibody-mediated cytotoxicity (CAMC) involving ICSA may lead to islet cell damage points to the possibility that a similar sequence of events may lead to the destruction of pancreatic B cells in vivo.

If these phenomena occur in Type 1 diabetes immediately after disease onset, changes in serum C_3 and C_4 levels or a negative correlation between CAMC activity and complement component levels should be found. This premise relies on the concept that at the onset of Type 1 diabetes mellitus, the classical complement pathway may be activated *via* the response of complement-fixing antibodies (whose titres are proportional to CAMC activity) to islet cell antigens. This activation may produce low serum C_4 levels; the resulting negative correlation between C_4 and CAMC

activity would become increasingly less distinct as the disease progresses.

This report describes studies of the role of complement components in a group of Type 1 diabetic children shortly after disease onset using serum ICSA, C_3 and C_4 assays and determinations of serum CAMC activity using rat pancreatic islet cells as target cells.

Subjects, materials and methods

Subjects

Clinical and immunochemical analyses

Fasting blood samples were collected using routine methods; the sera, separated and stored at −20 °C. All sera were assayed within

2 months of collection and heat-inactivated at 56 °C for 30 min prior to assay.

Haemoglobin A_1 assay

The percentage of Haemoglobin A_1 was determined by HPLC using a Fully Automated Haemoglobin A_{1c} Analyser (Kyoto Daiichi Kagaku, Kyoto, Japan).

ICSA assay

ICSA was assayed by indirect immunofluorescence using rat pancreatic islet cells (see below) according to the method of Lernmark [2]. Briefly, serial dilutions of sera $(1:4^n)$ were incubated with islet cells at 20 °C for 30 min in 100 μl of Medium 199 (M 199; Gibco, Grand Island, NY, USA), diluted with 12 ml M 199, concentrated by centrifugation (50 g, 5 min), incubated for 30 min at 20 °C with fluorescein-isothiocyanate (FITC)-conjugated rabbit anti-human-IgG antibody (Hoechst, Frankfurt, FRG), diluted 1:10 with M 199, and concentrated by centrifugation (50 g, 5 min) to a final volume of 100 μl. The cell suspensions were transferred to a Cunningumm chamber and their fluorescence evaluated at a magnification of 200× with an Olympus microscope equipped with an epifluorescence illuminator. A cell suspension was classified as 'ICSA-positive' if more than 30% of 100 randomly selected islet cells displayed a ring of fluorescence when exposed to serum at a 1:1 or greater dilution.

C_3 and C_4 assays

Serum C_3 and C_4 levels were determined exactly 2 weeks after blood samples were taken using Norpartigen antisera and the Partigen single radial immunodiffusion method (Hoechst, Frankfurt, FRG). The Norpartigen contained either only anti-C_{3c} antibody to antigen A which is common to β_{1C} and β_{1A} [3] or only C_4 antibody. In addition, only the C_{3c} fragment was used for the production of Norpartigen C_{3c} antiserum; the C_3 component used in these products was 100%-fragmented to C_{3c} for calibration of standard and control preparations. All of the C_4 Norpartigens were prepared using antisera produced by immunising several animal species with fragment C_4 (R. C. Schneider, Behringwerke, Marburg, FRG; personal communication).

Norpartigen antiserum and 5 μl of test serum were carefully placed in adjacent wells in the agar plate. The plate was covered and placed on a level surface for 48 h at room temperature. The diameters of precipitation rings formed in the agar were measured to 0.1 mm with a Partigen gauge and converted to serum C_3 and C_4 concentrations using a Partigen conversion table.

CAMC assay

Islet cells (see below) were incubated for 60 min at 37 °C in 1 ml M 199 supplemented with 124 μmol/l $Na_2{}^{51}CrO_4$ (New England Nuclear, Boston, Mass, USA), diluted with 10 ml M 199, centrifuged for 5 min (50 g), and resuspended at a concentration of 2×10^4 cells/ 50 μl in M 199 containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mmol/l L-glutamine (Gibco).

Islet cells were placed in 96-well, flat-bottom culture plates (Costar, Cambridge, Mass, USA) and 5 μl of ICSA-positive or -negative test serum was added to each well. The cells were incubated in 5% CO_2:95% air at 37 °C for 30 min. Subsequently, 50 μl of a 7.5% human AB serum solution was added to each well, and the cells reincubated for 2 h as described above. A 50 μl portion of the culture supernatant was assayed for radioactivity. Percent cytotoxicity was calculated as:

$$\frac{\text{experimental } {}^{51}\text{Cr-release} - \text{spontaneous } {}^{51}\text{Cr-release}}{\text{maximal } {}^{51}\text{Cr-release} - \text{spontaneous } {}^{51}\text{Cr-release}} \times 100$$

In vitro cultivation of rat pancreatic islet cells

Islets of Langerhans were isolated by collagenase digestion [4] from the pancreas of 5- to 7-week-old, 150–200, male Wistar rats. The isolated islets were preincubated for 60 min at 37 °C in a Ca^{2+}-free Krebs-Ringer bicarbonate (KRB) medium containing 16.7 mmol/l glucose, 0.5% bovine serum albumin (BSA fraction V; Sigma Chemical Co., St. Louis, Mo, USA), and 3 mmol/l ethylene glycol tetraacetic acid (EGTA) (Fluka AG, Buchs, Switzerland) [5], transferred to a Ca^{2+}- and EGTA-free KRB medium containing 16.7 mmol/l glucose, 0.1% BSA and 0.1% trypsin (1:250, Difco, Detroit, Mich, USA) and disrupted at room temperature with 10 aspirations each through 3 pipettes of decreasing tip diameter.

The islet cell suspension was diluted with 10 ml of trypsin-containing medium and incubated at 37 °C in a spinner flask. The suspension was sampled every 2–3 min and examined by phase contrast microscopy to determine the status of islet cell dissociation. Trypsin digestion was stopped after 5–6 min by adding a large quantity of cold (4 °C) sterile M 199 containing 16.7 mmol/l glucose, 10% heat-inactivated fetal bovine serum, 400 U/ml penicillin and 200 μg/ml streptomycin (standard culture medium). The mono-disperse cell suspension was centrifuged at 700 g at room temperature for 8 min, and the resulting cell pellet was resuspended in 10 ml of standard culture medium containing 2 mg/ml BSA.

Statistical analysis

All results are expressed as mean ± SEM. Serum complement levels and CAMC activity were tested for statistically significant differences using Student's paired and unpaired t-tests. Correlation coefficients (r) between serum C_3 or C_4 and CAMC activity were determined by Spearman's coefficient of rank correlation method with differences considered significant at the $p < 0.01$ level.

Results

Haemoglobin A_1

The normal range for Haemoglobin A_1 as determined by HPLC is 5.5–8.0%. However, of the 31 diabetic children assayed, only 6.53% of their haemoglobin A_1 values fell within the normal range. In addition, the population mean of the diabetic children (11.49 ± 0.4% was significantly different from the normal population mean $(p = 4.64 \times 10^{-2})$.

ICSA and its relationship to C_3 and C_4 activity

A mean of 5.97% of islet cells exposed to normal control serum were FITC-positive. In assays of Type 1 diabetic sera, 50% of the sera which resulted in less than 29% of the cells being FITC-positive had a CAMC activity of 10% or greater whereas the other 50% of the sera had CAMC values of 10% or less. Consequently, the percentage of CAMC-positive cells was not a reliable indicator of ICSA activity. However, 99.06% of Type 1 diabetic sera resulting in 30% or greater of the islet cells being FITC-positive had an ICSA activity of greater than 10%. Therefore, it was possible to discriminate between ICSA-positive and ICSA-negative children using this criterion. The percentages of false negative and false positive ICSA readings were 19.23

Table 1. Summary of islet cell surface antibody (ICSA), C_3 and C_4 assay results in children with Type 1 (insulin-dependent) diabetes mellitus

Time after onset of diabetes (months)	n	ICSA	Serum C_3 (mean ± SEM) (mg/dl)	Serum C_4 (mean ± SEM) (mg/dl)
Non-diabetic children	10	−	83.50 ± 8.0	33.50 ± 2.2
0–12	10	−	77.50 ± 4.5	33.50 ± 3.2
0–5[a]	9	+	66.78 ± 4.3	19.44 ± 1.8
6–12	12	+	84.08 ± 5.5	28.75 ± 2.8
0–12[b]	21	+	77.67 ± 4.1	24.76 ± 2.0
12–17[a]	9	+	76.67 ± 3.4	25.44 ± 2.4
12–24[b]	21	+	80.57 ± 3.3	27.19 ± 2.0

[a] Subset of ICSA-positive Type 1 diabetic children at 0–5 months after onset and 1 year later. [b] ICSA-positive Type 1 diabetic children at 0–12 months after onset and 1 year later

and 3.85, respectively, with low inter-examiner and inter-assay variability (data not shown).

The fact that BSA was present in the assay system did not adversely influence the results; it was present in both normal and diabetic sera at equivalent levels. Furthermore, the binding of BSA antibodies to islet cells is completely suppressed at BSA concentrations of 500 µg/ml [6] which is 4-fold lower than the 2 mg/ml used in this study.

A significant trend was found between the time after onset of Type 1 diabetes mellitus in ICSA-positive children and their serum C_3 and C_4 levels. The results of ICSA, C_3 and C_4 assays are summarised in Table 1. The mean serum C_4 level of all 21 ICSA-positive children was significantly lower than that of 10 ICSA-negative children. Furthermore, the mean serum C_4 level in 9 children with ICSA-positive Type 1 diabetes mellitus at 0–5 months after onset was also significantly lower than either the mean levels of the control children or the ICSA-negative Type 1 diabetic children. Although the mean serum C_3 level of 9 children with ICSA-positive Type 1 diabetes mellitus at 0–5 months after onset was also lower than the levels of either normal children or ICSA-negative Type 1 diabetic children, the difference was not statistically significant.

The mean serum C_3 and C_4 levels of 9 ICSA-positive Type 1 diabetic children at 0–5 months after onset were both significantly lower than those of 12 ICSA-positive diabetic children at 6–12 months after onset. Furthermore, all 21 ICSA-positive Type 1 diabetic children showed a trend towards increasing serum C_3 and C_4 levels 1 year later. Nine of these children at 0–5 months after the onset of diabetes had significantly lower initial mean serum C_3 and C_4 levels than 1 year later. However, there were no significant differences in mean serum C_3 and C_4 levels in 12 ICSA-positive Type 1 diabetic children determined at 6–12 months after onset and 1 year later.

In an effort to study the inter-relationship between ICSA reactivity and serum C_4 level, four children with Type 1 diabetes at 3–10 months after onset who con-
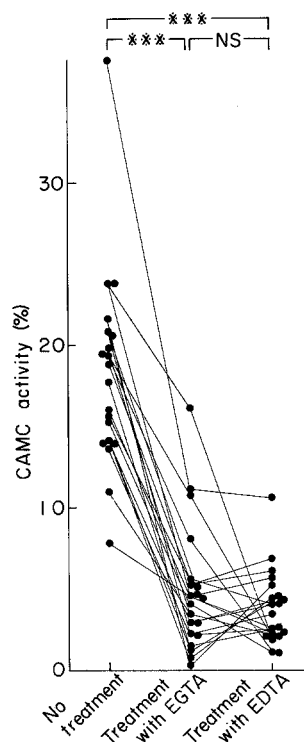
Fig. 1. Inhibition of complement-dependent antibody-mediated cytotoxic (CAMC) activity by treatment of the sera of Type 1 (insulin-dependent) diabetic children with ethylene glycol tetra-acetic acid (EGTA) or EDTA. *$p < 0.001$; $n = 20$
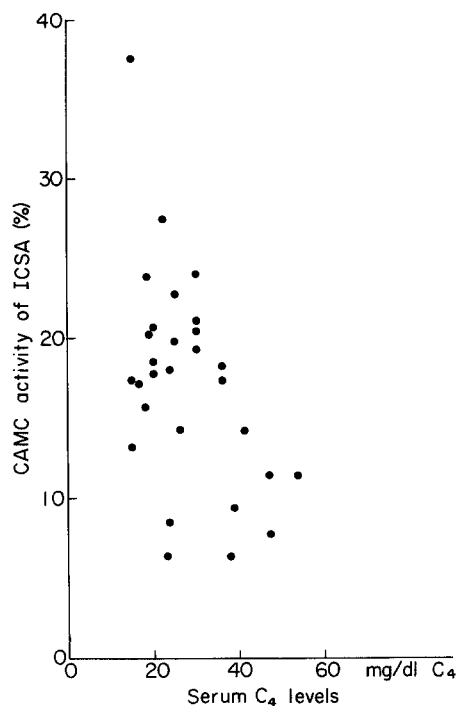
Fig. 2. Demonstration of the negative correlation between serum C_4 levels and CAMC activity of islet cell surface antibody (ICSA) in Type 1 diabetic children ($n = 29$, $r = -0.485$, $p < 0.01$)

verted from strongly ICSA-positive (1:16) to ICSA-negative 1 year later were examined. The mean serum C_4 level of all 4 children increased significantly (from

22.00 ± 4.8 mg/dl to 32.75 ± 5.4 mg/dl) during the course of the year.

CAMC assay

Rat pancreatic islet cells labelled with ^{51}Cr averaged $10,696.83 \pm 331.6$ ($n = 10$) counts/min (CPM); spontaneous (non-antibody-mediated) ^{51}Cr-release averaged $1,051.00 \pm 55.1$ ($n = 10$) CPM. In typical assays of CAMC activity and ICSA-induced release of ^{51}Cr in the presence of heat-inactivated human AB serum, an average of only $3.2 \pm 0.9\%$ ($n = 8$) of the counts were released into the medium.

Serum CAMC activity was confirmed [7] to be significantly higher in ICSA-positive children ($19.13 \pm 1.3\%$ $n = 21$) than ICSA-negative children ($11.82 \pm 1.6\%$, $n = 10$). In addition, we found the CAMC activity in untreated sera ($16.74 \pm 1.4\%$, $n = 20$) was completely inhibited by EGTA treatment ($4.95 \pm 0.9\%$, $n = 20$) (Fig. 1). No significant difference in CAMC activity was found between EGTA-treated sera ($4.95 \pm 0.9\%$, $n = 20$) and EDTA-treated sera ($4.00 \pm 0.5\%$, $n = 20$).

A negative correlation ($r = -0.485$) between serum C_4 concentration and CAMC activity (Fig. 2) observed initially disappeared one year later ($r = 0.078$). No correlation was found between serum C_3 level and CAMC activity during the course of the disease.

Discussion

Vergani et al. [8] reported that 22 of 86 Type 1 diabetic patients had below normal serum C_4 levels. However, they maintained that the depressed C_4 level was unrelated to activation of the complement system. They based their conclusion on the fact that depressed C_4 levels occurred not only in newly diagnosed diabetic patients but frequently in chronic diabetic patients as well as in five pairs of dizygotic twins with or without diabetes mellitus. In addition, there was no detectable evidence of activation of the complement system in these patients. Therefore, they believe that low C_4 values are irrelevant to the presence or absence of Type 1 diabetes mellitus.

Serum C_4 levels in Type 1 diabetic patients generally correlate with the number of C_4 null alleles [9, 10]. Moreover, two of three high risk supratypes (HLA-B8 Bfs C4AQ0 C4B1 DR3 and HLA-B18 BfF1 C4A3 C4BQ0 DR3) have been shown to contain null alleles at C_4 loci [11, 12]. Consequently, it is probable that C_4 null alleles are at least partially responsible for the relatively low C_4 levels which occur in Type 1 diabetic patients. However, this study demonstrates that Type 1 diabetic children have depressed serum C_3 and C_4 levels only during the initial stage of disease. It is unreasonable to believe that this phenomenon is due solely to C_4 null alleles; it seems more plausible that the low

initial levels are a consequence of activation of the classical complement pathway. In fact, it has been shown that complement activation ceases to occur as Type 1 diabetes mellitus progresses.

A highly positive correlation has been shown to exist between serum native C_3 and CH_{50} levels [13]. Therefore, native C_3 levels would be useful for clinical studies. Unfortunately, however, the quantitative assay of native C_3 using the single radial immunodiffusion method and anti-native C_3 serum results in overestimates; the complement component converts spontaneously from β_{1C} to β_{1A} in an agar substrate. Under the conditions of serum storage used in this study, complement components are known to be relatively stable. Consequently, the single radial immunodiffusion technique utilising Norpartigen containing only antibody to antigen A (anti-C_{3c} antibody) [3] or C_4 not only allows valid estimates of serum C_3 and C_4 levels [14] but estimates of complement activity as well [14, 15].

Vialletes et al. [16] reported depressed serum C_3 levels in 30% of Type 1 diabetic patients with recent onset and stated that it may be caused by increased complement consumption due either to insulitis or circulating immune complexes. Regarding the return of depressed serum complement component levels to normal values, it appears that while C_3 is restored relatively quickly, the recovery of C_4 is more gradual. In addition, patients with lupus nephritis [17-19] or rheumatoid arthritis [20, 21] often exhibit depressed serum C_4 levels with apparently normal C_3 levels.

It is also apparent that complement depletion resulting from activation of the classical complement pathway in the early stage of Type 1 diabetes mellitus is caused by depressed serum C_4 levels. It is reasonable to believe that the depressed serum C_4 level is attributable to increased complement consumption and reduced complement synthesis.

Studies employing a sensitive competitive equilibrium radioimmunoassay have shown that activated complement fragments, C_{3a}, C_{4a} and C_{5a}, are produced in vivo during the onset of Type 1 diabetes mellitus, which led to the belief that both the classical and alternative complement pathways may be activated in the early stages of the disease [22]. The consumption of C_3 and C_4 is caused by activation of the classical complement pathway triggered by a response of complement-binding antibody to antigen. The fact that serum CAMC activity in Type 1 diabetic children was almost totally inhibited by EGTA or EDTA strongly indicates that the alternative complement pathway was also activated along with the classical complement pathway. In fact, activation of the classical complement pathway is believed to be a consequence of binding of autoantibodies such as ICSA to islet cell surface antigens.

Although a significant negative correlation was observed between the serum C_4 concentration and CAMC activity at the early stage of diabetes, no such correlation was found one year later. These findings in-

dicate that at the early stage of Type 1 diabetes mellitus, the classical complement pathway is undoubtedly activated by ICSA binding to islet cell antigens at a level proportionate to CAMC activity. The fact that the initial negative correlation found between serum C_4 level and CAMC activity disappeared with time during the clinical course of disease provides additional evidence in support of this concept.

The demonstration that CAMC involving ICSA may lead to islet cell damage in vitro [1] points to the possibility that the destruction of pancreatic B cells might also result from a similar sequence of immunological events in vivo. If the classical complement pathway is activated similarly in vivo, significantly lower serum C_4 levels would occur in ICSA-positive patients than in ICSA-negative patients, and a negative correlation between serum C_4 levels and CAMC activity involving ICSA in Type 1 diabetes between serum C_4 levels and CAMC activity involving ICSA in Type 1 diabetes mellitus as found in this study would occur.

The clinical studies reported here suggest that CAMC induced by ICSA-related activation of the classical complement pathway may be deeply involved in the pathogenesis of Type 1 diabetes mellitus.

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