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Alternative complement pathway activity in sera from patients with sickle cell disease

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SUMMARY

The low molecular weight cobra venom factor (CoVF) was used to activate the terminal sequence of the alternative complement pathway in thirty-one sera from patients with sickle cell disease (SCD). The SCD sera were compared with normal sera as a source of the alternative complement pathway factors C3 proactivator (C3PA) and C3PA convertase. These factors are required for formation of the enzymatically active CoVF–C3PA complex which is capable of cleaving C3 and thus initiating generation of the cytolytic C5b–9 complex. CoVF cofactor activity was significantly less than normal in SCD sera as measured in an indirect lysis assay, indicating reduced C3PA or C3PA convertase activity in these sera. Qualitative (immunoelectrophoresis) and quantitative (radial immunodiffusion) measurement of C3PA showed, however, that this protein is normal or elevated in SCD sera. Taken together, the reduced CoVF cofactor activity and normal or elevated C3PA in SCD sera suggests that sera from patients with sickle cell disease have reduced C3PA convertase activity.

INTRODUCTION

Young sickle cell disease (SCD) patients are especially susceptible to *D. pneumoniae meningitis* (Robinson & Watson, 1966). While the basis of this susceptibility is not clearly defined, recent evidence suggests that at least one contributing factor is a relative deficiency in SCD sera of heat-labile opsonin activity for *Pneumococcus* (Winklestein & Drachman, 1968). This reduced opsonin activity occurs in SCD sera even though these patients show normal complement-mediated haemolysis in an assay which requires participation of all nine classical complement components (Johnston, Newman & Struth, 1973).

When Johnston and his colleagues (1973) examined opsonin activity in SCD sera under conditions which prevented activation of the classical complement sequence, they found that these sera did not fully activate and fix the essential opsonin C3 to pneumococci by the alternative pathway of complement activation. Since opsonin activity could be restored to SCD sera by addition of fresh normal serum, but not by serum heated at 50°C for 15 min, these investigators suggested that serum from SCD patients was deficient in a heat-labile alternative pathway factor.

The reaction mechanism of the whole alternative pathway involves a number of steps and numerous factors. We chose to investigate that part of the pathway in sera from SCD patients which represents the final steps of the sequence, and which can be activated by the

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low molecular weight cobra venom factor (CoVF) (Ballow & Cochrane, 1969). An indirect lysis assay system was used which is dependent upon participation of the alternative pathway factors, C3 proactivator (C3PA) and C3PA convertase, for formation of an enzymatically active CoVF–C3PA complex. This complex is capable of cleaving C3 and thus initiating the assembly of the cytolytic C5b–9 complex. Since C3PA is a heat-labile alternative pathway factor (Goodkofsky & Lepow, 1971), we also quantitatively and qualitatively examined this protein in SCD sera.

Our results show that: (1) CoVF activatable alternative pathway activity is significantly reduced in SCD sera; (2) this reduced activity is not due to either a quantitative or qualitative abnormality in these sera of the heat labile alternative pathway factor, C3 proactivator. By exclusion, these results suggest a partial deficiency of C3PA convertase in SCD sera.

MATERIALS AND METHODS

Thirty-one samples from twenty-six children with SCD (twenty-three HbSS; three HbSC), aged from 1 to 19 yr, were studied. Thirteen of these children were in painful crisis at the time the blood was drawn.* Serum from seventeen normal children (HbAA), aged 4 months to 13 yr, collected at Milwaukee Children's Hospital prior to elective surgery, were used as controls. Blood samples were obtained by venepuncture, allowed to clot for 1 hr at room temperature, followed by 1 hr at 0°C, centrifuged, the serum removed and frozen at −80° until just before use.

The methods for preparing veronal-buffered saline, containing 0·1% gelatin, 0·0005 M Ca^{2+} and 0·00015 Mg^{2+} (GVB^{2+}), dextrose–veronal-buffered saline ($DGVB^{2+}$) and 0·04 M ethylenediaminetetracetate (EDTA) buffer have been described (Nelson *et al.*, 1966). Total haemolytic complement activity (CH_{50}) was measured in SCD and normal sera using the method of Shulman (1958).

Cobra venom cofactor activity was measured using the indirect lysis technique described by Hunsicker (Hunsicker, Ruddy & Austen, 1973). Dilutions of each test serum were prepared in $DGVB^{2+}$. A 0·1-ml aliquot of either a serum dilution or buffer was incubated with 0·1 ml $DGVB^{2+}$ containing 5 u of CoVF (Cordis Laboratories, Miami, Florida) for 30 min at 37°C in a shaking water bath. Each reaction mixture then received 5×10^8 sheep erythrocytes (Gibco Diagnostics, Madison, Wisconsin) in 0·04 M EDTA, and 0·4 ml guinea-pig sera (Pel Freeze Biologicals, Rogers, Arkansas) diluted 1 : 4 in 0·04 M EDTA. Incubation was continued for 60 min at 37°C. The reaction was terminated by addition of 6·8 ml saline and centrifugation. Optical density of released haemoglobin was read at 414 nm. For each sample tested, the serum dilution required to generate a CoVF complex capable of inducing 50% lysis of the unsensitized erythrocytes was calculated. The reciprocal of this value is referred to as cobra venom activatable haemolysis of CoVF–AH_{50} (Brai & Osler, 1972). A standard serum was included in each experiment to ensure accurate comparison of the CoVF-AH_{50} values obtained.

C3PA protein levels in patients and normal sera were measured by radial immunodiffusion using antisera directed against the major antigenic determinant of this molecule (C3 activator) (Behring Diagnostics, Somerville, New Jersey) which was incorporated into 1·5% Nobel agar (Difco, Detroit, Michigan) as described (Allison, 1971). Reference sera containing known amounts of C3 proactivator were purchased from Custom Reagents, Chula Vista, California. Normal and SCD sera were subjected to immunoelectrophoresis at 100 V for 40 min in veronal buffer, pH 8·6. The C3PA was precipitated with the antisera to C3A.

The significance of differences between the mean of normal controls and SCD patients in or out of crisis was tested by Student's *t*-test. Correlations between CoVF-AH_{50} titres and C3PA levels were analysed by determination of linear regression coefficients.

RESULTS

The mean total haemolytic complement activity in the SCD sera did not differ significantly from the normal controls ($P > 0·1$) (Table 1). The one SCD serum which showed a CH_{50} titre far below the normal range was not among those with the lowest results in either the CoVF–AH_{50} assay or the C3PA determinations.

As shown in Fig. 1, the mean CoVF–AH_{50} titre for normal children was 10·7. In contrast the mean CoVF–AH_{50} titres were 7·6 and 8·3 for SCD patients in crisis and out, respectively.

* Sickle cell crisis refers to an episode of pain in one or more parts of the body presumably related to increased sickling in peripheral small vessels.

TABLE 1. Total serum haemolytic complement levels

Group	Sera tested	CH_{50} units
Sickle cell disease	15	109 (27–147)*
Normal	6†	101 (89–116)‡

* Numbers in parentheses represent ranges. In the SCD group, one of the fifteen patients had a CH_{50} of 27. The range for the remaining fourteen was 82–147.

† CH_{50} values were obtained on six of the seventeen serum samples from normal children used in these experiments.

‡ The normal CH_{50} range in our laboratory is 89–132 CH_{50} units.

These titres were significantly depressed as compared to the normal controls ($P<0{\cdot}001$). There was no significant difference between CoVF–AH_{50} titres of the two groups of SCD patients ($P>0{\cdot}05$).

The mean C3PA level in normal children was 211 μg/ml. The mean C3PA levels in sickle cell disease patients in or out of crisis at the time the sera was drawn were 281 μg/ml and 226 μg/ml, respectively. While the C3PA in the crisis patients was significantly elevated ($P<0{\cdot}05$), there was no significant difference between the amount of C3PA in the normals and SCD patients out of crisis ($P>0{\cdot}1$). By immunoelectrophoretic analysis the C3PA in SCD sera did not differ in mobility from that in normal sera (Fig. 3).

There was no correlation (r did not differ significantly from zero) between CoVF–AH_{50} titre and C3PA protein level in the sera from the normal controls ($r = 0{\cdot}38$, $P>0{\cdot}1$); the sera from the SCD patients in crisis ($r = 0{\cdot}06$, $P>0{\cdot}1$) or the sera from SCD patients out of crisis ($r = 0{\cdot}19$, $P>0{\cdot}1$).

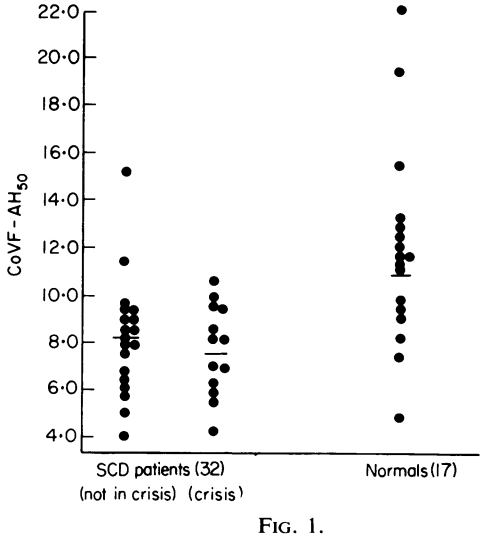

FIG. 1.

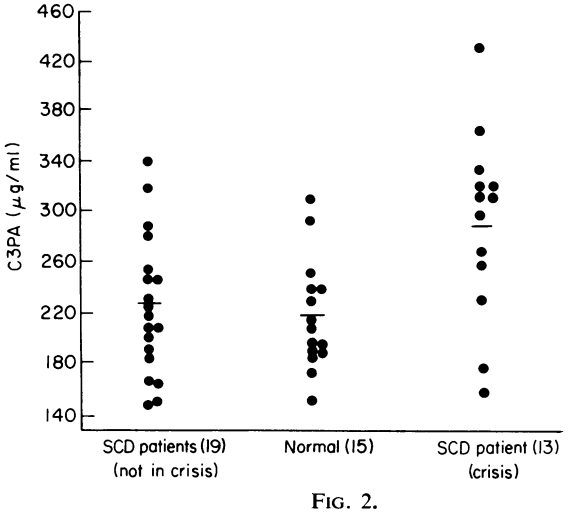

FIG. 2.

FIG. 1. CoVF–AH_{50} titres in SCD and normal sera.

FIG. 2. Quantitative radial immunodiffusion measurement of C3PA in SCD and normal sera.

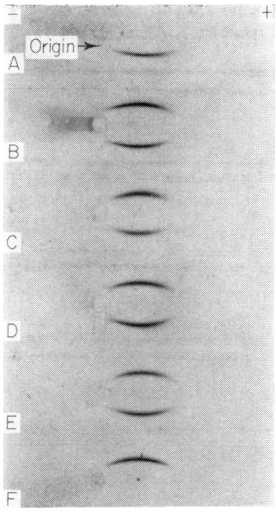

FIG. 3. Immunoelectrophoretic analysis of C3PA in SCD and normal sera. Wells A and F contained normal serum; B and D, SCD serum (in crisis) and; C and E, SCD serum (not in crisis). Anti-C3A was added to each trough.

DISCUSSION

CoVF was used to examine the alternative complement pathway in sickle cell disease sera. When introduced into serum, CoVF forms a complex with a well-defined alternative pathway protein, C3 proactivator (Cooper, 1973). In the presence of C3PA convertase and Mg^{2+}, this complex becomes stable and achieves enzymatic activity which is capable of cleaving C3 and thus initiating the assembly of the cytolytic C5b–9 complex. Magnesium is required for formation of the C3-cleaving enzyme but not for activation of the later complement components.

In the assay system used, human serum provides the alternative pathway factors required for formation of the CoVF–C3PA complex. A constant amount of C3–9 is supplied by guinea-pig sera diluted in EDTA. The EDTA prevents further interaction of the CoVF and C3PA while allowing activation of the later guinea-pig complement components. C3PA and C3PA convertase are the only known alternative pathway factors required for formation of the enzymatically active cobra venom factor complex (Cooper, 1973).

Our findings indicate that sera from SCD patients in or out of painful crisis generate less haemolytic activity than normal sera after mixture with the low molecular weight cobra venom factor, the terminal complement components and unsensitized sheep erythrocytes (Fig. 1). This significantly reduced activity is not, however, due to either; reduced levels of C3 proactivator in SCD sera (Fig. 2); or the presence in SCD sera of an altered form of the C3PA molecule as determined by immunoelectrophoresis (Fig. 3). The elevated C3PA values in SCD patients in crisis is consistent with the recent observation that C3PA is an acute phase reactant (Schutte *et al.*, 1974).

Within the range of C3PA measured, the correlation coefficient for the normal controls of CoVF–AH_{50} titre versus C3PA protein does not differ significantly from zero. Therefore, within this range of C3PA and for the levels of C3PA convertase that were actually encountered yet undetermined in these experiments, it is highly likely that CoVF–AH_{50} titre is a reliable assay for C3PA convertase activity. We therefore propose that the observed difference between the ability of the sera from the normal and patient population to support CoVF activatable haemolysis may reflect reduced C3PA convertase activity in the SCD sera.

A precursor form of the C3PA convertase molecule has been recently defined by Fearon, Austen & Ruddy (1974). These authors have shown that it is the active form of the molecule which participates in generation of the enzymatically active CoVF–C3PA complex. Both the precursor and active form of C3PA convertase are present in normal serum. A partial deficiency of C3PA convertase in SCD sera may therefore be due to either less of the active form of this molecule or less C3PA convertase total protein. While heat lability of human C3PA convertase has not been reported, 70% of the activity in guinea-pig serum which is analogous to human C3PA convertase is destroyed by heating at 56°C for 30 min (Dierich *et al.*, 1974).

Deficient complement activity in human serum usually reflects either under-production of a specific component or over-utilization due to a consumption phenomenon. Recently red blood cell stroma have been shown to activate the alternative complement pathway (Poskitt, Fortwengler & Lunskis, 1973). Since chronic haemolysis is a constant feature of SCD (Diggs, 1956) it is possible that the abnormal alternative pathway activity observed in SCD sera may reflect consumption *in vivo* of alternative pathway factors by fragments of lysed sickled erythrocytes.

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