Sickle Cell Disease. A Serum Defect in the Thromboplastin Generation Test

By Henry H. Henstell, Miriam Kligerman and Lowell E. Irwin

In the present investigations, it has been found that individuals with sickle cell disease (SCD) have a defective generation of thromboplastin.\textsuperscript{1,2} It is noted that this deficiency arises from an excessive loss of a labile serum factor with thrombin-like activity.

Materials

1. Plasma, for all testing except the serum thrombin, was prepared from whole blood anticoagulated with dry balanced oxalate.\textsuperscript{3} The cells were sedimented by centrifuging at 2000 rpm for 10 minutes at 5 C.

For use in the serum thrombin tests, nonanticoagulated plasma was prepared from blood drawn by the two syringe technic. The blood (second syringe) was immediately centrifuged in the cold and the plasma removed with a plastic pipette. Such plasma, in plastic, in the cold, remained unclotted for a minimum of 1 hour.

2. Substrate plasma was pooled normal, oxalated, citrated or ACD plasma, stored frozen in 5–10 ml volumes.

3. Barium sulfate absorbed plasma was prepared by agitating 100 mg. BaSO\textsubscript{4} powder with 1 ml. plasma for 3 minutes at room temperature. The BaSO\textsubscript{4} was removed by centrifuging at 6000 rpm for 5 minutes at 5 C.

4. Exhausted plasma, deficient in factors XI and XII was prepared by the method of Soulier\textsuperscript{4} from pooled normal, intact plasma obtained with the usual "silicone" technics. It was lyophilized in small volumes and stored at 5 C.

5. Serum, unless otherwise stated, was prepared from clotted whole blood, incubated for 2 hours at 37 C. and separated by centrifugation at 2000 rpm for 10 minutes at 5 C.

6. Heated normal serum was pooled serum which had been held at 50 C. for 30 minutes.\textsuperscript{5} It was stored frozen in small volumes.

7. Hemophilia B serum was obtained from a donor who had 15 per cent of normal factor IX activity. It was lyophilized in small volumes and stored at 5 C.

8. Prothrombin, partially purified, was prepared from hentonite absorbed plasma.\textsuperscript{6} The prothrombin was concentrated by absorption to BaSO\textsubscript{4} followed by elution with citrate.\textsuperscript{7} It was lyophilized in small volumes and stored at 5 C.

9. Prothrombin-free beef plasma was obtained from Hyland Laboratories, Los Angeles, California.

10. Tissue thromboplastin was obtained from Ortho Pharmaceuticals, (Acuplastin\textsuperscript{®}).

11. Russell Viper Venom (Stypven\textsuperscript{®}) was obtained lyophilized, from Burroughs Wellcome and Co. It was reconstituted in 0.9 per cent NaCl.

12. Cephalin was a 10 mg./ml suspension in 0.9 per cent NaCl of animal cephalin powder, obtained from Nutritional Biochemicals Corp.

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13. Partial thromboplastin time cephalin (PTT cephalin) was a chloroform extract of Difco rabbit brain thromboplastin.8

14. Platelet suspensions were prepared from platelet-rich plasma derived from normal human blood, anticoagulated with EDTA, and centrifuged at 500 rpm for 3-5 minutes. The platelets were sedimented at 13,000 rpm at 5 C., resuspended in cold 0.9 per cent NaCl, and resedimented three times. They were suspended finally in 0.9 per cent NaCl in half the original volume of plasma.

15. Fibrinogen stock solution was a 1 per cent solution of outdated Merck, Sharp and Dohme human fibrinogen. The clottable protein was 0.884 per cent as determined by Blombäck's method.9 Different batches of fibrinogen were checked against the same normal bloods to insure that the same antithrombin curves were obtained. The difference in percentage of clottable protein between batches was negligible. The fibrinogen solution was stored frozen in small volumes under a partial vacuum. No deterioration was noted over the 3-month period of use.

16. Thrombin solutions were prepared from Parke-Davis Topical Thrombin (bovine). The contents of a single vial were weighed and the rated units/mg. calculated. For each day's testing a weighed portion of thrombin was dissolved in 0.9 per cent saline in a polypropylene tube immediately before use. Dilutions of the stock thrombin were also in plastic tubes. The pipettes used were silicone coated.

17. Euglobulin was prepared by a 1 to 20 dilution of plasma which was acidified to pH 5.1 with 1 per cent acetic acid. After chilling for 1 hour, the precipitate was removed by centrifugation and taken up in a mixture of veronal buffer (8 parts) and balanced oxalate solution (2 parts)3 in a volume equal to the original plasma.

18. Veronal buffer was 0.028 M at pH 7.5.

All preparative procedures, unless otherwise stated, were in glass.

METHODS

1. Thromboplastin Generation Test (TGT)
   a. Standard Measurement
      A modified Biggs and Douglas10 test system was used
      Incubation mixture:
      0.1 ml. BaSO₄ adsorbed test or control plasma
      0.85 ml. Veronal buffer
      0.05 ml. Test or control serum
      0.4 ml. 0.9 per cent NaCl
      0.1 ml. Cephalin or platelets
      0.5 ml. M/40 CaCl₂
      Clotting mixture:
      0.1 ml. Substrate plasma
      0.1 ml. Incubation mixture
      0.1 ml. M/40 CaCl₂
   b. Preincubation of Plasma with Thrombin
      Preincubation mixture (3 min. at 37 C.):
      0.1 ml. BaSO₄ absorbed control plasma
      0.2 ml. thrombin solution in Veronal buffer, or Veronal buffer
      The following reagents were added in rapid succession to complete the incubation mixture of the TGT:
      0.65 ml. Veronal buffer
      0.05 ml. Test or control serum
      0.4 ml. 0.9 per cent NaCl
      0.1 ml. Cephalin
      0.5 ml. M/40 CaCl₂
      Clotting mixture as above in Section 1a
   c. Preincubation of Plasma with Diluted Serum
Preincubation mixture (3 min. at 37 C.):
0.1 ml. BaSO4 absorbed control plasma
0.2 ml. Veronal buffer diluted test or control serum, or Veronal buffer
The remainder of the test system was as in section 1b, except that only test serum was added in the incubation mixture.

d. Thrombin Added to the Substrate plasma
Preincubation, incubation and clotting mixtures were as in Section 1b with the following exceptions:
1. The preincubation mixture contained only plasma and veronal buffer
2. An equal mixture of M/20 CaCl2 and thrombin (0.02 U diluted to 2 ml. in 0.9 per cent NaCl) was substituted for the M/40 CaCl2 in the clotting mixture.

2. Prothrombin activity in plasma was measured according to the methods of Quick11 and/or Ware and Stragnell,12 and reported as per cent of normal plasma activity. Normal is 80–120 per cent. For serum prothrombin, the Ware and Stragnell method was used. It was reported as per cent of normal plasma activity. Normal is less than 30 per cent.

3. Recalcified clotting time of plasma was measured at 37 C. in a system consisting of 0.1 ml. plasma, 0.1 ml. 0.9 per cent NaCl, and 0.1 ml. M/40 CaCl2. The normal range is 90–120 seconds.

4. Factor IX activity was measured by the correction of a Biggs and Douglas TGT system containing normal plasma and hemophilia B serum. Graded amounts of test serum were added to this system. The correction was expressed as per cent of normal activity by comparison with the correction by similar graded additions of pooled normal serum.

5. Heat labile factor (HLF) activity was measured similarly by the correction of a TGT system containing normal plasma and heated normal serum. The test serum correction was expressed as per cent of normal by similar comparison with pooled normal serum correction curves.

6. Total contact factor activity was measured as the influence of the celite-activated sample on the recalcified clotting time of exhausted plasma. It was expressed as per cent of normal activity by comparison with the corrective capacity of fresh normal plasma.

7. Factor X activity was measured as the correction of the clotting time of a Factor X-deficient system comprising partially purified prothrombin, prothrombin-free beef plasma, cephalin, CaCl2 and Stypven. Optimum concentration was determined for each batch of each reagent. It was quantitated as in #6.

8. Antithrombin III (AT III) was measured according to the method of Hensen and Loeliger.13

9. Serum thrombin activity was measured in the serum derived from the chilled, non-anticoagulated plasma which was allowed to clot in a glass tube at 37 C. The clot was immediately wound out. At intervals of 8, 15, 30, 45 and 60 minutes, the ability of the incubating serum to clot human fibrinogen at 37 C. was measured. On each day of testing the thawed fibrinogen stock solution was diluted with an equal volume of Veronal buffer. The clotting system consisted of:
0.1 ml. 0.50 per cent fibrinogen solution
0.1 ml 0.01 M CaCl2
0.1 ml. serum
Equivalent mixtures of fibrinogen, CaCl2 and 0.9 per cent NaCl remained uncleotted after 8 hours incubation at 37 C.

10. Normal control samples: The individual normal samples used for standards in the above tests were from donors who had been previously tested and demonstrated to contain the specific activities close to 100 per cent of normal.

11. Variations in plasma oxalate concentration in SCD patients had no effect on the TGT and other coagulation test systems.

All samples and reagents for the above tests, with the exception of thromboplastin, Stypven, Cephalin and CaCl2, were stored in a melting ice bath prior to use in testing.
RESULTS

These data summarize the results of 79 studies of 39 patients.

Pattern of the Defect

Thromboplastin generation tests (TGTs) were performed in 39 patients with sickle cell disease, (SCD), of whom 24 had homozygous, and 15 heterozygous SA and SC disease. On each day of testing a normal sample was run concurrently. The barred area of figure 1 shows the range of these normal TGTs.

In every instance of homozygous SCD (fig. 1*) and in 4 of the 15 cases of heterozygous SCD (Curves 2, 4, 14 and 18; fig. 1) the TGTs were abnormal. In the majority of cases of heterozygous disease, however, the TGTs were either normal (4 cases), or defective only in a system sensitized by using half the usual concentration of serum (7 cases).

The pattern of the TGTs was unaffected by the type of thromboplastic agent used. The same defective TGT was obtained with normal platelets, patient's platelets or PTT cephalin. Hence, the defect was in no way related to the nature of thromboplastin, or to factors which might be present in association with platelets.

The results of tests other than the TGTs are given in tables 1 and 2. Table 1 lists the findings in the homozygous patients and in the 4 heterozygous patients with marked TGT abnormality. Clinically severe disease, as indicated by the low hematocrit values and the high reticulocyte percentages, occurred in association with the abnormal TGTs. In contrast, the patients with heterozygous disease, and minimally disturbed TGTs, had clinically mild disease, with comparatively normal hematocrit and reticulocyte values (table 2). However, within the TGT defective group, there were no parallel changes in the degree of TGT abnormality and the hematocrit and reticulocyte values (last 4 columns, table 1). This lack of correlation would seem to indicate that the mechanisms underlying the TGT abnormality are distinct from the hemolytic processes as measured by the hematocrit and reticulocyte data.

Repetitive study of the TGT in 9 homozygous SCD patients indicated, moreover, that the degree of the defect was not constant. Temporal variations in TGT in one individual are shown in figure 2. On each day of testing, a normal individual was also tested. The range of these normals is indicated by the shaded area. At no time, was any TGT in a homozygous SCD patient within the normal range. This is consistent with the group findings (fig. 1). The TGTs were all well beyond any possible variation in the normal test pattern as measured in this laboratory.

Nature of the Defect

Reference to table 1 reveals that there was no consistent abnormality in prothrombin activity or in prothrombin conversion in SCD. The crossmatching

*Curves are given only for the 19 patients who had the studies detailed in table 1.
technic revealed that the TGT defect resided in the serum component. Results characteristic of the findings in 29 cases are illustrated in figure 3. No plasma defect was demonstrable. On the contrary, the patients’ plasmas (curve B) commonly showed a degree of generative activity which was greater than normal (curve A). The significance of this observation is not clear.

These data limit the possible clotting factors involved to Factors IX, X, XI, XII, thrombin and, perhaps, other heat labile factors (PPA?). Reference to table 1 indicates that there was no abnormality in either the Factor XI-XII complex, or in Factor X which could explain the defective TGT. Factor IX was reduced to significantly low levels in only 2 of 11 instances. This could scarcely account for a consistent and severe defect in generation. In addition, a Factor IX deficiency manifests itself in a reduced quantity of thromboplastin generated. In these studies, the duration of the lag phase, but not the quantity, was affected. The participation of Factor IX is even less likely in light of the following data.

Consequently, attention was focused upon thrombin or other labile serum factors. In the following experiment (fig. 4), the TGT was run with SCD serum incubated in contact with the clot, at 37°C. for varying periods of time: for 2 hours (curve A), for 1 hour (curve B) and with no incubation of the serum (curve C). The generation of thromboplastin was progressively more defective with increasing periods of incubation. A greater reduction in thromboplastin generation occurred with SCD serum incubated for 1 hour.
Table 1.—Clotting and Other Tests in SCD Patients with Marked TGT Serum Defect

<table>
<thead>
<tr>
<th>Pt.</th>
<th>Sex</th>
<th>Age</th>
<th>Tests</th>
<th>Prothrombin (%)</th>
<th>Retrol. Clot. Plasma (Sec.)</th>
<th>First Stage Factors (%)</th>
<th>AT III (%)</th>
<th>Hb Type</th>
<th>Hemat. (%)</th>
<th>Retics. (%)</th>
<th>Curve #</th>
<th>30 Sec. Intercept (Min.)</th>
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<td>3</td>
<td>44-74 44-92</td>
<td>6-9</td>
<td>180</td>
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<td>90</td>
<td>&lt;10</td>
<td>SS</td>
<td>23-26</td>
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<td>124</td>
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<td>80</td>
<td>90</td>
<td>&lt;10</td>
<td>SA</td>
<td>39</td>
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<td>91</td>
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<td>135</td>
<td>106-141</td>
<td>45-105</td>
<td>95</td>
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<td>50</td>
<td>47</td>
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<td>SS</td>
</tr>
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<td>165</td>
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<td>15-68</td>
<td>25-75</td>
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<td>100</td>
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<td>75</td>
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*On steroid medication.
†Ware and Stragnell method.
‡Activated contact factors.
‖Heat labile factor.
||Antithrombin III.
Table 2.—Clotting and Other Tests in SCD Patients with a Normal TGT or a "Mild Defect"

<table>
<thead>
<tr>
<th>Pl.</th>
<th>Sex</th>
<th>Age</th>
<th>Tests</th>
<th>Prothrombin (%)</th>
<th>Recalc. Clot. Pl.</th>
<th>AT III (%)</th>
<th>Hb (Type) (%)</th>
<th>Retics. (%)</th>
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<td>Plasma</td>
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<td>102</td>
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<td>90</td>
<td>1:0</td>
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</table>

*Seen only on dilution of serum to half the usual concentration in the TGT.

The last 6 patients are children of L. E.

(curve B) than with normal serum incubated for twice as long (curve D).

This dependence upon a labile serum factor is more precisely illustrated in figure 5, which is representative of 8 studies in 6 patients. Normal and SCD sera were incubated at 37 C., in the absence of the clot, for 8, 20, 60 and 120 minutes prior to use in the TGT. The sera were diluted to 5 per cent concentration with a severely defective SCD serum which had been incubated at 37 C. for 24 hours. The generative capacity of the diluted test SCD serum, incubated for 8 minutes (dashed curve) was equivalent to that of normal serum incubated for 20 minutes (solid curve). The SCD serum incubated for 20 minutes was equivalent to normal serum activity after 60 minutes of incubation. The activity in SCD serum, hence, deteriorated faster than that in normal serum by a factor of approximately 3. At 60 minutes incubation, the SCD serum had lost all capacity to correct the defective diluent serum within the limits of the test system.

**Serum Thrombin and Heat Labile Factor (HLF)**

The implication of thrombin is suggested more directly by the results of serum thrombin clotting studies in which it is seen that fibrinogen clotting activity of SCD serum was lost at a markedly faster rate than normal (fig. 6). Twenty-four normal individuals were tested of whom 15 were white (8 males, 7 females) and 9 were negro (6 males and 3 females). No differences were observed due either to race or sex.

The amount of thrombin generated in this test system did not differ significantly from normal. The clotting of euglobulin derived from SCD plasma also resulted in normal yields of thrombin. Euglobulin is equivalent to plasma in its clotting factors, but is deprived of progressive antithrombin. Furthermore, the rate of loss of thrombin (progressive antithrombin) is not influenced by the amount of thrombin present. Hence, the above observations cannot be explained by any possible differences in the amount of thrombin present.

SCD serum was also incapable of correcting the defective thromboplastin generation induced by heating normal serum at 50 C. for 30 minutes. This degree of heating is sufficient to destroy thrombin. In 8 of 12 observations,
the SCD serum had less than 10 per cent of normal corrective activity. In only two instances was the corrective capacity normal (table 1, HLF).

The defective generation of thromboplastin of each of the 9 SCD sera was completely corrected by preincubating the normal plasma with trace amounts of thrombin (fig. 7). The least amount of thrombin which would optimally accelerate generation was first determined in a normal system (solid lines). This was found to be 0.02U, an amount which had no effect on the substrate plasma clotting. The addition of this amount of thrombin to the defective SCD TGT (fig. 7, dashed curves) resulted not only in complete correction, but also in a rate of generation almost equal to that of the thrombin accelerated normal system. This amount of thrombin similarly corrected the TGT defect induced by heating normal serum at 50 C. for 30 minutes as well as the defect of the prolonged incubated SCD serum of the previous studies. In contrast, the thrombin did not correct the defective generation of Factor VIII-deficient plasma. Although recognizing that acceleration of one aspect of the coagulation system may obscure deficiencies of other factors, it is unreasonable that the minimal amount of thrombin used would have completely obliterated the severe SCD serum defect unless the thrombin was substitutive.

It is generally accepted that thrombin accelerates the generation of thromboplastin via activation of plasma Factors VIII and V. The following experiments indicate that SCD serum is markedly deficient in such plasma activating capacity. Figure 8 shows the influence on the TGT of preincubating the normal plasma component with small amounts of SCD or normal serum, prior to the addition of the standard amount of SCD serum. The results of the two experiments shown are typical of the 6 SCD sera studied. Normal plasma was

Fig. 2.—Temporal variations in the Thromboplastin Generation Test.
preincubated either with buffer (curve 1) or with two concentrations of normal serum (curves 2 and 3). This increased serum concentration did not significantly affect the TGT when added after preincubation of the plasma with buffer. Similarly, the plasma was preincubated with varying amounts of SCD serum. For clarity, only the most informative curve is shown (curve 4). In Series 1, 0.01 ml. of SCD serum had less ability to activate the plasma than did 0.004 ml. of normal serum. In Series II, the activation ability of 0.01 ml. of SCD serum was between that of 0.001 ml. and 0.005 ml. of normal serum. Hence, the potency of the SCD sera was approximately one-third that of normal serum. These data correlate well with the relative TGT generative capacities of the serially incubated SCD and normal sera described above.

**DISCUSSION**

The present data indicate the existence of a defect in the capacity of serum from individuals with sickle cell disease to support normal generation of thromboplastin.

The deficiency is noted in every case of homozygous SCD studied. In heterozygous disease, there is a wide range, from pronouncedly defective to normal TGTs. No correlation could be found between the clinical severity of the disease and the degree of TGT abnormality. This lack of correlation suggests that the phenomenon underlying these findings is not related to the recognized manifestations of the disease which are primarily hemolytic in nature.
Fig. 4.—The Thromboplastin Generation Test with sera incubated for varying periods of time.

Fig. 5.—Dependence of the Thromboplastin Generation Test on the degree of serum incubation.
Fig. 6.—The serum thrombin clotting time in homozygous sickle cell disease.

No deficiency in Factors IX, X, XI, XII, prothrombin conversion or in the amount of thrombin generated existed which could account for a defective thromboplastin generation. However, the serum was characterized by an excessively rapid loss, upon incubation, of a heat labile factor; of the ability to clot fibrinogen; and of the ability to activate plasma Factors VIII and V. These activities characterize thrombin sufficiently well to suggest that the abnormality in SCD is an excessively rapid loss of thrombin, or a thrombin-like factor, from the serum.

The most reasonable explanation for the rapid loss of thrombin appears to be accelerated neutralization by a progressive antithrombin. This might be due either to increased antithrombin III (II?), not detectable by the usual procedures, or to an abnormal sensitivity of the SCD thrombin to antithrombin.

It is not yet known if this abnormality is unique for sickle cell disease. In a paper by Connor, Warner and Carter, an excessive loss of a labile serum factor (thrombin?) was noted in patients with otherwise unexplained, clinically significant bleeding. These included patients with gastrointestinal bleed-
Fig. 7.—Correction of the defective SCD TGT by preincubating the plasma with thrombin.

Fig. 8.—Influence of preactivation of the plasma by serum on the SCD TGT CP preincubated 3 min. with CS or CP.
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ing, nosebleeds, easy bruising, operative and postoperative bleeding, subdural hematomas, hereditary telangiectasia, thrombasthenia and myelofibrosis. Many had a family history of bleeding. In addition, it was commonly found in the newborn, in liver disease, with polycythemia vera and may contribute to the defective TGT with coumarin drugs.

In spite of the apparent similarities to the present studies, major differences exist between Connor’s data and ours. None of the mentioned disorders are particularly characteristic of SCD. The serum, in order to develop Connor’s defect, must be incubated for a minimum period of 3 hours, 1 hour of which is in contact with the clot; less time will not expose the defect. Moreover, the defective TGT was not demonstrable when platelets were substituted for the soybean phosphatide as a source of factor 3.

In SCD, the defect is demonstrable with serum incubated for only 2 hours and even for 1 hour. It is not affected by platelets when used as a source of factor 3 in TGT. Although there are similarities in the end result, there appear to be differences in the ways in which the defect is brought about. Further study of the relationship between the two processes is indicated.

Summary

1. A defective ability of sickle cell disease (SCD) serum to support thromboplastin generation was demonstrated.
2. The serum defect was associated primarily with homozygous SCD, although defects of varying severity were also noted in the heterozygous forms.
3. The deficiency appears to be due to an accelerated loss of thrombin or thrombin-like factors from serum.

Summary in Interlingua

1. Esseva demonstrate un defecto, in morbo a cellulas falciforme, in le potentia del sero de supportar le generation de thromboplastina.
2. Le defecto in le sero esseva associate primarimente con homozygotic morbo a cellulas falciforme, sed su occurrentia a vane grados de severitate esseva etiam notate in formas heterozygotic del morbo.
3. Il pare que le defecto es causate per un acceleration del perdita de thrombina o de factores simile a thrombina ab le sero.

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References


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