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Deficiency of serum bactericidal activity against *Salmonella typhimurium* in sickle cell anaemia

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SUMMARY

Systemic salmonellosis is a recognized complication of sickle cell anemia (SCA). In our initial study of SCA host defences against salmonella, we evaluated the bactericidal activity of serum against *Salmonella typhimurium*. When compared to controls, sera from eight out of nineteen SCA patients were deficient in bactericidal function. Levels of factor B, haemolytic complement and agglutinating antibody were similar in SCA and control sera. However, abnormalities that might theoretically account for the decreased antibacterial activity were observed in many SCA sera. These abnormal findings included: (a) defective function of the alternative complement pathway (decreased bacterial killing in the presence of Mg EGTA); (b) low serum C3 concentration; and (c) decreased total iron-binding capacity (TIBC), with a resultant increase in per cent saturation of iron-binding capacity. Of these deficiencies only the abnormal alternative pathway function was significantly associated with decreased serum bactericidal activity. A suggested function of serum bactericidal activity is prevention of bacteraemia by susceptible organisms. Thus diminished serum bactericidal capacity may increase the risk of *Salmonella* bacteraemia in some individuals with sickle cell disease.

INTRODUCTION

Bacterial infections occur with increased frequency in sickle cell disease (Hook, 1961; Robinson & Watson, 1966; Kabins & Lerner, 1970; Barrett-Connor, 1971). Furthermore, infection is the leading cause of death in children with this illness. Salmonellosis, especially osteomyelitis, is a common and well-known infection in persons with sickle cell anaemia (SCA). The tendency of blood-borne salmonellae to infect abnormal or devitalized tissues (Hook, 1961) may account for the localization in bone, but not the frequency of systemic salmonellosis in patients with sickle cell disease.

Based on studies in animals, both impaired reticuloendothelial function secondary to erythrophagocytosis (Kaye, Gill & Hook, 1967) and the iron overload state (Weinberg, 1974) have been suggested as explanations for the frequent salmonella infections in haemolytic states (including SCA.) Defective host-defense mechanisms have been described in SCA (Winkelstein & Drachman, 1968; Pearson, Spencer & Cornelius, 1969; Johnston, Newman & Struth, 1973), but direct evidence that these findings are relevant to protection against *Salmonella* infection or to clinical salmonellosis is lacking. Therefore, as an initial step in further investigation of SCA host defenses against *Salmonella*, the present study evaluates both the bactericidal activity of serum and those serum factors which contribute to bacterial killing.

MATERIALS AND METHODS

Subjects and serum. Twenty-two outpatients with sickle cell disease (documented by haemoglobin electrophoresis) were stable and without infection at the time of study. Their ages ranged from 5 to 23 years, with a mean age of 13 years. Control

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sera were obtained from children at the time of hospitalization for elective surgery and from healthy, adult medical and laboratory personnel. The age range of twenty-six controls was 3 to 50 years, with a mean age of 23 years. Since the quantity of blood obtained from small children was limited, it was not possible to perform each of the studies noted below on all serum specimens.

Blood collected by venipuncture was allowed to clot in sterile, acid-washed glass tubes. Serum was collected after centrifugation and frozen at −70°C until utilized in the studies described below.

Bacteria. A recent clinical isolate of *Salmonella typhimurium* was stored at −70°C. For serum bactericidal assays organisms were grown overnight in trypticase soy broth (TSB) (BBL, Division of Becton, Dickinson and Co., Cockeysville, Maryland), and an aliquot of this culture was then transferred to fresh TSB and cultured for an additional 2 hr in a shaking water bath at 37°C. These log-phase bacteria were washed in Hanks' balanced salt solution (HBSS) (BBL) and resuspended in the same solution.

Bactericidal assays. The ability of control and SCA serum to kill *S. typhimurium* was investigated in the following manner (Davis & Wedgewood, 1965; Root, Ellman & Frank, 1972). The assay mixtures, consisting of 0·9 ml serum (SCA or control), 0·1 ml bacteria in HBSS ($1–4 \times 10^3$ organisms), and 0·1 ml sterile saline, were placed in 16 × 75 mm glass tubes with screw caps. Another set of tubes was prepared in an identical manner with the exception that the 0·1 ml of sterile saline contained ethylene glycol tetra-acetic acid (EGTA) (Sigma Chemical Co., St Louis, Missouri) and magnesium chloride (Fisher Scientific Co., Fair Lawn, New Jersey). The final concentration of EGTA was 0·009 M, and that of magnesium (Mg^{2+}) was 0·004 M. Each serum assay mixture was run in duplicate or triplicate.

The serum mixtures were placed in a CO_2 incubator at 37°C and rotated 8–10 times/min (Multi-Purpose Rotator, Scientific Instruments, Springfield, Massachusetts). At 0, 15, 30, 60 and 120 min samples of these serum mixtures were removed, diluted appropriately in saline and plated on eosin–methylene blue agar (BBL). Bacterial counts were performed after incubation at 37°C for 24 hr.

Complement determinations. Haemolytic complement activity of serum, manifested by lysis of antibody-sensitized sheep erythrocytes (Cordis Laboratories, Miami, Florida) was evaluated by a standard technique (Mayer, 1961). Optical density was read at 541 nm in a Beckman DU spectrophotometer. Serum complement activity was expressed as 50% haemolytic units (CH_{50} u).

Serum concentrations of C3 were quantified by single radial immunodiffusion in agar (Immuno-Plates, Hyland Division, Travenol Labs, Costa Mesa, California). Serum levels of factor B (C3 proactivator/activator, $\beta 2$ glycoprotein II) of the alternative complement pathway were evaluated in a similar manner (M-Partigen Plates, Behring Diagnostics, Somerville, New Jersey).

Serum antibody determinations. Serum agglutinating antibody against *S. typhimurium* was determined by a previously described technique using microtitre equipment (Cooke Engineering Co., Alexandria, Virginia) (Lehmann *et al.*, 1968). Antibody titres were expressed as the reciprocal of the highest serum dilution ($\log_2$) causing macroscopic agglutination of organisms.

Serum iron and iron-binding capacity. Serum iron and latent iron-binding capacity were quantified by the method of Persijn, Van Der Slik, & Riethorst (1971), with 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (Ferro-Zine, Hach Diagnostics, Ames, Iowa) as the colour reagent. Other values were then calculated in the following manner.

Total serum iron-binding capacity (TIBC) = serum iron + latent serum iron-binding capacity.

Per cent saturation of serum iron-binding capacity = serum iron/TIBC.

Statistical analysis. Differences between experimental groups were evaluated with Student's *t*-test or χ^2 (fourfold table) analysis.

RESULTS

Bactericidal assays

The ability of control sera to kill *S. typhimurium* is shown in Fig. 1. Serum bactericidal activity was completely abolished by heat inactivation (56°C for 30 min), by the addition of 0·01 M disodium ethylene diamine tetra-acetate (EDTA) or by absorption with zymosan (2 mg/ml of serum) (Nutritional Biochemical Corporation, Cleveland, Ohio) (Mayer, 1961). These observations indicate that complement system activity was required for bacterial killing.

Studies of serum antibacterial activity in SCA patients demonstrated two types of response (Fig. 1). Eight out of nineteen SCA sera were deficient in their capacity to kill *Salmonellae* (i.e. bacterial survival was >45% after 30 min and/or >10% after 60 min incubation) (Fig. 1). The remaining SCA sera exhibited bactericidal activity similar to that of control sera. When the entire group of SCA sera was considered, there was a significant difference (after 15 or 30 min incubation) between the ability of SCA and control sera to kill *S. typhimurium* (Table 1).

The role of the alternative complement pathway in bacterial killing was evaluated by adding Mg EGTA to serum at a concentration which chelates the available calcium (Fine *et al.*, 1972). Under these

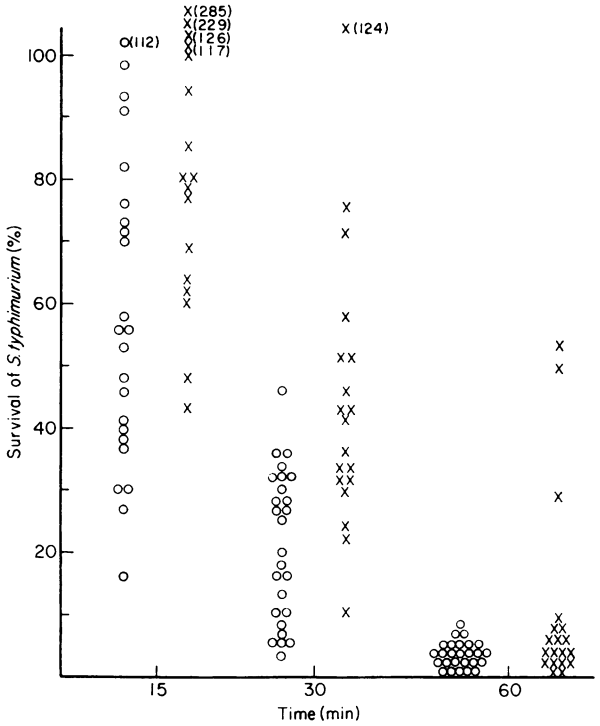

FIG. 1. Bactericidal activity of sickle cell anaemia (×) and control (○) sera against *S. typhimurium* at the times indicated.

$$\text{Per cent of bacteria surviving} = \frac{\text{viable bacteria at 15, 30, or 60 min}}{\text{viable bacteria at 0 time}}.$$

Values in parentheses greater than 100% indicate bacterial growth.

conditions the alternative pathway (which requires Mg^{2+}) (Götze & Müller-Eberhard, 1971; Sandberg & Osler, 1971), but not the classical pathway (which requires Ca^{2+}) of complement activation is able to function. The addition of Mg EGTA to control serum caused only a slight decrease in bactericidal activity (Table 1 and Fig. 2). Evidence that bacterial killing in chelated serum was due to alternative complement pathway activation rather than to the action of EGTA itself is shown in Fig. 3. EGTA (with or without Mg^{2+}) in HBSS allowed the growth of *S. typhimurium* for at least 1–2 hr. Similarly, EGTA without Mg^{2+} failed to inhibit bacterial growth in serum. However, the addition of EGTA plus Mg^{2+} permitted bacterial killing in serum. Thus supplemental magnesium is needed for demonstration of antibacterial activity in serum containing EGTA. This observation indicates that the serum concentration of ionized magnesium (10^{-6} M) in the presence of 0·01 M EGTA without supplemental Mg^{2+} (Fine *et al.*, 1972) is inadequate for optimal alternative pathway function.

As with total serum bactericidal activity, evaluation of alternative complement pathway function in SCA sera revealed two patterns of response (Fig. 2). Five out of seventeen SCA sera were unable to kill *S. typhimurium* efficiently in the presence of Mg EGTA (i.e., >60% bacterial survival after 30 min and/or >15% survival after 60 min incubation), indicating a defect in the alternative pathway of complement activation. The twelve remaining Mg EGTA-chelated SCA sera killed bacteria in a normal manner. Overall results are summarized in Table 1.

Complement determinations

Quantifications of C3, factor B and haemolytic complement in control and SCA sera are presented in Table 2. C3 levels were significantly lower in sera from patients with SCA than in control sera. Both factor B levels and haemolytic complement activity were similar in control and SCA sera.

TABLE 1. Total and alternative complement pathway-mediated serum bactericidal activity—sickle cell anaemia and controls

Experimental group	Per cent survival of *S. typhimurium*			
	Time (min)			
	15	30	60	120
Total bactericidal activity (untreated serum)				
Sickle cell anaemia	99·9±15·5*† (17)	45·1±5·7‡ (19)	9·8±3·7 (19)	5·2±2·6 (19)
Controls	58·4±5·3 (23)	21·4±2·4 (26)	2·5±0·4 (26)	0·7±0·2 (26)
Alternative pathway activity (presence of Mg EGTA)				
Sickle cell anaemia	86·3±15·1 (8)	57·2±26·8 (17)	50·8±39·9 (17)	11·9±9·4 (17)
Controls	69·2±8·0 (10)	25·6±3·9 (16)	4·6±1·3 (16)	0·8±0·2 (16)

* Mean±s.e.m. (number of sera tested in parentheses).
† Significantly greater than controls ($P < 0·02$).
‡ Significantly greater than controls ($P < 0·001$).

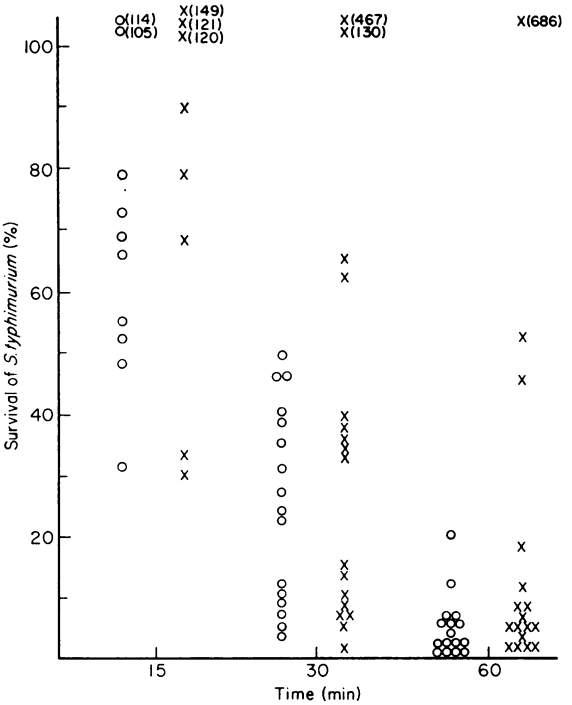

FIG. 2. Bactericidal activity of sickle cell anaemia (×) and control (○) sera containing Mg EGTA against *S. typhimurium*.

$$\text{Per cent of bacteria surviving} = \frac{\text{viable bacteria at 15, 30, or 60 min}}{\text{viable bacteria at 0 time}}.$$

Values in parentheses greater than 100% indicate bacterial growth.

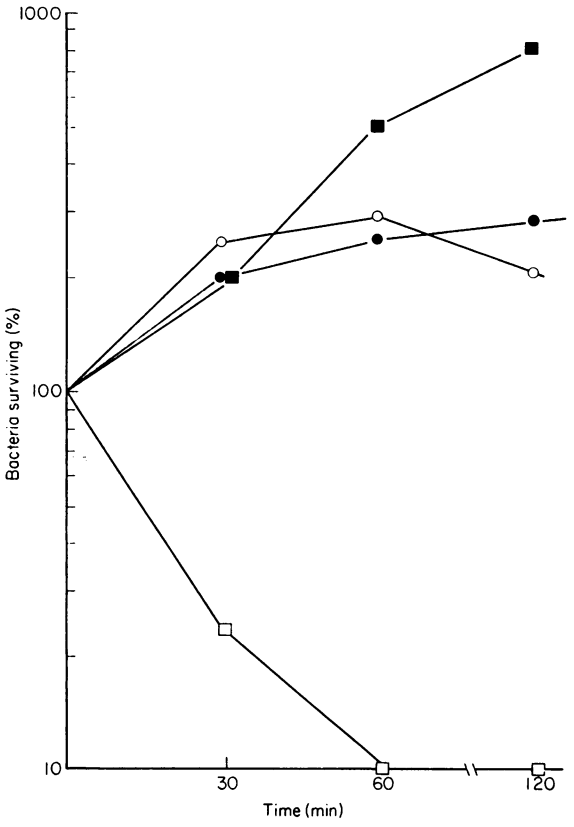

FIG. 3. The influence of EGTA on the viability of *S. typhimurium* incubated in control serum or in Hanks' balanced salt solution for the indicated periods of time.

$$\text{Per cent of bacteria surviving} = \frac{\text{viable bacteria at 30, 60, or 120 min}}{\text{viable bacteria at 0 time}}.$$

Values in parentheses greater than 100% indicate bacterial growth. (■) Mg EGTA in Hanks' solution; (●) EGTA in Hanks' solution; (○) EGTA in serum; (□) Mg EGTA in serum.

TABLE 2. Serum complement and antibody determinations—sickle cell anaemia and controls

Experimental group	No.*	C3 (mg/100 ml)	No.	Factor B (mg/100 ml)	No.	Haemolytic complement (CH_{50} units)	No.	Antibody against *S. typhimurium* (log_2 titre) Total	2-MRA†
Sickle cell anaemia	14	117·6±8·7‡§ (87–215)	14	22·7±2·1 (14–46·5)	16	36·7±2·0 (19–49)	22	3·4±0·4 (1–8)	2·0±0·4 (0–7)
Controls	23	143·0±5·8 (100–200)	23	21·3±0·8 (11–26·8)	23	37·1±1·3 (28–54)	21	3·1±0·3 (1–6)	1·8±0·3 (0–4)

* Number of sera tested.
† 2-mercaptoethanol-resistant antibody (IgG).
‡ Mean±s.e.m. (range in parentheses).
§ Significantly less than controls ($P < 0·025$).

TABLE 3. Serum iron, total iron-binding capacity (TIBC) and per cent saturation of TIBC—sickle cell anaemia and controls

Experimental group	No.*	Iron (μg/100 ml)	TIBC (μg/100 ml)	Per cent saturation of serum TIBC
Sickle cell anaemia	15	113·5±8·1† (70–188)	293·9±8·6‡ (227–349)	38·6 (26–65)
Controls	19	109·6±6·7 (47–174)	340·2±8·3 (280–395)	32·2 (15–51)

* Number of sera tested.
† Mean±s.e.m. (range in parentheses).
‡ Significantly less than controls ($P< 0·001$).

Serum agglutinating antibody

Most control and SCA sera had low titres of agglutinating antibody against the test organism (Table 2). There was no difference between antibody levels in the two groups of sera.

Serum iron and iron-binding capacity

Serum iron and TIBC were examined because of the known influence of the iron–iron-binding capacity interaction on serum bactericidal activity (recently reviewed by Bullen, Rogers & Griffiths, 1972; Weinberg, 1974). As can be seen in Table 3, the TIBC of SCA sera was significantly lower than that of control sera. As a result, the per cent saturation of serum binding capacity was slightly elevated in SCA sera.

The study of serum iron and TIBC has subsequently been expanded to include thirty SCA patients and twenty-nine age-matched controls. In this larger study, iron values were still similar in both groups of sera. The TIBC of SCA sera was lower than that of control sera (298·0±7·9 *vs* 354·3±7·5 μg/100 ml). Furthermore, per cent saturation of iron-binding capacity was increased in SCA sera as compared to controls (38·0±2·0 *vs* 27·7±1·1). These differences between SCA and control sera were highly significant ($P<0·001$).

Correlation of serum studies

The results of bactericidal assays with SCA sera are listed in Table 4, along with the corresponding determinations of alternative pathway activity, C3 concentrations and serum iron and TIBC levels. Each SCA serum with decreased total bactericidal capability also exhibited abnormal alternative pathway function and/or low C3 levels. The relationship between decreased total bactericidal activity and alternative pathway dysfunction was significant ($\chi^2 = 5·242$, 1 d.f., $P<0·025$).

DISCUSSION

The predisposition to *Salmonella* infection in SCA has not been satisfactorily explained. As a step in further evaluation of anti-*Salmonella* defence mechanisms in SCA, we examined serum bactericidal activity against this organism. Those specific factors known to influence serum bactericidal capacity, including antibody, complement activity and the iron–TIBC interaction, were studied in detail. We included an evaluation of the alternative pathway for complement activation, which mediates the bactericidal activity of normal serum against certain Gram-negative bacilli, especially in the absence of specific antibody (Wardlaw & Pillemer, 1956; Götze & Müller-Eberhard, 1971; Root *et al.*, 1972; Reed & Albright, 1974).

Sera from eight out of nineteen sickle cell disease patients in the present study were deficient in killing *S. typhimurium*. Additional evaluation revealed three abnormalities which might account for decreased

TABLE 4. Serum bactericidal studies in patients with sickle cell anaemia

Patient	Serum bactericidal activity: Total	Serum bactericidal activity: Alternative pathway	C3 (mg/100 ml)	Iron/TIBC (g/100 ml)
1	↓*	↓†	135	136/270 (50)‡
2	↓	↓	215	n.d.§
3	↓	N	87	70/273 (26)
4	↓	↓	110	n.d.
5	↓	↓	115	n.d.
6	↓	N	110	97/282 (34)
7	↓	↓	n.d.	n.d.
8	↓	N	95	89/227 (39)
9	N	N	140	89/349 (26)
10	N	N	90	120/306 (39)
11	N	N	135	n.d.
12	N	N	105	153/342 (45)
13	N	n.d.	95	96/311 (31)
14	N	N	n.d.	n.d.
15	N	N	n.d.	114/306 (37)
16	N	N	n.d.	188/288 (65)
17	N	N	n.d.	147/291 (51)
18	N	N	n.d.	116/342 (34)
19	N	n.d.	105	80/263 (31)
Controls	—	—	143±6¶	110±7/340±8 (32±2)

N = Normal; ↓ = decreased.

* Decreased total serum bactericidal activity = bacterial survival in serum > 45% at 30 min and/or > 10% at 60 min.

† Decreased alternative pathway bactericidal activity = bacterial survival in serum +Mg EGTA > 60% at 30 min and/or > 15% at 60 min.

‡ Serum iron/total iron-binding capacity (per cent saturation of TIBC in parentheses).

§ n.d. = Not done.

¶ Mean ± s.e.m.

serum antibacterial activity. These findings were: diminished function of the alternative complement pathway, decreased C3 concentration and low serum iron-binding capacity. The association between decreased serum bactericidal activity and abnormal alternative pathway function was statistically significant. C3 levels in SCA sera with decreased bactericidal activity, while lower than in control sera, were similar to concentrations in SCA sera with normal bactericidal activity. The third abnormality, low iron-binding capacity, did not appear to correlate with bactericidal activity.

Decreased pneumococcal opsonizing activity associated with deficient function of the alternative pathway in sickle cell disease sera was described by Johnston *et al.* (1973). This deficiency has been confirmed by two additional studies (Koethe, Casper & Rodey, 1976; Wilson, Hughes & Lachmann, 1976), but other investigators reported normal alternative pathway function in SCA sera (Strauss, Forristal & West, 1975; Provisor, Allen & Baehner, 1976; Bjornson, Gaston & Zellner, 1976). Whatever the reason for these discrepancies, our study demonstrates a defect in the alternative pathway of SCA sera and shows that this defect correlates with decreased killing of *Salmonella*, an important pathogen in sickle cell disease.

The nature of the alternative pathway defect is not known. Serum concentrations of alternative pathway factor B were normal in SCA sera. In our studies (including this report and additional investigations) diminished serum C3 concentration has been confirmed in a total group of thirty-four SCA patients.

However, the decreased C3 concentration does not explain the alternative pathway dysfunction, since these parameters do not appear to correlate (Table 4). Previous studies have yielded conflicting report of factor B concentrations (Koethe *et al.*, 1976; Wilson *et al.*, 1976) and C3 levels (Johnston *et al.*, 1973; Ringelhann & Konotey-Ahulu, 1973) in SCA.

The other potential cause of decreased bactericidal activity in SCA serum was the low total iron-binding capacity. Since serum iron-binding capacity is due to avid binding of this metal by transferrin (Bullen *et al.*, 1972; Weinberg, 1974), the reduction of TIBC indicated that transferrin concentrations were decreased in SCA sera. As a result of this low transferrin level, the per cent saturation of iron-binding capacity was increased in some SCA sera. An increase in, or total saturation of, transferrin iron-binding capacity will diminish or abolish the antibacterial activity of normal serum (Bullen *et al.*, 1972; Weinberg, 1974). In fact, the addition of sufficient $Fe(NH_4)_2(SO_4)_2$ to saturate the binding capacity of transferrin will completely eliminate the ability of normal serum to kill *S. typhimurium* (W. L. Hand & N. L. King, unpublished observations). Furthermore, parenteral administration of haemoglobulin or iron-containing compounds to animals will increase the severity of experimental infections (Bullen *et al.*, 1972; Weinberg, 1974). Since SCA and other haemolytic anaemias are associated with an increased frequency of salmonellosis, some investigators have suggested that these infections are related to the iron-overload state (Weinberg, 1974). However, most SCA patients have normal serum iron levels (our study, Finch *et al.*, 1970), probably because of rapid iron turnover in their active bone marrows. In addition, we found that the modest increase in per cent saturation of iron-binding capacity in SCA sera did not account for diminished bactericidal activity (Table 4). Accumulation of iron in the reticuloendothelial system and in areas of infarction in SCA may interfere with local antibacterial defenses, but this possibility has not been evaluated.

Is there a unifying explanation for the observed serum deficiencies in SCA? Although definite proof is lacking, we suggest that in SCA a decreased synthesis of transferrin and of those complement components produced in liver and spleen may be responsible for the serum deficiencies. Transferrin is produced by macrophages (Haurani, Meyer & O'Brien, 1973) and by liver tissue (probably including hepatocytes) (Jeejeebhoy *et al.*, 1975). C3 is synthesized in the liver, possibly in both hepatocytes and macrophages (Stecher & Thorbecke, 1967; Alper *et al.*, 1969; Ruddy, Gigli & Austen, 1972). Since many complement system factors are produced by macrophages of liver and spleen (Ruddy *et al.*, 1972), synthesis of one or more alternative pathway components may also take place in these same cells (Johnston *et al.*, 1973). The postulated liver and splenic dysfunction in SCA could presumably be related to sickling of red cells with associated microcirculatory stasis and/or to marked phagocytosis of erythrocytes by macrophages. Demonstrations of splenic dysfunction ('functional asplenia') in SCA (Pearson *et al.*, 1969) and of impaired reticuloendothelial system function in animals with naturally occurring or induced haemolytic anaemia (Kaye *et al.*, 1967) are compatible with this concept.

The significance of the compromised ability of SCA serum to kill *Salmonella* is uncertain. However, it is of interest that the majority of Gram-negative bacilli isolated from the blood of patients with bacteraemia are resistant to the bactericidal effect of normal serum (Roantree & Rantz, 1960; Vosti & Randall, 1970; Simberkoff, Ricupero & Rahal, 1976). It has been suggested that the major protective function of normal serum bactericidal activity is prevention of blood stream invasion by susceptible organisms (Roantree & Rantz, 1960; Vosti & Randall, 1970). Therefore, deficient serum anti-*Salmonella* activity may increase the risk of *Salmonella* bacteraemia in sickle cell disease.

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