Pseudosickling of Hemoglobin Setif

By Samuel Charache, Eva Raik, David Holtzclaw, Patricia J. Hathaway, Elizabeth Powell, and Patricia Fleming

Hemoglobin Setif produces pseudosickling of red cells in vitro; the nature of the process and the conditions that "trigger" it are unknown. Studies of red cells, hemolysates, purified hemoglobin solutions, and artificial mixtures of Hb A and Setif suggest that pseudosickling is produced by intracellular crystallization of insoluble hemoglobin. Increased toxicity of the surrounding medium accentuates the process, probably by causing a rise in intracellular osmolality in the renal medulla is similar to that which produces pseudosickling in vitro, carriers do not have renal dysfunction of the type found in patients with sickle cell disease.

PSEUDOSICKLING of red cells containing Hb Setif (α 94 Asp → Tyr) was first described by Drupt et al12 and has been reported by others.3 The appearance of deformed cells does not resemble either truly sickled cells or the sickled cells of deer and other species: their contorted, elongated form gives an impression of twisted rods rather than the anisotropic domains characteristic of classic sickling disorders. Like the red cells of deer and unlike true sickle cells, they have not been found in vivo, and they have not been recognized to produce a clinical disorder.

Conditions required for deformation of Hb Setif-containing red cells have not been defined. Drupt found that incubation of a small volume of blood (50 μL) in an empty 5-mL plastic tube at 37°C yielded optimal results,4 and he and his coworkers showed that the phenomenon requires oxyhemoglobin, not deoxyhemoglobin.5 They suggested that dehydration of red cells might be involved in sickling, and Raik et al3 showed that sickling could not be related to variations in the salt composition of isotonic suspending solutions. The requirement for oxyhemoglobin may perhaps be explained by the site of the amino acid substitution6: replacement of Asp α94 (G1) by tyrosine would be expected to disrupt a hydrogen bond that stabilizes the oxy, but not the deoxy, conformation of the hemoglobin molecule.6

If we are to truly understand the pathogenesis of sickle cell disease, the absence of clinical illness and of in vivo sickling in almost all persons with sickle cell trait is as important as their presence in patients with sickle cell anemia. Normal βA globin chains, either in hybrid molecules (αβ4αβ5) or in homotetramers (α4β2) can copolymerize with Hb S,10 and AS erythrocytes can sickle, but they usually do not at normal blood PO2, and most heterozygotes remain symptom free. Heterozygous carriers of Hb Setif are also symptom free1-3; their freedom from more than minor hematologic abnormalities (see below) is usually attributed to the low proportion of the abnormal hemoglobin in their red cells (12% to 24%). Raik et al studied a compound heterozygote (II-1),3 with Setif/alpha and beta thalassemia, whose red cells contained 30% of the abnormal hemoglobin; he too showed neither vaso-occlusive crises nor hemolysis. To further characterize the behavior of red cells containing Hb Setif and to compare them with more familiar hemoglobinopathies, we have further defined the conditions required for pseudosickling. Suggestions are made concerning the nature of the process and the lack of clinical consequences.

PATIENTS

The compound heterozygote (FIK, II-1),3 his mother PEY, and his brother FER were studied. All were carriers of Hb Setif; FIK had microcytic red cells (Coulter Counter) with target cells and hypochromia, as did his mother and brother to a lesser degree (Table 1), but Howell-Jolly bodies and siderocytes were not seen. The mother had been treated for menorrhagia and had had recurrent urinary tract infections with hematuria. Serum electrolytes, urea nitrogen, and creatinine were normal in all three patients, except for minimally elevated chloride in PEY (109 mmol/L). After overnight dehydration PEY was unable to concentrate her urine, but her sons were normal in this regard. Ophthalmologic examination (by Dr M. Hargrave) was normal in all three patients, except for a pigmented area in the temporal retina of FIK.

MATERIALS AND METHODS

For initial studies blood was obtained from the Setif/thalassemia compound heterozygote referred to earlier and a normal control. After collection in Sydney in ACD anticoagulant solution, the samples were shipped to Baltimore in ice and arrived within 48 hours with no evidence of hemolysis. Red cells were washed with 0.15 mol/L NaCl solution, gassed with carbon monoxide, and stored at 70°C until further use. Studies of sickling and oxygen affinity were done at Westmead Hospital, near Sydney, using blood collected in Na2EDTA from three members of the affected family.

Pseudosickling was evaluated by incubating red cells in buffered potassium phosphate (4 to 5 mmol/L) or Tris HCl (0.01 mol/L) buffered NaCl or KCl solution for varying periods of time and at various temperatures. Osmolality was adjusted by alteration of NaCl or KCl concentration, and was measured with an Advanced Osmometer. After incubation, cells were fixed in 3.0% glutaraldehyde in phosphate buffered 0.15 mol/L NaCl solution pH 7.3. For estimating the proportion of deformed cells, samples were coded so that the observer did not know how they had been treated. All cells were counted by a single observer, and cells from different experiments (Table 2) were counted separately. Four hundred cells were counted and were considered as sickled only if they had the typical "sickled" shape (Fig 1). Crenated cells were also enumerated; it seemed likely that some of them represented transitional forms between discocytes and sickled forms, for their numbers decreased as

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overnight by ultrafiltration. A precipitate formed only in samples of solubility of Hb Setif, the hemoglobin concentration of the supernate, the specimen was mixed and equilibrated for several hours, and a small amount of buffer was added to the precipitate. If the supernatant was too small (about 0.2 mL) to be handled (typically, 6 or 10 mL), the concentration remained more or less constant with repeated centrifugation (eg, concentrators and centrifuged at 10,000 rpm (RCF = 12,100 g) for ten minutes to sediment any precipitate. Supernatant fluid was decanted, a 15 to 25 μL sample was removed for measurement of hemoglobin concentration, and the remainder was returned to the concentrator and the process repeated until the volume of supernatant fluid was too small (about 0.2 mL) to be handled (typically, 6 or 7 cycles). In such experiments if the supernatant hemoglobin concentration remained more or less constant with repeated centrifugation (eg, Hb Setif in Fig 2B), that concentration was considered the “solubility” of the hemoglobins. If the concentration rose continuously, one could only say that “solubility” exceeded the final concentration achieved. If one started with a dilute solution (Fig 2A), the final concentration achieved might well be less than the solubility.

For experiments involving deoxyhemoglobin, carboxyhemoglobin

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**Table 1. Carriers of Hb Setif**

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age</th>
<th>Hb A (%)</th>
<th>MCV (fL)</th>
<th>Hb MCV (%)</th>
<th>Hb F (%)</th>
<th>Urine/Serum Osmolarity (mosm/kg H2O)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEY</td>
<td>F</td>
<td>52</td>
<td>80</td>
<td>14.3</td>
<td>4.9</td>
<td>5.7 1.3 0.6 277/290</td>
</tr>
<tr>
<td>FIK</td>
<td>M</td>
<td>28</td>
<td>3.2</td>
<td>39.5</td>
<td>12.6</td>
<td>14.0 2.8 0.5 1081/291</td>
</tr>
<tr>
<td>FER</td>
<td>M</td>
<td>26</td>
<td>14.0</td>
<td>78</td>
<td>16.4</td>
<td>5.7 6.6 1.9 0.7 1092/283</td>
</tr>
</tbody>
</table>

Abbreviation: MCHSC, mean corpuscular hemoglobin concentration in buffer of given osmolality, calculated from hemoglobin, microhematocrit value, and proportion of Hb Setif.

Urine and serum osmolarity were measured after overnight abstension from water.

Proportions of Hb Setif and A2 were measured by elution from strips of cellulose acetate after electrophoresis at pH 8.6; 1.0% A2 was seen in sample from FIK; but no “split” A2 was seen in samples from PEY or FER. The % Hb Setif in FIK is higher than previously reported; his MCHSC in isotonic buffer (285 mosm/kg H2O) was approximately 10.2 g/dL.

the proportion of clearly sickled cells increased. Apparent differences in sickling properties from day to day (compare FER in Table 2) were more likely due to variation in the observer than in the red cells. Red cell indices for cells suspended in hypertonic buffer were based upon automated (Coulter Counter) hemoglobin and red cell determinations and microhematocrits centrifuged for 15 minutes. Centrifugation for longer periods did not result in greater packing of the red cells. Cells were considered oxygenated when studied in nitrogen, added to a closed tube, a drop of red cell suspension was added, additional N2 was blown over the surface, and the tube was capped.

For purification of Hb Setif, CO-equilibrated buffers and hemolysates were used. Fractions were separated by chromatography on diethyl aminoethyl (DEAE)-Sephadex14 checked for purity by electrophoresis at pH 8.6, rechromatographed if necessary, and concentrated by ultrafiltration through cellophane membranes after reaggassing with CO. Concentrated (5 to 10 g/dL) purified solutions of Hb A or Setif were stored under CO at -70°C. In an attempt to measure solubility, samples of Hbs A and Setif were further concentrated overnight by ultrafiltration. A precipitate formed only in samples of Hbs A or Setif (5 to 10 g/dL) were dialyzed overnight at 5°C against 5 mmol/L potassium phosphate in 150 mmol/L KCl (pH 7.2 unless specified otherwise) saturated with carbon monoxide. Dialyzed solutions were diluted with buffer to a chosen hemoglobin concentration (usually 4 g/dL), centrifuged, and regassed with carbon monoxide. These solutions were added to Centricon-10 (Amicon Corporation, Scientific Systems Division, Danvers, MA) filters (10 kd nominal cutoff). Each concentrator was filled with 2 mL of hemoglobin and then centrifuged at 6,000 rpm (RCF = 4,340 g) in a Sorvall SS-34 rotor at 5 to 10°C. In most experiments, the centrifuge was stopped at five-minute intervals, the hemoglobin solutions above the filters were mixed, and centrifugation was resumed. At 15- to 20-minute intervals the hemoglobin solutions above the filters were removed from the concentrators and centrifuged at 10,000 rpm (RCF = 12,100 g) for ten minutes to sediment any precipitate. Supernatant fluid was decanted, a 15 to 25 μL sample was removed for measurement of hemoglobin concentration, and the remainder was returned to the concentrator and the process repeated until the volume of supernatant fluid was too small (about 0.2 mL) to be handled (typically, 6 or 7 cycles). In such experiments if the supernatant hemoglobin concentration remained more or less constant with repeated centrifugation (eg, Hb Setif in Fig 2B), that concentration was considered the “solubility” of the hemoglobins. If the concentration rose continuously, one could only say that “solubility” exceeded the final concentration achieved. If one started with a dilute solution (Fig 2A), the final concentration achieved might well be less than the solubility.

For experiments involving deoxyhemoglobin, carboxyhemoglobin

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**Table 2. Sickling of Hb Setif-Containing Red Cells**

<table>
<thead>
<tr>
<th>Osmolarity (mosm/kg H2O)</th>
<th>Effect of Osmolarity</th>
<th>Effect of pH</th>
<th>Effect of Time of Incubation</th>
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<tr>
<td>285</td>
<td>23</td>
<td>0</td>
<td>6.4 4 0.2 0.5 6.0 0.5</td>
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<tr>
<td>331</td>
<td>36</td>
<td>0.6 6.6 11 1</td>
<td>1 1 1 0.2</td>
</tr>
<tr>
<td>370</td>
<td>37</td>
<td>7.1 19 2.1</td>
<td>1.5 24 2 0</td>
</tr>
<tr>
<td>393</td>
<td>37</td>
<td>7.5 44 2.2</td>
<td>2 41 2 2</td>
</tr>
<tr>
<td>459</td>
<td>45</td>
<td>7.8 64 5</td>
<td>4 59 5 6</td>
</tr>
<tr>
<td>494</td>
<td>39</td>
<td>36 19</td>
<td>6 87 16 17</td>
</tr>
</tbody>
</table>

Note: Cells incubated at 23°C in 0.04 mol/L PO4/NaCl buffer: 24 hours, pH 7.3; 459 mosm/kg, pH 7.3; two hours, 436-451 mosm/kg H2O. See text for description of patients FIK, FER, and PEY.

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Fig 1. Pseudosickling of Hb Setif-containing red cells. (A) Light microscopy, Nomarsky optics. (B, C) Scanning electron microscopy.
was first converted to the oxy form by placing it in an IL 237 Tonometer (Instrumentation Laboratories, Inc., Lexington, MA) and exposing it to oxygen and light from a 200-watt bulb for 30 minutes at 5°C. The oxyhemoglobin was then converted to the deoxy form by an additional 30-minute exposure to nitrogen. Most of the sample became deoxygenated (84% to 92%) with negligible methemoglobin production (<2.4%). Each concentrator was filled with 1.5 mL of the deoxygenated solution, a few crystals of Na dithionite were added, and the solution was overlaid with a small amount of mineral oil. The concentrators had been preflushed with nitrogen, and they were enclosed in 50-mL centrifuge tubes that also had been preflushed with nitrogen, and oxygen. Saturation was measured with the CO-Oximeter, and pH and P02 with an IL-1312 blood gas analyzer (Instrumentation Laboratories).}

Hemoglobin concentrations were determined by conversion to cyanmethemoglobin with Drabkin's reagent. Percentages of oxyhemoglobin, carboxyhemoglobin, and methemoglobin were determined using an IL 282 CO-Oximeter (Instrumentation Laboratories). Deoxyhemoglobin was taken as the sum of these three subtracted from 100. Oxygen dissociation curves were constructed by equilibrating blood with gas mixtures containing 5% CO2, nitrogen, and oxygen. Saturation was measured with the CO-Oximeter, and pH and P02 with an IL-1312 blood gas analyzer (Instrumentation Laboratories).

RESULTS

Pseudosickling (Fig 1) was increased by increased osmolality (Table 2) and was greater at 37°C than at 23°C. None was seen after 20 hours incubation at 0°C. There was little difference between tris or phosphate buffers, and there was not much difference if cells were suspended in NaCl or KCl (data not shown). Sickling was enhanced in alkaline buffer, and decreased in acid (Table 2). Although MCHC increased promptly when cells were suspended in hypertonic buffer (within an hour), sickling did not become marked until several hours had passed (Table 2). Consistently, FIK showed more marked sickling and PEY less; the concentration of Hb Setif within their red cells was highest in FIK and lowest in PEY (Table 1). Very little sickling (which could not be distinguished from crenation) was seen if cells were deoxygenated before incubation.

The sediment collected after overnight ultrafiltration of purified Hb Setif contained long birefringent needle-like crystals (Fig 3). As noted in "Methods," Hb A did not precipitate under the conditions. The sediment after brief ultracentrifugation in Centricon tubes contained tiny clusters of birefringent needles that readily redissolved in buffer.

When dilute (<1 g/dL) solutions of carboxy Hbs A or Setif were centrifuged on Centricon-10 filters, hemoglobin concentration above the filter rose from <1 to 7 to 8 g/dL (Figure 2A). Trace amounts of precipitate formed in samples containing Hb Setif. When the starting solution was more concentrated (Fig 2B), a difference became evident. The hemoglobin concentration of samples containing Hb A rose steadily, exceeding 30 g/dL; considerable amounts of precipitate formed in samples containing Hb Setif at concentrations above 5 g/dL, and the supernatant hemoglobin concentration rose very slowly. It should be emphasized that equilibrium was never achieved in these experiments, and the "solubilities" described below represent differences in the behavior of Hb A and Setif rather than absolute numbers.

Since it seemed likely that a concentration gradient formed above the filter during a 15- to 20-minute "run" (the concentration being highest immediately above the filter), experiments were repeated, stopping the centrifuge every five minutes, mixing the supernatant fluid, and resuming centrifugation. Little difference was seen in comparison with earlier experiments. When precipitates were redissolved and the experiment repeated, results were similar, suggesting that the process observed was reversible precipitation rather than denaturation. Experiments were repeated after dialyzing samples to pH 6.6 or 7.5 (data not shown). There was no difference between the two samples of Hb A (27 g/dL final
that FIK's curve diverged from that of the control at high (68%) or low (8%) saturation.

DISCUSSION

Hb Setif, an alpha chain variant, usually comprises 12% to 15% of hemolysates, reflecting the usually low proportion of alpha chain variants and perhaps the instability of the abnormal hemoglobin. The high proportion in the blood of FIK (39.5%) reflects the coexistence of one or more thalassemia genes, as could be true for higher proportions observed in Saudi Arabian patients. The lower proportion in PEY's blood (in comparison to FER) may reflect coexisting iron deficiency.

The most remarkable property of Hb Setif is its capacity to induce sickling of red cells in vitro. "Sickling" has been described in human red cells containing Hb Barts, in addition to those containing Hb S, and crystals have been described in red cells containing Hb C. The initial patient with Hb Iαthalassemia was a compound heterozygote whose red cells contained 70% Hb Iα; clinically she exhibited no vaso-occlusive manifestations, her fresh red cells were not sickled, and the rheologic properties of her blood were unremarkable. Sickling of Hb Barts has been described only once, and that in normal red cells is a laboratory curiosity. Red cells containing crystals of Hb C can be found in freshly collected blood, and crystallization can be induced readily if the cells are incubated in 3% NaCl solution. Vaso-occlusive crises do not occur, but decreased deformability of the red cells has been implicated in the genesis of hemolytic anemia. Hb C is less soluble than Hb A, but the difference is not striking (35 v 4 g/dL).

Red cells of deer, some sheep, some mongooses, and other species can be induced to undergo sickling-like changes in vitro. Deoxygenation of the sample is not required; other factors involved include high pH, the presence of EDTA, and in vitro dehydration. Only in angora goats are distorted red cells thought to exist in vivo, and there polymerized hemoglobin was not found in freshly collected cells. Efforts to produce in deer disability related to the sickling phenomenon have been unsuccessful.

Our experiments with Hb Setif here were carried out with concentrated ("nonideal") solutions. Results are difficult to interpret because a significant portion of the solution is occupied by the solute, and the properties of the solute hemoglobin as a polyelectrolyte may alter its behavior. Equilibrium conditions probably were never achieved, and the "solubilities" recorded are only semiquantitative. With those reservations we interpret our data to show that Hb Setif is much less soluble than hemoglobin A, that mixtures of Hb A and Setif also show decreased solubility, and that conditions that raise the intracellular hemoglobin concentration foster pseudosickling. Of some interest, solubility is lower and (as in deer) sickling is enhanced at pH above 7.4 (Table 2).

The site of amino acid substitution in Hb Setif is an important contact point between α and β chains in the oxy
conformation. Loss of the contact should reduce oxygen affinity; contrary to expectation, oxygen affinity is only slightly perturbed, but mild instability is produced. The nature of the substitution, tyrosine for aspartic acid, might be expected to produce further anomalies because the side chains of the two amino acids differ in size and polarity. Aubert et al suggested that the difference produces a change in tertiary structure of the α chain in the vicinity of the substitution and suggested that the normal αβ contact within the molecule might be replaced by contacts between α chains in adjacent molecules. Such contacts could explain self-association into crystals, if the sites on the molecules were dissimilar, but it seems more likely that the change in tertiary structure is more widespread than they proposed, and that virtually all portion(s) of the external surfaces of the molecule could be involved.

Our observation that precipitates from mixtures of Hb A and Setif always yield Hb A on analysis could reflect entrapment of the latter hemoglobin. No attempt was made to wash the precipitates because of erratic results obtained when washing precipitates of pure Hb Setif (see "Methods"). The magnitude of the apparent interaction under certain conditions (70% Hb A, Table 2) suggests that mixed tetramers (ααββ) may be important units in crystals within red cells, as they are in polymers forming in mixtures of hemoglobin S with other hemoglobins. Existing as mixed tetramers in the crystal, they would be expected to reassociate into hemoglobins A and Setif during electrophoresis.

Edelstein et al recently suggested that self-association of Hb Setif, when oxygenated, could in turn affect oxygen binding. By analogy to Hb S, which associates in the T (deoxy) conformation and has anomalously low affinity in concentrated solution, one would expect self-association of Hb Setif (in the R or oxy conformation) to increase affinity. As with Hb S, the difference might only be present at high hemoglobin concentration (ie, red cells or artificially prepared solutions of the pure hemoglobin). Raik et al found normal affinity of FIK’s blood, and we found a minimal difference from normal, but further studies at varying concentrations of the purified abnormal hemoglobin would seem indicated.

At the temperature of the centrifuge (5°C to 10°C) Hb Setif began to precipitate (crystalize?) from crude hemolysates at Hb Setif concentrations about 6 g/dL and precipitated from solutions containing 25% Hb Setif at a total hemoglobin concentration of 23 g/dL (Fig 4), well below the MCHC of red cells. If blood from the three patients studied is compared at equal sickling (about 20%, Table 2) at 23°C, the intracellular Hb Setif concentration in those cells is similar (5.7, 5.7 and less than 10.2 g/dL, Table 1), despite the difference in temperature.

Pseudosickled cells appear to contain bundles of twisted fibers (Fig 1, and Fig 28 in ref. 4). Those fibers (and earlier stages of molecular aggregation) probably increase red cell rigidity, and one might expect to find infarctions in the hypertonic red cell medulla similar to those seen in sickle cell disease or trait. Neither FIK nor FER showed evidence of such infarctions; hematuria and hyposthenuria in their mother was considered by her physician to be a consequence of recurrent infection. Absence of lesions could reflect a difference in solubility due to the difference in temperature between our in vitro studies (5°C to 23°C) and 37°C, but it seems more likely that the lag between red cell shrinkage and sickling (Table 2) may account for absence of clinical findings: erythrocytes probably escape from the vasa recta before significant numbers of polymers or crystals have formed. A similar argument has been used to explain absence of lesions from vascular beds with a very low PO2 (ie, the myocardium) in sickle cell anemia, but “delay times” in that disease are probably much shorter than the lag times seen here.

### ACKNOWLEDGMENT

Dr Wilbur Hughes gave us the use of his laboratories at the Institute of Clinical Pathology and Medical Research at Westmead Hospital; we are most grateful to him and his staff for their friendliness and cooperation. We wish to thank Dr M. Hargrave for examining the patients’ eyes, Claire Norwood for measuring the proportion of Hb Setif in a large number of samples, the laboratory of Dr Ronald Nagel of the Albert Einstein College of Medicine for scanning electron microscopy, and Dr Gary Ackers for helpful discussion. We are indebted to Dr F. Drupt, who provided us with a copy of his doctoral thesis; the insights it provided were most useful in the present work.

### Table 3. Composition of Artificial Mixtures of Hb A and Setif During Ultrafiltration

<table>
<thead>
<tr>
<th>Initial Composition of Mixtures</th>
<th>Hb Concentration</th>
<th>% Setif</th>
<th>Hb Concentration</th>
<th>% Setif</th>
<th>Hb Concentration</th>
<th>% Setif</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Solution g/dL</td>
<td></td>
<td>Solution g/dL</td>
<td></td>
<td>Solution g/dL</td>
<td></td>
</tr>
<tr>
<td>Time (min)</td>
<td>Solution</td>
<td>Ppt</td>
<td>Solution</td>
<td>Ppt</td>
<td>Solution</td>
<td>Ppt</td>
</tr>
<tr>
<td>25</td>
<td>7.0</td>
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<td></td>
<td>19.1</td>
<td>35</td>
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<td>47</td>
</tr>
</tbody>
</table>

NOTE. Carboxyhemoglobin solutions of initial concentration 4 g/dL, in 5 mm PO2 0.15 mol/L NaCl, pH 7.2 were subjected to ultrafiltration through Centricon-10 filters (see text).

Abbreviation: Ppt, precipitate.
REFERENCES

Pseudosickling of hemoglobin Setif

S Charache, E Raik, D Holtzclaw, PJ Hathaway, E Powell and P Fleming