Hemoglobin Mississippi (β^{44}ser → cys)

Studies of the Thalassemic Phenotype in a Mixed Heterozygote with β^+-Thalassemia

Martin H. Steinberg,* Junius G. Adams III,* W. Tully Morrison,*
D. Jeanette Pullen,‡ Robert Abney,‡ A. Ibrahim,§ and Ronald F. Rieder§
Veterans Administration Medical Center and Departments of Medicine and Pediatrics,‡*
University of Mississippi Medical Center, Jackson, Mississippi 39216; and Department of Medicine,§
State University of New York-Downstate Medical Center, Brooklyn, New York 11203

Abstract

Hemoglobin Mississippi (HbMS: β^{44}ser → cys) has anomalous properties that include disulfide linkages with normal β-, δ-, γ-, and α-chains, and the formation of high molecular weight multimers. While heterozygotes for HbMS are clinically and hematologically normal and carriers of the β^+-thalassemia gene in our family had mild microcytic anemia, the proband with HbMS-β^+-thalassemia had a hemoglobin level of 7 g/dl, mean corpuscular volume (MCV) of 68 fl, reticulocytes of 2–6%, HbF of 18%, marked anisocytosis and poikilocytosis, and splenomegaly, all features of thalassemia intermedia. With oxidant stress, her erythrocytes developed multiple dispersed Heinz bodies, but HbMS was only mildly unstable. HbMS was susceptible to proteolytic degradation in the presence of ATP. The unexpectedly severe clinical findings in HbMS-β^+-thalassemia may result from the proteolytic digestion of HbMS, as well as the excessive α-chains characteristic of β^+-thalassemia, which combined provide the increment of cellular damage that results in the phenotype of thalassemia intermedia.

Introduction

The thalassemias are characterized by varying degrees of reduced globin synthesis, while hemoglobinopathies are a result of the production of globin chains whose amino acid sequence is abnormal. Thalassemic hemoglobinopathies have features of both: they have alterations in the primary structure of globin, but are also associated with the reduction of globin chain synthesis and phenotype of typical thalassemia (1, 2). The first examples of this type of disorder were the Lepore hemoglobins, products of hybrid genes formed by nonhomologous crossingover between the δ- and β-globin alleles (3–6), and the α-globin chain termination codon mutants, characterized by elongated α-globin chains, and typified by Hb Constant Spring (7). Some thalassemic hemoglobinopathies are a result of mutations causing hyperunstable β- or α-globin chains (8–10), while coding region mutations that permit alternative splicing of pre-mRNA, and are associated with reduced normal globin synthesis have also been well characterized (11–13). In some cases it is not yet clear why there is reduced gene expression, leaving the molecular causes of the thalassemia phenotype still speculative (1, 14–17).

We have described a β-globin variant, hemoglobin Mississippi (HbMS)[1] $\alpha_2\beta_2^{44}$(CD3)ser → cys, that had the anomalous properties of forming disulfide linkages with β^A, δ-, γ-, and α-globin chains, as well as high molecular weight multimers.[2] In this report we describe in detail the hematological features associated with HbMS. The proband of our study, a mixed heterozygote for HbMS and β^+-thalassemia, has the phenotype of thalassemia intermedia.

Methods

Hematological studies. Standard hematological techniques were used. Blood counts and erythrocyte indices were obtained using electronic cell counters (Coulter Electronics, Hialeah, FL). HbA_2 was measured by cellulose acetate electrophoresis and spectrophotometric analysis of eluted fractions (18), as well as by DEAE-Sephadex chromatography (18). HbF level was determined by the 1-min alkali denaturation method (19) and the $^G\gamma$- and $^A\gamma$-chains separated by high performance liquid chromatography (HPLC) (20).

Globin biosynthesis studies were done as previously described (8, 21).

Heinz bodies were induced by incubating 0.1 ml of fresh blood with 2.0 ml of acetylphenylhydrazine (APH) solution in phosphate buffer, and examining the cells at increments of 15 min (22). Whole blood was mixed 1:4 (vol/vol) with a fresh solution of new methylene blue (NMB) prepared by adding 0.5 g of NMB and 1.6 g Na oxylate to 100 ml of distilled H_2O, incubated at 37°C, and examined at hourly intervals for 4 h, and again at 24 h.

Hemoglobin stability studies are described in detail elsewhere.[2]

Electron microscopy. Blood was drawn in heparinized tubes and red cells were centrifuged and fixed in 4% glutaraldehyde, buffered with cacodylate (pH 7.3) at 4°C, for 30 min. Cells were then washed in buffer and post-fixed for 30 min in 1% OsO_4 similarly buffered. For transmission electron microscopy (TEM), cells were then dehydrated in graded ethanol and embedded in Epon 812. Thin sections were obtained with a diamond knife and stained with uranyl acetate and lead citrate. For scanning electron microscopy, glutaraldehyde fixed cells were placed on poly-L-lysine coated cover slips, fast fixed in buffered OsO_4, dehydrated in graded ethanol, critical point dried, coated with gold-palladium, and studied in a JEOL 100 Cx TEMSCAN (JEOL USA, INC., Peabody, MA). All samples were collected, processed, and examined at the same time.

Proteolysis of HbMS. Rabbit reticulocytes were isolated as previously described from phenylhydrazine-treated animals (23). The cells were washed three times with 0.1 mM NaCl, 15 mM KCl, 25 mM Tris pH

Address reprint requests to Dr. Steinberg, (151), VA Medical Center, Jackson, MS 39216.

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1. *Abbreviations used in this paper:* APH, acetylphenylhydrazine; HbMS, hemoglobin Mississippi; MCV, mean corpuscular volume; NMB, new methylene blue; PCV, packed cell volume.

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7.8, and lysed with 1.6 vol of hypotonic buffer (10 mM Tris, pH 7.8, containing 0.5 mM dithiothreitol). Leupeptin (Sigma Chemical Co., St Louis, MO) 10 μg/ml, an inhibitor of certain other proteases, was added to protect the ATP-dependent proteolytic enzyme from possible degradation by other enzymes in the lysate. The cells were then homogenized using the A and B pestles of a Dounce homogenizer. The hemolysate was centrifuged at 15,600 *g* for 15 min followed by centrifugation of the supernate at 100,000 *g* for 60 min. 25 μl of the 100,000 *g* supernatant were assayed for proteolytic activity in a 50-μl reaction mixture in the presence or absence of 1 mM ATP and an ATP generating system consisting of 50 μg/ml creatine phosphokinase and 12 mM creatine phosphate as described (24). An ATP trap consisting of 10 mM glucose and 0.5 U of hexokinase was added to reaction mixtures lacking added ATP to consume any ATP that might be generated (25).

Hemolysates from the father (II-5), mother (II-10), proband (III-3), and the control subject (c) were utilized as substrates for the proteolytic activity. In addition to studying total hemolysate, the hemolysate prepared from washed erythrocytes was fractionated by gel filtration on a 2.5 × 60-cm column of Sephadex G-200 equilibrated with 10 mM Tris-HCl, 140 mM Na Cl, 5 mM KCl 1.5 mM $MgCl_2$, pH 7.4. The hemolysates were dialyzed against borate buffer, pH 9.0 (3.1 g boric acid/liter, 30.95 g sodium borate/liter) and rendered radioactive by reductive methylation using [^{14}C]formaldehyde (0.25 mCi in 0.14 mg) followed by treatment with sodium borohydride as described previously (26). 3 μl of a solution containing 0.9 mg/ml or 4.3 mg/ml of the radioactive hemoglobin was used to assay susceptibility to the ATP-dependent protease. Generation of TCA-soluble ratioactivity was measured and the degradation of labeled protein was expressed as the percentage of the acid-soluble counts divided by the added acid precipitable counts × 100. In addition, globin was prepared from the unfractionated radioactive lysates and similarly tested using 3-μl samples containing 2 mg globin/ml.

Results

Case reports. The proband, a 6.5-yr-old child of Chinese ancestry, was referred for the evaluation of anemia. She was asymptomatic and had shown nearly normal growth and development. Physical examination showed pallor of the mucous membranes and splenomegaly. The mother and two siblings of the proband, ages seven and one-half and three years, were well and had no abnormalities on physical examination. The father was also normal.

Recently, profound anemia, thrombocytopenia, and reticulocytopenia developed, in association with a febrile illness characterized by skin eruption. The clinical picture was consistent with fifth disease (erythema infectiousum). Transfusions were given and eventually her blood counts returned, to their prior levels. A similar febrile illness, but without hematological sequelae, later occurred in the two siblings of the proband.

Hematological findings. The pedigree of the family along with the level of HbA_2 and HbF and the results of globin biosynthesis studies are shown in Fig. 1 while the hematological data of the proband's immediate family are presented in Table I. II-5 had no detectable hematological abnormalities except for an occasional coarsely stippled erythrocyte, although he was the carrier of Hb MS. II-10 had typical heterozygous β^+-thalassemia on the basis of erythrocyte indices, HbA_2 level, and the α/β biosynthesis ratio. The two siblings of the proband who had heterozygous β^+-thalassemia had blood counts and HbA_2 levels virtually identical to that of the mother. The proband had a moderately severe anemia with features resembling β-thalassemia intermedia, but the globin biosynthetic ratio was similar to that of the mother. The blood film (Fig. 2) was reminiscent of that seen in the more severe β-thalassemias and was strikingly different from other family members. In addition, rare "ghost" cells, typified by a fine membrane without significant cytoplasm, were seen in the proband.

In five additional adult heterozygotes for Hb MS, the packed cell volume (PCV) was 39.7±2.1, mean corpuscular volume (MCV) 92.6±8.4 fl, and reticulocyte count, 1.3±0.5%. The levels of HbA_2 and F were normal.

Stability studies, reported elsewhere,[2] were normal in iso-

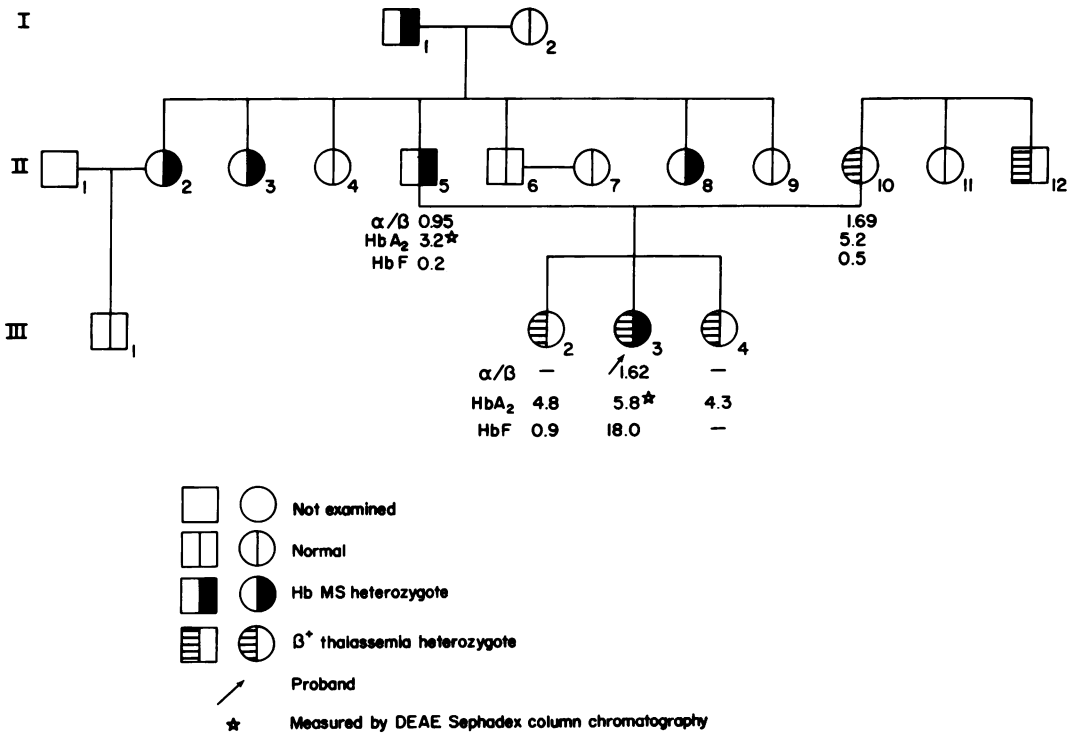

Figure 1. Family pedigree. The proband, III-3, is a mixed heterozygote for HbMS and β^+-thalassemia. The α/β biosynthesis ratios, HbA_2 and HbF levels are shown beneath each individual of the proband's immediate family.

Table I. Hematological Data—Hb Mississippi

	Hb*	PCV	RBC‡	MCV	MCH§	Reticulocytes
	g/dl		*10^9/liter*	*fl*	*pg*	*%*
II-5	15.4	47	5.3	89	29	0.6
II-10	10.5	34	5.5	61	19	0.4
III-2	11.4	33	5.5	61	21	—
III-3‖	6.9–7.6	25	3.7	68–73	20	2.5–5.7
III-4	10.6	30	4.9	61	22	—

* Hemoglobin concentration; ‡ Red blood cells; § Mean corpuscular hemoglobin; ‖ Proband.

propanol buffer at 37°C and in phosphate buffer at 50°C. At 60°C in phosphate buffer there was a 50 and 25% reduction in recoverable globin at 1 h in the proband and father, respectively.

Identification of Hb MS. These results are presented in detail elsewhere.[2] In brief, globin chain separation of the hemolysate prepared from the erythrocytes of the proband showed three abnormal β-globin peaks. When each peak was cleaved with trypsin and its constituent peptides were separated by HPLC, the sole abnormality was a serine to cysteine substitution at position 44 (CD3). The unusual electrophoretic behavior of Hb MS is described elsewhere.[2]

Heinz bodies. After incubation with APH, single large precipitates were seen in most cells of the proband at 15 min. The mother and father of the proband, and the control did not show precipitated hemoglobin. By 60 min occasional small, and multiple precipitates were seen in the cells of all family members and the control, and all cells of the proband had multiple small Heinz bodies with occasional cells still containing single large inclusions. By transmission electron microscopy, the Heinz bodies induced by APH were predominantly circumferential in the cells of the II-10 and II-5, as well as the control (Fig. 3, *B–D*), but were more numerous often aggregated and distributed throughout the cells of the proband (Fig. 3 *A*).

When whole blood was incubated with NMB, hemoglobin precipitates first appeared in cells of the proband after 3 h and were more numerous at 4 h. By 24 h both the proband (III-3)

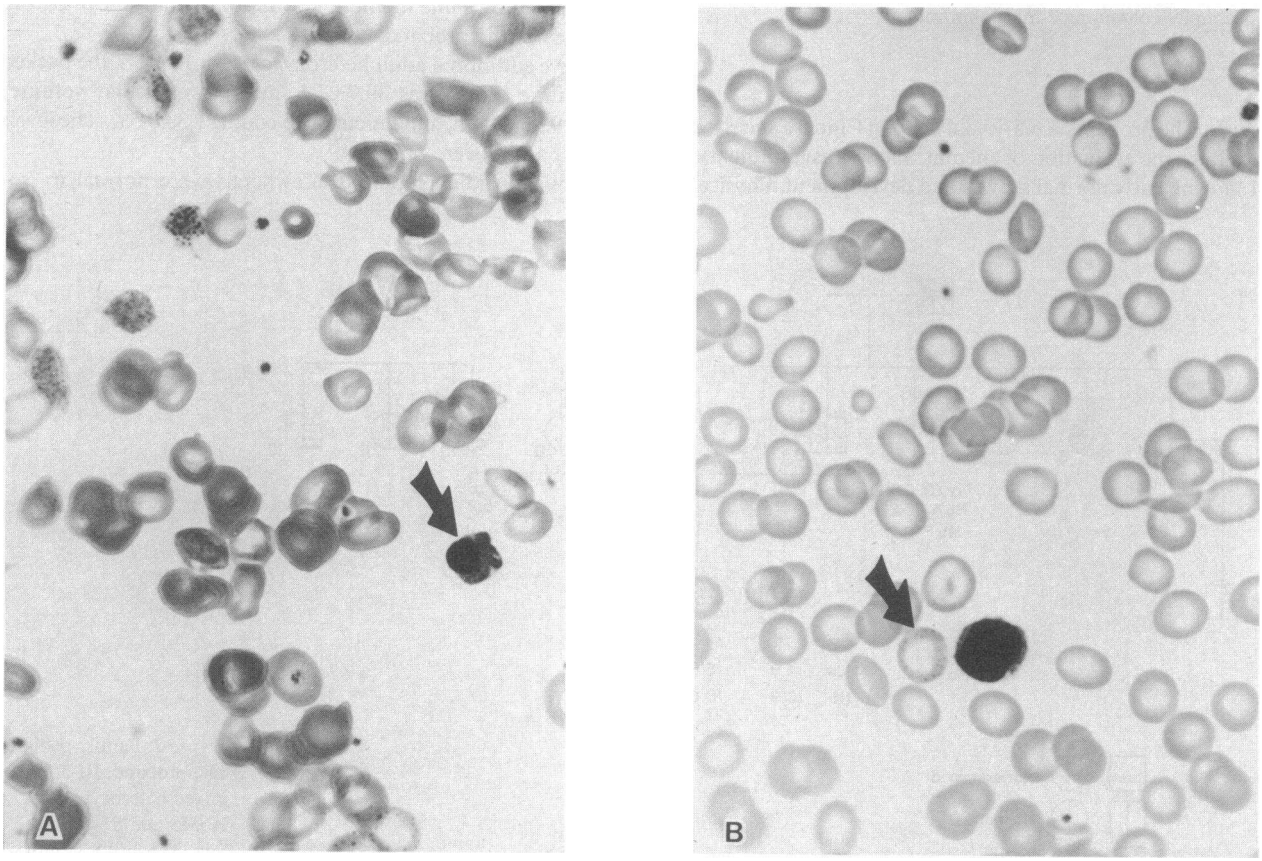

Figure 2. Peripheral blood films. Shown are the proband, III-3, with HbMS/β^+-thalassemia (A) and her 1 yr older sibling (B), III-2, with β^+ thalassemia. While hypochromia, microcytosis, and basophilic stippling (*arrow*) are present in III-2, the proband shows considerable poikilocytosis, anisocytosis, nucleated RBCs (*arrow*), and large numbers of heavily stripped cells. These findings are more typical of thalassemia intermedia, or major, than of simple heterozygous β-thalassemia. The blood film of (II-5, not shown) a carrier of HbMS alone, was entirely normal.

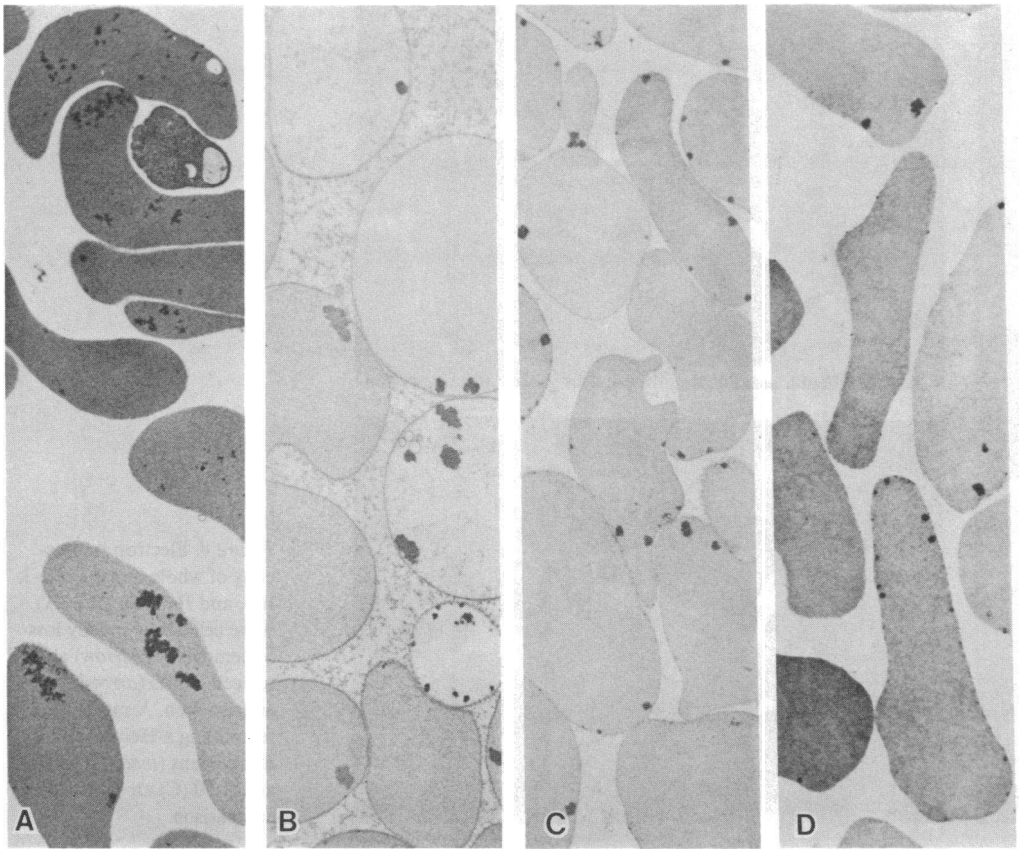

Figure 3. Heinz body distribution. Electron micrographs showing Heinz bodies induced following 60 min of incubation of whole blood with acetylphenylhydrazine A, III-3; B, II-10; C, II-5; D, control.

and her father (II-5) had large multiple inclusions. No inclusions were seen in the mother (II-10), a normal control and controls with α-thalassemia and $\alpha + \beta$-thalassemia up to 24 h of incubation.

Erythrocyte glucose-6-phosphate dehydrogenase deficiency was not present in any family member.

Electron microscopy. Transmission and scanning electron microscopy of fresh whole blood reflected the morphological changes seen by light microscopy. However, some cells of the proband (III-3) showed inclusions (Fig. 4 *A, inset*) resembling Heinz bodies. The most interesting finding in III-3 and in II-5, were the presence of erythrocytes that contained little hemoglobin and appeared as partial ghosts (Fig. 4 *A*). These cells were easily seen in III-3, and in II-5, but examination of at least 300 cells in II-10 and the normal control revealed only a single cell of a similar type.

Susceptibility to proteolysis. The percent of hemoglobin degradation that occurred when ^{14}C-labeled hemolysate of the proband and the normal control were incubated with a rabbit reticulocyte lysate in the presence or absence of ATP is shown in Fig. 5. With ATP, there is a greater than threefold increase in the percent protein degradation when the proband's hemolysate is compared with the control. A plateau occurred between 1 and 2 h of incubation and reached $\sim$ 4%. In the absence of added ATP, degradation of [^{14}C]Hb of the proband was about three times that of the control, but the plateau level was $< 2\%$.

The effect of hemolysate concentration upon degradation of [^{14}C]Hb in the proband, mother, father and control is shown in Table II. While the effects of concentration upon degradation seemed minimal, it appeared as if the hemolysates both parents of the proband had increased susceptibility to proteolysis when compared to the control, while the proband had about twice the level of degradation as did either of her parents. Also shown in Table II is the percent proteolysis when unfractionated globin, prepared from the hemolysate, served as the substrate for proteolysis.

Gel filtration separated the hemolysates of II-5 and III-3 into a high molecular weight fraction that eluted with the void volume and a normal hemoglobin peak.[2] The high molecular weight fraction contained HbMS, as well as HbA and HbF, while the normal hemoglobin peak contained only HbA and HbF.[2] The results, shown in Table III, indicate a three- to fourfold increase in the susceptibility to proteolysis in the high molecular weight fraction that contains HbMS multimers, when compared with the peak that contains only tetrameric normal hemoglobin. Differences in substrate preparation, including the gel filtration of hemolysates, as well as the use of a different preparation of proteolytic lysate, account for the differences in magnitude of proteolysis between these experiments and those using unfractionated hemolysates.

Discussion

HbMS in the simple heterozygote is indetectable by hematological examination and is not associated with clinically evident abnormalities. The β^+-thalassemia gene in this kindred, as yet uncharacterized, causes mild anemia with microcytosis and hypochromia, is associated with normal HbF levels, and causes the typical imbalance in globin chain synthesis. Yet the proband in this family, a mixed heterozygote for HbMS and β^+-thalas-

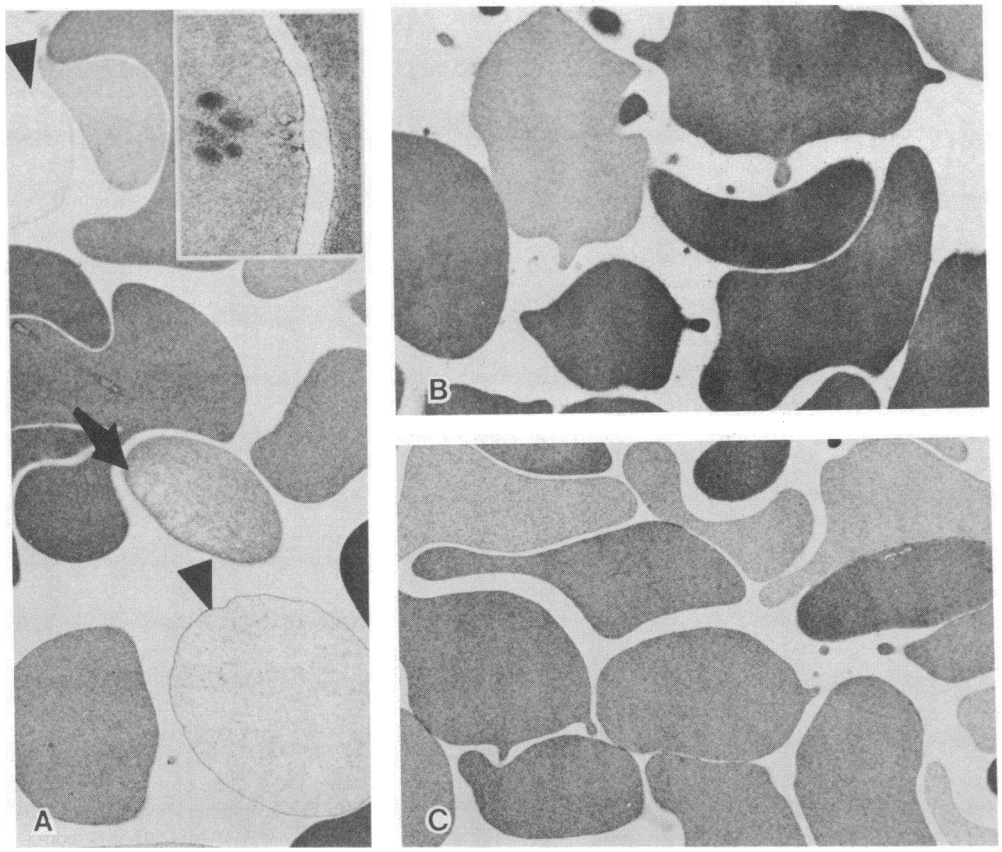

Figure 4. Electron microscopy of whole blood in III-3, II-5, and II-10. In III-3 (A) some cells show patchy loss of hemoglobin (*arrow*) and red cell ghosts (*arrowheads*) are also seen. A rare inclusion resembling a Heinz body is also present (*inset*). II-5 (B) and II-10 (C) are shown for comparison.

semia, had the phenotype of thalassemia intermedia typified by splenomegaly, moderately severe anemia with marked morphological abnormalities of the erythrocytes, and an elevated HbF level. While chronic transfusion is not yet needed, blood transfusions were used when our patient developed a severe aregenerative crisis, likely due to fifth disease. Thus, while HbMS itself cannot be considered a "thalassemic variant," its interaction with β^+-thalassemia appears to cause thalassemia intermedia. While there are relatively few examples of mixed heterozygotes for abnormal hemoglobins and β^+-thalassemia (27, 28), this interaction does not seem to be associated with thalassemia intermedia. HbS-β^+-thalassemia, is the most prevalent example of mixed heterozygosity for a variant hemoglobin and thalassemia.

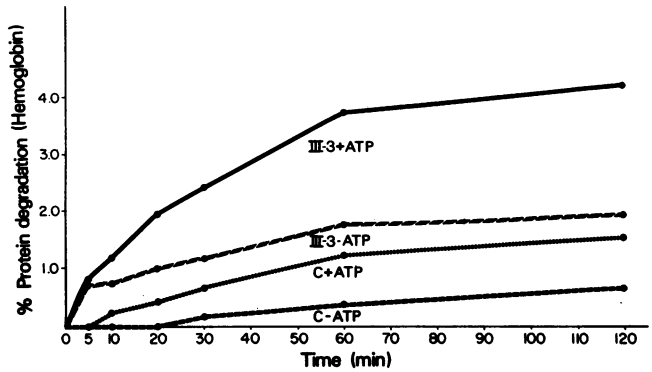

Figure 5. APT dependent proteolysis. Time course of protein (hemoglobin) degradation in the presence and absence of ATP in hemoglobin of the proband (III-3) and a normal control (C).

In affected individuals the hemoglobin concentration is 10–11 g/dl, mean cell volume ~ 70 fl, HbF 5%, and HbS, ~ 75% (27, 28). The β-thalassemia genes in Blacks are often "mild," with less suppression of β-globin synthesis than seen in Mediterranean populations (29). HbC-β^+-thalassemia, also predominant in Blacks, is a very mild anemia with a hemoglobin of concentration 12–13 g/dl (27, 28). Patients with HbE-β^+-thalassemia have a more severe disease (27, 28) but this is, at least in part, a result of the intrinsically thalassemic nature of the β^E gene (11, 12). Isolated cases of β^+-thalassemia with HbD and Hb Saki have been associated with relatively mild disease (27). In fact, other than HbS-β^0-thalassemia, which resembles sickle cell anemia, even mixed heterozygotes with β-globin variants and β^0-thalassemia also have mild clinical and hematological abnormalities (27, 28). While HbMS is unstable, its instability seems very mild compared to the unstable variants associated with hemolytic

Table II. Proteolysis of Hb Mississippi

	Hemolysate				Globin	
	0.84–0.90 μg/μl		4.20–4.53 μg/μl		2 μg/μl	
	+ATP	−ATP	+ATP	−ATP	+ATP	−ATP
II-5	2.04*	0.80	2.06	0.67	10.20	2.82
II-10	1.83	0.61	2.01	0.78	8.96	2.60
III-3	3.67	1.10	4.87	1.48	16.70	4.55
C	1.17	0.29	1.20	0.49	10.15	2.44

* Percent proteolysis.

Table III. Proteolysis in Gel Filtration Fractionated Hemolysate

	High molecular weight fraction		Normal hemoglobin fraction	
	1 μg/μl		1 μg/μl	
	+ATP	−ATP	+ATP	−ATP
II-5	32.4*	16.7	5.2	2.3
III-3	27.4	10.0	11.1	4.4
C	—	—	6.9	1.4

* Percent proteolysis.

anemia (30). Heinz bodies are nearly undetectable in freshly obtained blood, although the presence of an enlarged spleen makes it likely they would be removed from the circulating red cells (31). The instability of HbMS is insufficient to cause hemolytic disease in the simple heterozygote, and the hematological abnormalities of the proband are those seen in thalassemia and not the hemolytic anemia associated with unstable hemoglobins (32). Additionally, the interactions of β-thalassemia with unstable hemoglobins are not typified by severe anemia (27). The evidence therefore suggests that the instability of HbMS alone cannot explain the anemia of the proband.

HbMS appears to be sensitive to oxidative denaturation as suggested by the pattern and course of Heinz body formation under conditions of oxidative stress. This is evident when either APH, a potent oxidizing agent, or NMB, a considerably milder oxidant stress, is used. The morphology of Heinz bodies induced in cells of the proband is quite different than seen other family members or controls. In the severe β-thalassemias, the redundant α-chains that form Heinz bodies do not appear to be membrane bound, but form within the cytoplasm (32). In contrast, Heinz bodies of HbH disease, formed of excess β-globin chains, appear in apposition to the membrane, although their exact mechanism of binding is not clear (33). Recent studies also suggest that the red cells of HbH disease and β-thalassemia acquire different abnormal characteristics (34). Heinz bodies were rarely seen in fresh blood of our patient. The observation that Heinz bodies induced by APH had a distinctly different form and location in the proband, when compared to other family members, or the control, suggests that either a greater portion of the probands hemoglobin is susceptible to oxidant stress, or that the sites and mechanisms of hemoglobin denaturation differ in the proband and her father. Attempts to quantify the proportion of HbMS present indicate that the proband has about 40% HbMS while the father has ~ 14%,[2] however because of the anomalous properties of this variant, such measurements must be tentative.

HbMS is more susceptible to proteolytic digestion either in the presence of absence of ATP. Erythrocyte proteases are present in both cell cytoplasm and membrane, and those that are soluble are active at neutral pH and are ATP-dependent enzymes (23, 35, 36). They are capable of degrading abnormal proteins (23, 37, 38) and are likely to hydrolyze proteins that become redundant (39), such as the free α- and β-chains that appear in the thalassemias (40–47). These proteases may preferentially attack α-globin chains (46) and denatured globin chains, and play a role in the pathophysiology of the thalassemias and unstable hemoglobins. This ATP-dependent proteolytic system is found only in nucleated erythroid cells and reticulocytes and is not present in mature erythrocytes. However, erythrocytes have been shown to have the capacity to degrade hemoglobin that has been damaged by oxidant agents in a process that is independent of ATP (48). The unfractionated hemolysate of the proband was about three times as susceptible to proteolytic digestion as that of the control, when used as a substrate for a reticulocyte derived ATP-dependent proteolytic system. Purified globin extracted from the hemolysate showed a similar increment in degradation (Table II). The greater amount of globin digestion when compared to hemoglobin may be due to the stabilizing effects of heme upon the tetramer (30). Heterozygotes with HbMS or β^+-thalassemia had intermediate levels of hemoglobin degradation. These results are consistent with the lower HbMS concentrations in the simple heterozygote and the excessive α-globin chains present in the β^+-thalassemia carrier. However, when globin was used as a substrate, both simple heterozygotes and the control showed similar levels of protein degradation. When the hemolysate of II-5 and III-3 was fractionated into a normal hemoglobin fraction and a fraction that contained high molecular weight multimeric hemoglobin, this latter peak was three- to fourfold more susceptible to proteolytic digestion than the normal peak. The normal hemoglobin fraction and the gel filtration prepared control hemolysate were equally susceptible to proteolysis. These experiments indicate that HbMS has increased susceptibility to proteolytic degradation. An increased tendency to undergo digestion has been previously demonstrated in work with artificially generated abnormal hemoglobins in the rabbit (23) and more recently in the cases of the markedly unstable Hbs Gun Hill and Leiden (49, 50).

Our studies suggest the following hypotheses in explanation of the unexpectedly severe clinical characteristics of HbMS-β^+-thalassemia. When the gene for HbMS is present in trans to a β-thalassemia gene, cell damage is promoted as a result of the higher concentration of this mildly unstable variant. In addition, the thalassemia phenotype becomes more pronounced in the presence of relatively low levels of HbMS. In vivo, the increased susceptibility of the hemolysate of the proband to degradation by ATP-dependent proteases, and possibly proteases that do not require ATP and have the capacity to hydrolyze oxidant-damaged hemoglobins, is most likely a function of the presence of two substrate species; HbMS and free α-globin chains. Together, their destruction provides the increment of cellular damage that results in the phenotype of thalassemia intermedia.

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