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36. Requests for reprints should be addressed to: Paul W. K. Wong, M.D., Department of Pediatrics, Presbyterian-St. Luke's Medical Center, Rush Medical School, Chicago, Ill. 60612 (USA).
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Calcium
cystic fibrosis
mucin

Effects of Calcium on Intestinal Mucin: Implications for Cystic Fibrosis

J. F. FORSTNER [24] AND G. G. FORSTNER

Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada

Extract

A major feature of the disease cystic fibrosis is the excessive concentration of mucus within ducts and glands of mucous-producing organs. Some mucous secretions also show an elevation in calcium concentration. Using purified rat intestinal goblet cell mucin as a model mucin, we have investigated the effect of millimolar additions (1–25 mM) of $CaCl_2$ on the physical properties of the mucin. Isotonicity of incubation media was preserved in order to mimic in vivo conditions. $CaCl_2$ (8–15 mM) caused a 15–33% decrease in viscosity, no change in electrophoretic mobility in acrylamide gels, and a 20–30% decrease in solubility of the mucin. Solubility changes were reversed by the addition of EDTA (20 mM) to incubations. Insolubility was also produced in incubations of mucin with a mixture of soluble intestinal contents (NaCl washings). These findings strongly suggest that the mucin became smaller and more dense as calcium was added, a process most probably achieved by loss of intramolecular water.

Speculation

It is hypothesized that elevated concentrations of calcium within glycoprotein secretions of patient with cystic fibrosis may substantially increase the density and insolubility of mucins, promoting the formation of mucous "plugs."

Mucous secretions of patients with cystic fibrosis are excessive in amount in salivary glands, bronchi, intestine, gall bladder, and cervix (7, 12, 14). The calcium concentration in many of these secretions is also high, ranging from "slightly elevated" to over 100-fold in excess of normal (12). It has been hypothesized that Ca may contribute to the formation of mucous "plugs" in glands and ducts by causing polymerization, aggregation, or gel formation of otherwise normal mucin macromolecules. A few studies have shown that Ca is responsible for aggregation of small salivary proteins (4, 5, 13), but no studies have been performed to discover whether Ca changes the physical properties of purified mucin macromolecules of intestinal or bronchial origin.

Over the past few years a native goblet cell mucin has been purified in our laboratory from rat small intestine (9, 11). It resembles mucins purified by others from several human and animal organs (1, 4, 6, 17–19), in that it is a polydisperse viscous glycoprotein rich in carbohydrate, containing high quantities of serine, threonine, and proline within the peptide core.

We have used rat goblet cell mucin as a model for the investigation of Ca-mucin interaction. Previously we showed that rat mucin binds Ca ions, especially under conditions of low ionic strength, but that during binding Ca caused only minor physical changes in the mucin (8). In the present study we have measured the effects of Ca upon some of the physical properties of mucin in isotonic media in order to mimic more closely in vivo conditions. Our results indicate that Ca may alter the three-dimensional architecture of mucin so as to increase its density and exclude water. It is postulated that this mechanism may be involved in the formation of mucous plugs in cystic fibrosis.

METHODS

The preparation of goblet cell mucin (GCM) from rat intestine, radioactive labeling of the mucin using precursor [$^{14}C_1$]glucosamine, the techniques of polyacrylamide disc gel electrophoresis, analytic ultracentrifugation, and measurement of solubility of the mucin have been described in detail in earlier publications (8, 9, 11). Minor modifications and specific details are included as appropriate under Results.

Intestinal washings, when required for solubility experiments, were collected by washing the entire small intestine of each of 10 rats with approximately 20 ml 0.15 M NaCl. The washings were pooled, centrifuged at 4° at 30,000 × g for 15 min to remove particulate material, and the supernatant solutions saved. The soluble supernatant fluid was stored frozen in a dilute state or else concentrated isotonically 10-fold by an Amicon Dia-Flo ultrafilter

and then stored frozen as a concentrate. Both dilute and concentrated washing solutions were thawed immediately before use in solubility experiments with goblet cell mucin.

The water used in the preparation of all solutions was rendered ion free as described earlier (8). Chloride salts of various cations (Ba, Zn, Mn, Mg) were made using anhydrous powders (oven dried to constant weight) of the chloride salts. The usual buffers in solubility studies included 0.01 M Tris-HCl in 0.14 M NaCl, pH 7.4, or 0.155 M Tris-HCl, pH 7.4. In a few instances the buffer used was 0.15 M Hepes, pH 7.4, and the results were identical with those obtained using Tris or Tris-NaCl buffers.

Viscosity of goblet cell mucin was measured in a Cannon-Ubbelohde semimicro capillary viscometer at 20° as described earlier (8) and in a Brookfield Synchrolectric model LVT rotary viscometer over a wide range of shear rates (22–230 sec $^{-1}$). Units of viscosity were centistokes and centipoises for capillary and rotary viscometry, respectively, and were converted into units of viscosity "number" by the following equation: $\eta sp/c = \frac{\eta - \eta_0}{\eta_0} \div c$, where η_0 is the viscosity of the solvent, η is the mucin viscosity in that solvent, and c is the concentration of goblet cell mucin in grams, dry weight per 100 ml. Viscosity of the mucin at zero shear was obtained from viscosity data over a range of shear rates extrapolated to zero.

RESULTS

EFFECT OF Ca ON VISCOSITY OF GOBLET CELL MUCIN

The viscosity of mucin in 0.155 M Tris-HCl, pH 7.4, was examined over a range of mucin concentration and shear rate values using both capillary and rotary viscometry (Fig. 1). In the absence of Ca the viscosity varied with both shear rate and mucin concentration. In general, the viscosity was higher at low shear rates (- - -) and underwent a sharp increase at concentrations of mucin above 0.5 g/100 ml, at which visible gelation began. At the higher shear rates of capillary viscometry (——) gelation was not visible. In both cases the addition of CaCl$_2$ (5–25 mM) caused a decrease in viscosity, which was approximately 15–18% for zero shear and 33% for high shear conditions in 10 mM CaCl$_2$ (Fig. 1). At the higher concentrations of mucin, Ca appeared to retard gel formation at zero shear.

Under no conditions of goblet cell mucin concentration, temperature variation (20°–37°), shear rate, or CaCl$_2$ concentration
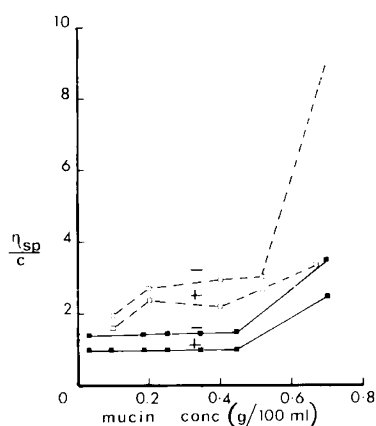

Fig. 1. Viscosity of goblet cell mucin. Rotary viscometry was performed over a range of shear rates (22–230 sec $^{-1}$) extrapolated to give zero shear rate values for viscosity (□—□). η_{sp}/c refers to viscosity number. Capillary viscometry values (■——■) were obtained using a Cannon-Ubbelohde semimicroviscometer. + and – refer to the presence or absence, respectively, of 10 mM CaCl$_2$ in the buffer solvent which was either 0.155 M Tris-HCl, pH 7.4, or 0.01 M Tris-HCl-0.14 M NaCl, pH 7.4. Each viscosity value was the average of triplicate determinations which did not exceed a range of more than ±0.05 viscosity number units.
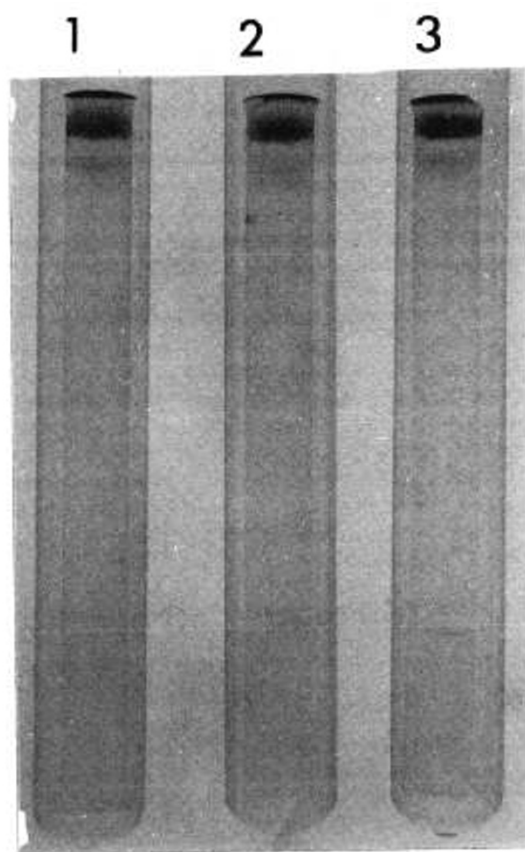

Fig. 2. Polyacrylamide disc gel electrophoresis of mucin. Goblet cell mucin (32 μg dry wt) was mixed with bromphenol blue dye in 60% sucrose with or without CaCl$_2$ (8 mM) in a final volume of 0.05 ml. Samples were applied to 4% acrylamide gels made in 0.15 M Tris-borate buffer, pH 8.6, with or without the addition of 8 mM CaCl$_2$. Gels were run for 55 min at 2 mA/tube and subsequently stained with periodic acid-Schiff reagent. In *gel 1* there was no Ca in the mucin sample or the gel; in *gel 2* there was CaCl$_2$ in the gel and the buffer during electrophoresis, and in *gel 3* there was CaCl$_2$ in the mucin sample, the gel, and the buffer during electrophoresis.

(1–50 mM) did Ca cause an elevation in viscosity suggestive of gelation or polymerization of the macromolecule.

POLYACRYLAMIDE DISC GEL ELECTROPHORESIS

In an attempt to rule out dissociation by Ca, which might have accounted for the lowering of viscosity, goblet cell mucin was subjected to electrophoresis in 4% polyacrylamide gels at pH 8.6 in the presence and absence of CaCl$_2$ in the gel and buffer systems (Fig. 2). As described earlier (9), the basic pattern consisted of one broad periodic acid-Schiff-positive band near the origin and two faint bands below. In the presence of CaCl$_2$ (3, 8, and 25 mM), the mucin did not undergo any detectable change in band widths, intensity of stain, or mobility.

SEDIMENTATION VELOCITY STUDIES

Goblet cell mucin (2.5 mg dry wt/ml) was subjected to boundary ultracentrifugation in 0.01 M Tris-HCl-0.14 M NaCl buffer, pH 7.4, containing 0, 1, 5, and 10 mM CaCl$_2$. In the absence of Ca, the Schlieren optical pattern consisted of a single peak with a leading shoulder (previously published (10)). No change in pattern was observed in the presence of CaCl$_2$. Thus, there was no indication, by either gel electrophoresis or sedimentation pattern, that Ca had caused the mucin to dissociate into lighter or smaller fragments.

It was observed, however, that the addition of CaCl$_2$ to GCM samples caused a decrease (18% at 10 mM CaCl$_2$) in the measurable concentration of GCM, as determined from Schlieren

peak areas (Table 1, *column 3*). This happened despite the original addition of exactly 2.5 mg mucin/ml to each cell, and was associated with a small but significant increase in calculated $s_{20,w}$ values (*column 4*). It was suspected that some of the mucin may have aggregated with $CaCl_2$ and sedimented rapidly to the bottom of the ultracentrifuge cell before the attainment of maximum rotor speed. Thus only the remaining soluble mucin was detected in Schlieren tracings. By comparing test samples with control GCM samples (no added $CaCl_2$) having the same GCM concentration, $s_{20,w}$ values were seen to be identical (Table 2). Thus the remaining soluble GCM in Ca-containing cells possessed sedimentation properties characteristic of the intact control mucin. Since Ca appeared to have primarily affected GCM solubility, separate experiments were conducted to examine solubility more directly.

SOLUBILITY

A soluble mixture of mucin (8.33 mg dry wt/ml) in 0.01 M Tris-HCl-0.14 M NaCl, pH 7.4, became visibly insoluble at 4, 25, and 37° after the addition of $CaCl_2$ (final concentration 10 mM). Because of the scarcity of mucin, however, and the subjectivity of visual inspection for solubility, a quantitative microcentrifugation technique (8) was employed. Briefly, this method involved the incubation of ^{14}C-radioactively labeled mucin (0.25 mg) in isotonic buffer to which $CaCl_2$ was added, followed by centrifugation of aliquots (0.06 ml) in microhematocrit capillary tubes. Solubility was calculated as a percentage of control (Ca-free) samples by measuring the radioactivity of supernatant and pellet fractions.

Table 1. *Sedimentation velocity of goblet cell mucin (GCM)*[1]

	$CaCl_2$, mM	GCM added, mg/ml	GCM measured, mg/ml %	$s20,w$
Control	0	2.5	2.5	11.95
Test	1	2.5	2.5 (0)	11.96
Control	0	2.18	2.18	12.20
Test	5	2.5	2.18 (13)	12.17
Control	0	2.05	2.05	12.54
Test	10	2.5	2.05 (18)	12.52

[1] $CaCl_2$ and GCM were mixed to give the final concentrations shown in *columns 1* and *2*. Sedimentation runs were performed at 20° in a Beckman model E analytic ultracentrifuge using double-sector cells at 48,000 rpm. The solvent was 0.01 M Tris-HCl-0.14 M NaCl, pH 7.4. GCM concentrations in *column 3* were determined from total peak areas of Schlieren tracings. $s_{20,w}$ values had standard deviations not greater than ±0.06. Parentheses indicate the reduction in measured concentration of GCM as a percentage of control (no $CaCl_2$) samples containing 2.5 mg GCM/ml.

Table 2. *Relative effectiveness of several cations on mucin solubility*[1]

Chloride salt of cation (25 mM)	Effectiveness ± range
Zn	1.24 ± 0.01
Mn	1.13 ± 0.05
Mg	1.06 ± 0.02
Ca	1.00 ± 0.00
Ba	0.94 ± 0.02

[1] Incubations of ^{14}C-labeled mucin in 0.01 M Tris-HCl-0.14 M NaCl, pH 7.4, were carried out as described in text. The effect of Ca (25 mM) was to cause a decrease in solubility of 30%. This value was arbitrarily set at 1.00 and other divalent cations were compared. Each value represents the average of four incubations ± the total range obtained.
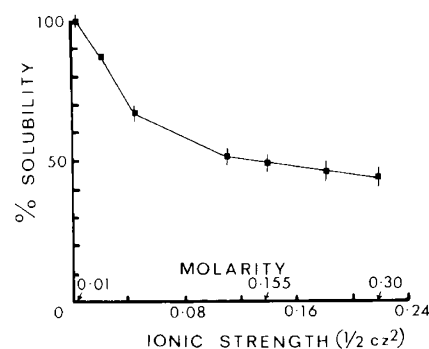

Fig. 3. Solubility of goblet cell mucin. Radioactively labeled (^{14}C) mucin was incubated in Tris-HCl buffers of increasing molarity for 30 min at room temperature. Each incubation contained 0.67 mg dry wt of mucin, about 500-1,000 cpm, and was 0.2 ml in volume. After centrifugation at 12,500 × g for 5 min, radioactivity in the pellet and supernatant solutions was determined and solubility was calculated as a percentage. Ionic strength was determined from the formula $0.5 cz^2$. Each value represents the mean of values obtained from three separate incubations. Each vertical bar represents the total range of values obtained. Radioactivity determination was performed on duplicate samples by scintillation counting in Bray's solution. Total recovery of mucin radioactivity in pellet plus supernatant was 100% ± 2%.
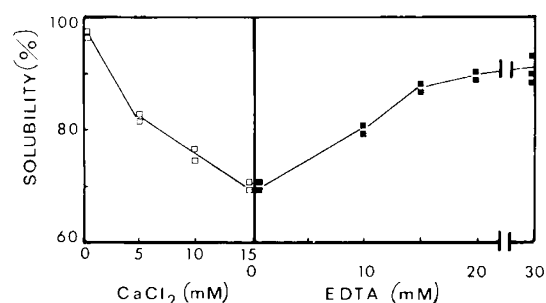

Fig. 4. Solubility of mucin with Ca and EDTA. ^{14}C-Labeled goblet cell mucin (0.25 mg) was incubated in 0.01 M Tris-HCl-0.14 M NaCl, pH 7.4, containing increasing amounts of $CaCl_2$ and solubility determined as described in the legend to Figure 4. The effect of Ca is shown on the left. Separate incubations all containing 15 mM $CaCl_2$ plus varying amounts of Na_2 EDTA were performed, and solubility values are shown on the right. Each value is the average of two incubations.

Effect of Ionic Strength on Solubility. Figure 3 shows that the solubility of mucin in Tris-HCl buffer, pH 7.4, decreased progressively with increasing molarity of buffer until, at 0.155 M, solubility reached 50% of control values. Between 0.155 and 0.3 M Tris, however, only a small (3.5%) drop in solubility occurred. In 0.01 M Tris-HCl-0.14 M NaCl, pH 7.4, solubility was 70-80% of the control (not shown). In subsequent experiments to test the specific effects of $CaCl_2$ on mucin solubility in various buffers, ionic strength was equalized in samples by adding extra buffer ions to control (Ca-free) incubations in order to compensate for the addition of $CaCl_2$ (1-25 mM) to test samples.

Effect of Ca on Mucin in Isotonic Buffer. $CaCl_2$ was added in increasing concentrations to incubations of goblet cell mucin in either 0.155 M Tris-HCl or 0.01 M Tris-HCl-0.14 M NaCl, pH 7.4. Figure 4 (*left*) shows that Ca caused a progressive decrease in solubility down to about 70% of the control (Ca-free) incubation. This was true for both buffers. It is of interest to note that the degree of insolubility produced by 10 mM $CaCl_2$ was approximately 22%, which is very close to the previously measured decrease (18%) (Table 1) in soluble GCM during analytic ultracentrifugation. The two findings support the interpretation that Ca caused a decrease in the solubility of mucin during sedimentation velocity runs.

In subsequent experiments the mucin was incubated in isotonic buffer containing 15 mM $CaCl_2$ to which increasing Na_2 EDTA was added (Fig. 4, *right*). With increasing EDTA, the solubility of mucin was gradually restored to about 90% of control levels.

Effect of Ca on Mucin in Intestinal Luminal Washings. In order to find out whether intestinal mucin would respond to Ca in the same fashion within a more physiologic environment, the following experiment was performed. ^{14}C-Labeled goblet cell mucin was incubated with or without $CaCl_2$ for 30 min at 37° in either dilute or 10-fold concentrated intestinal washings. Figure 5 indicates that in each case Ca caused a progressive drop in solubility to approximately 50% of control values. Thus goblet cell mucin became partly insoluble with Ca in a medium containing isotonic NaCl and normal soluble luminal contents, which are presumed to include bacterial products, digested food products, pancreatic and other enzymes, bile salts, cellular debris, and other proteins, lipids, glycoproteins, and ions.

Specificity of Ca Ions. It was of interest to determine whether the solubility of mucin was affected by other divalent or monovalent cations. As seen in Table 2, the Ca effect was arbitrarily designated as 1.00 for 25 mM $CaCl_2$, and the effectiveness of other cations related to it. Zn, Mn, and Mg were all found to be slighly more effective, and Ba slightly less effective than Ca. Tris-HCl and NaCl were much less effective. Thus monovalent cations had very little effect, but all of the divalent cations tested were effective and there did not seem to be a high degree of specificity for Ca.

DISCUSSION

Our results indicate that calcium, in concentrations known to obtain in some secretions in cystic fibrosis (13), crucially modifies the behavior of a model epithelial mucin under isotonic conditions, making it denser, less viscous, and more insoluble. The fact that viscosity fell but the molecule clearly became heavier, as indicated by its solubility characteristics, strongly suggests that the mucin became smaller and more dense as calcium was added, a process most probably achieved by loss of intramolecular water. Insolubility, which might be taken as the most dramatic and advanced evidence of this process, was not seen in hypotonic media (8). Clearly, therefore, the divalent cation effect required a near isotonic background, and these conditions are precisely those which are encountered in secretions *in vivo* (12). Insolubility was also not limited to simple test tube conditions, but could be demonstrated in the presence of complex organic contents of the
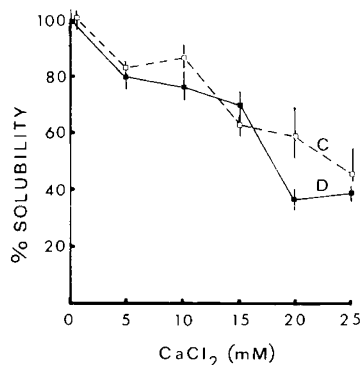

Fig. 5. Solubility of mucin in intestinal washings. Intestinal washings were obtained from 10 rats using 20 ml 0.15 M NaCl/rat. The washings were centrifuged at 30,000 × g for 30 min to remove particulate matter and the supernatant material either stored or concentrated isotonically 10-fold, and then stored at −20°. Incubation of mucin in the intestinal washing solutions was carried out as described in the legend of Figure 5 with increasing $CaCl_2$ additions. Values represent the average (and total range of values) of three incubations. C and D refer to solubility in concentrated or dilute intestinal washings.

intestinal lumen. It seems likely, therefore, that excessive calciun if present within cystic fibrosis mucous secretions, could substar tially increase the density and reduce the solubility of mucins Whether other divalent cations play an important physiologic rol in altering mucins is open to conjecture. Only calcium is likely t be present in sufficient concentration extracellularly to have an appreciable effect.

Decreased hydration and size, increased density, and loss of solubility indicate that the mucin sheet may become more granular and less gelatinous in the presence of calcium. These changes mav explain the "hyperpermeability" described by Gibson *et al.* (12) for cystic fibrosis gastrointestinal mucosa *in vivo*, and for salivary mucous films *in vitro*. Increased granularity might interfere with the spreading quality of the mucin over the epithelial surface, possibly decreasing its uniformity and leaving denuded hyperpermeable areas of intestinal surface.

The significance of our findings lies in the fact that mucins normally account for the viscosity of bronchial and intestinal secretions, and are major components of obstructive plugs in cystic fibrosis (14). Previous authors (5, 14) have shown that small molecular weight glycoproteins in saliva are aggregated by additional calcium, and that the aggregation is reversed by EDTA. Warton and Blomfeld (22) have described hydroxyapatite crystals in saliva of cystic fibrosis. Although these components may contribute to the turbidity which is characteristic of cystic fibrosis saliva (7, 13), they bear little resemblance to highly glycosylated mucin macromolecules. Whether rat intestinal mucin is appropriate as a model for human intestinal or bronchial mucin has not been unequivocally demonstrated. Rat mucin does, however, possess most of the features characteristic (9) of intact epithelial mucins isolated from many organs, including a bronchial mucin from cystic fibrosis patients (19).

In addition to the implications for cystic fibrosis, the present study raises certain questions relevant to the normal function of mucins. It has been hypothesized, for example, that mucous provides protective "waterproofing" for epithelial surfaces (16, 20). Anatomically, the mucous blanket in the intestine occupies a zone through which all solutes destined for absorption must penetrate. Therefore, agents such as Ca ions which alter mucous structure might be expected to influence water or solute transport. This may be analogous to the effects of Ca upon ion fluxes through artificial phospholipid membranes (15) or collagen membranes (2), where Ca produces a "tightening" of structure and neutralization of membrane negative charge.

The interaction of Ca and intestinal mucin may also have physiologic significance within normal goblet cells of the intestine. Warner and Coleman (21) have shown recently by electron probe analysis that goblet cells contain a major share of the total intestinal Ca. The reason for this distribution is not clear, but in view of the present work, it is possible that Ca helps to keep mucin dense or compact within the secretory granules of the cell. Once released into the lumen, the mucin would then be diluted, the Ca removed, and the mucin would swell and spread over the mucosal surface.

SUMMARY

Millimolar additions (8–25 mM) of $CaCl_2$ to rat intestinal goblet cell mucin in isotonic buffer media caused a 15–33% decrease in viscosity, no change in electrophoretic mobility, and a 20–30% decrease in solubility of the mucin. If elevated Ca concentrations similarly affect the solubility of human mucins, they could contribute to mucous "plug" formation in cystic fibrosis.

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24. Requests for reprints should be addressed to: J. F. Forstner, M.D., Research Institute, The Hospital for Sick Children, 555 University Ave., Toronto, Ontario M5G 1X8 (Canada).
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Erythrocytes   sickle cell anemia
hemoglobin   α-thalassemia

Sickle Cell Syndromes. I. Hemoglobin SC-α-Thalassemia

GEORGE R. HONIG,[52] UNSAL GUNAY, R. GEORGE MASON, LOYDA N. VIDA, AND CHRISTINE FERENC

University of Illinois Sickle Cell Center and Department of Pediatrics, The Abraham Lincoln School of Medicine, University of Illinois Medical Center, Chicago, Illinois, USA

Extract

Hematologic and globin synthesis studies were performed in a black American family in which the genes for α-thalassemia and hemoglobins (Hb) S and C were segregating. The following distribution of these abnormalities was found: father, sickle cell trait + α-thalassemia; mother, HbC trait + α-thalassemia, propositus, HbSC + α-thalassemia; older sibling, α-thalassemia trait; and younger sibling, hemoglobin H disease.

The child with HbSC-α-thalassemia demonstrated more severe anemia and a more hemolytic picture than is typical of HbSC disease. Her erythrocytes exhibited decreased osmotic fragility in comparison with HbSC erythrocytes, but had an indistinguishable oxygen equilibrium curve and 2,3-diphosphoglycerate (2,3-DPG) level. Erythrocyte sickling in the patient, however, was significantly reduced, with less than 35% sickle forms observed at nearly complete oxygen desaturation.

The sibling with hemoglobin H disease exhibited 26% Bart's (γ_4) hemoglobin at birth, a level comparable with that seen in infants with HbH disease in Far Eastern populations. At age 5 months typical findings of mild hemoglobin H disease appeared, with HbH making up 6.5% of the total hemoglobin.

Speculation

The presence of α-thalassemia in the proband of this study appeared to modify her HbSC disease so as to reduce its clinical severity as well as its pathologic potential. The ameliorative effect of α-thalassemia would appear to be related to a reduction in the intracellular hemoglobin concentration in the patient's erythrocytes, but other factors may also be responsible for these changes. Further study of genetically modified sickle hemoglobinopathy syndromes may ultimately aid in the development of effective means for the treatment of these disorders.

The clinical expression of sickling disorders may vary widely, even among individuals having an apparently identical form of sickle cell disease. This variability has stimulated investigation of the role of genetic as well as nongenetic factors in the hematologic