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DEVELOPMENTAL HEMATOLOGY OF HOMOZYGOUS HB C DISEASE.

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Little is known about the changes of hematological values accompanying the growth and development of young Hb C homozygotes (CC) and their clinical correlates. We have obtained complete blood counts and hemoglobin composition on 18 CC patients who were identified through cord blood testing and examined at intervals of at least six months. Iron deficiency and α or β thalassemia were excluded by determinations of Free Erythrocytic Porphyrin and Ferritin levels, by DNA analysis and when necessary by family studies. Reference values for normal (AA) subjects and from SS children of a comparable age group were obtained. The Hb levels of the CC patients were intermediate between those of AA and SS children. The average Hb levels declined slightly in accordance with an increase of the reticulocyte counts. However, the erythrocyte counts of CC children overlapped those of AA children despite the different Hb levels between them. They were also quite higher than those of the SS children. The CC patients had a distinct microcytosis throughout the first decade although their iron and α globin gene status was normal. The "inappropriate erythrocytosis" together with the microcytosis suggested a "thalassemic" feature of erythropoiesis in young Hb C homozygotes. Indeed, the proportion of Hb A_2 exceeded 4.0% in CC children over two years old and 10/18 patients had splenomegaly. Thus, the clinical features of Hb C disease in children may be related to deficient β^C globin biosynthesis as well as the effects of Hb C on RBC membranes.

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ANALYSIS OF THE REGION 5' TO THE $^G\gamma$ GENE IN A PATIENT WITH $^G\gamma$-β^+-HPFH/β^S.

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The normal change from fetal to adult hemoglobin includes a switch in production of γ- to β-globin chains as well as a switch from a high to low $^G\gamma/^A\gamma$. We have cloned and sequenced the region 5' to $^G\gamma$ genes from both chromosomes of a black patient heterozygous for β^S and non-deletional $^G\gamma$-β^+-HPFH. We analyzed the region from 400 base pairs 5' to the cap site of $^G\gamma$ to 580 base pairs 3' to the poly A site on the HPFH chromosome. We found a T to C sequence change at position -175 to the cap site. The mutation at position -202 found in other blacks with this phenotype, was not present. T at -158 to the $^G\gamma$ cap site is frequently associated with a high $^G\gamma/^A\gamma$, but this gene has a C at this position. Since other mutations associated with high Hb F or high $^G\gamma/^A\gamma$ were not found, the -175 mutation may be responsible for this patient's phenotype. Analysis of the β-like cluster from the β^S chromosome showed it has the Benin haplotype. This haplotype is associated with low Hb F and low $^G\gamma/^A\gamma$. The DNA sequence was determined from -350 to -60 to the $^G\gamma$ cap site, and a A to G sequence change was found at position -309. Most of the sequence changes associated with increased expression of the γ gene are further downstream from the -309 change we found. This variant may be associated with inhibition of expression of the γ gene. We are currently screening appropriate controls with specific oligonucleotide probes to determine if the two DNA variants described here are neutral polymorphisms. In addition, sequence analysis of the $^G\gamma$ promoter region of the other two major haplotypes associated with β^S is in progress.

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TIN-PROTOPORPHYRIN (Sn-PP), AN INHIBITOR OF HEME OXYGENASE ACTIVITY, CAUSES CHANGES IN HEME METABOLISM IN PRIMARY CULTURES OF HEPATOCYTES.

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Sn-PP has been shown *in vivo* to reduce neonatal hyperbilirubinemia via decreased hemeoxygenase (HO) activity and increased bile secretion of heme (Proc. Natl. Acad. Sci. 78:6466, 1981) as well as to saturate tryptophan pyrrolase and to decrease aminolevulinic acid synthase (ALAS) activity in the liver (J. Clin. Inv. 76:2436, 1985). To initiate investigations on the mechanism(s) of inhibition of enzymes of heme metabolism we examined the effect of Sn-PP and of succinyl acetone (SA), an inhibitor of aminolevulinic acid dehydrase (ALAD) on the activities of ALAS, the first and rate-limiting enzyme of heme synthesis and HO, the rate-limiting enzyme of heme degradation in primary cultures of rat hepatocytes. Liver cells of 150-175g Sprague-Dawley rats were cultured, following perfusion *in situ* with collagenase. 1 x 10^6 cells were incubated in 1.5ml medium 199 for 24h in the presence of 30uM heme, 30uM Sn-PP or 1mM succinyl acetone. The cellular heme content was measured by the method of Morrison (J. Anal. Chem. 37:1124, 1965), ALAS activity according to Sinclair and Granick (Anal. Biochem. 79:380, 1977) and HO activity according to Maines and Kappas (J. Biol. Chem. 250:4171 as modified in J. Biol. Chem. 257:4000, 1982). The percent change in heme (nmole/mg protein) content of the cells from control (no addition) was +350% with addition of heme, +200% with Sn-PP, and -90% with SA. The ALAS activity was not detectable with addition of heme, decreased 50% with Sn-PP and increased several fold with SA. Heme and SA caused a large increase in HO activity and Sn-PP significantly reduced its activity. In summary, the primary hepatocyte culture system exhibits the effect of inhibitors of heme metabolism observed *in vivo* and should thus prove useful for investigations into the mechanism of their action.

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AN AUTOMATED KINETIC METHOD FOR DETERMINING FETAL HEMOGLOBIN LEVELS.

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We have developed a cost-effective automated method for determining fetal hemoglobin (Hb F) levels. Under our experimental conditions, we found that following complete alkali denaturation of hemoglobin A, the rate of change in absorbance at 414 nm is directly proportional to Hb F concentration. Unlike most of the inaccurate alkali denaturation procedures where up to 18% of Hb F is lost during the first few minutes of the reaction, our method is dependent only upon Hb F concentration. Our method is linear, accurate, and precise from 1 to 100% Hb F. It is essential to perform analyses on an instrument that is highly accurate and precise with respect to sample and reagent delivery, wavelength adjustment, absorbance measurement, and temperature control (COBAS-FARA, Roche Analytical Instruments, Inc. Nutley, NJ). Precision studies for 15 repeated analyses of three different specimens reveal the following results: Level 1, mean = 3.8, SD = 0.2, CV = 5.3%; Level 2, mean = 15.1, SD = 0.1, CV = 0.7%; and Level 3, mean = 50.2, SD = 1.1, CV = 2.2% (where mean and SD are in %Hb F). Up to 29 different hemolysates can be analysed simultaneously in less than 20 minutes. Our method eliminates the laborious pipetting, timing, filtering, and precipitating steps required in most alkali denaturation procedures. The wide dynamic range and high precision of our assay allows all samples to be analyzed at one time regardless of fetal hemoglobin concentration.