Composition of \(\gamma\)-Chains in the Hemolysates of an Umbilical Cord Blood and Erythropoietic Bursts From Carriers for Hemoglobin-F Malta-I (G\(\gamma\)117, His\(\rightarrow\)Arg)

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By using a globin isoelectric focusing technique, we measured the levels of G\(\gamma\), Malta-I G\(\gamma\), and A\(\gamma\) globin chains in the hemolysates of an umbilical cord blood of a carrier for hemoglobin F Malta-I (G\(\gamma\)117, His\(\rightarrow\)Arg), a G\(\gamma\) variant. Malta-I G\(\gamma\) focused slightly cathodal to \(\beta\)-chains. Malta-I G\(\gamma\), normal G\(\gamma\), and A\(\gamma\) chains comprised 21.8\%, 57.5\%, and 20.7\% of the total \(\gamma\)-chains, respectively. Thus, we documented for the first time the reduced level of mutant G\(\gamma\) chain relative to normal G\(\gamma\) chain in a hemoglobinopathy of fetal hemoglobin. We then analyzed the levels of the biosynthetic rates of the \(\gamma\)-chains in the erythropoietic bursts derived from an adult carrier of the same family using a methylcellulose clonal cell culture method. The relative proportions of Malta-I G\(\gamma\), normal G\(\gamma\), and A\(\gamma\) chains in the total \(\gamma\)-chains were 22.1\%, 28.5\%, and 49.4\%, respectively. The ratio (0.78) of Malta-I G\(\gamma\)/normal G\(\gamma\) in the synthesized \(\gamma\)-chains of the adult carrier was significantly higher than that (0.38) in the hemolysate of the cord blood. This difference may be due to shortened lifespan of F cells containing Malta-I G\(\gamma\) or to some intrinsic differences in the regulation of \(\gamma\)-globin gene expression between a normal newborn and cell culture milieu. Finally, we observed significant variations in the ratios of normal G\(\gamma\):A\(\gamma\), Malta-I G\(\gamma\):A\(\gamma\), and Malta-I G\(\gamma\):normal G\(\gamma\) in individual bursts in culture. Coefficients of variations for each ratio were 0.38, 0.28, and 0.14, respectively. These values were significantly higher than the coefficients of variations in the clonal expression of allelic \(\beta\)-globin genes. This observation may suggest instability of \(\gamma\)-gene expression in culture. Alternatively, it may suggest that determination of the composition of \(\gamma\)-chains in F cells takes place continuously during burst formation, which is analogous to the commitment to become F cells in culture.

NORMAL human \(\gamma\)-chains consist of two molecular species; one with glycine (G\(\gamma\)) and the other with alanine (A\(\gamma\)) at position 136. In general, the ratio of G\(\gamma\):A\(\gamma\) is 3:1 at birth (newborn ratio) and most often 2:3 in the small amount of hemoglobin-F (HbF) present in the erythrocytes of adults (adult ratio). Since both glycine and alanine are neutral amino acids, the quantitation of the \(\gamma\)-chain variants previously required large amounts of hemoglobin (Hb) and the use of tryptic digestion and peptide analysis of the \(\gamma\)-chains. Recently, a simple technique for the electrophoretic separation of the two \(\gamma\)-chains became available. Earlier, we discovered that it was possible to reactivate synthesis of significant amounts of an HbF variant (HbF Malta-I) in cultures of peripheral blood mononuclear cells from an adult carrier. Application of this technique to a cord blood hemolysate and clonal cell cultures of erythropoietic precursors derived from carriers of HbF Malta-I (G\(\gamma\)117 His\(\rightarrow\)Arg) provided us with a unique opportunity to investigate the interrelationship of the functions of the allelic and nonallelic \(\gamma\) structural genes.

**MATERIALS AND METHODS**

**Blood Samples**

Blood samples from two members (A, B) of a family known to carry HbF Malta-I were used in the studies. Carrier A is a healthy adult described in our previous report, and his blood was used in clonal cell culture for examination of augmentation of \(\gamma\)-chain biosynthesis. Umbilical cord blood erythrocytes from carrier B, the third offspring of the family, contained HbF Malta-I. The cord blood was analyzed for identification and determination of the levels of specific \(\gamma\)-chains by isoelectric focusing. Unfortunately, there was a delay in acquisition of the cord blood samples and thus it could not be used for biosynthetic studies. We also cultured peripheral blood mononuclear cells from a carrier of HbC disease for analysis of \(\beta\)-chain synthesis in individual bursts.

**Globin Isoelectric Focusing of Hemolysates**

For determination of the isoelectric location of Malta-I G\(\gamma\) chain, HbF Malta-I was prepared from the hemolysate of carrier B and was subjected to Hb isoelectric focusing on an LKB Multiphor under the conditions we described previously. After electrophoresis, portions of the gels containing HbF Malta-I were carefully excised and the variant Hb eluted by a small amount of phosphate-buffered saline (PBS). Hemoglobin-A\(_2\) was prepared from the hemolysate of an individual heterozygous for \(\beta\)-thalassemia by using an Isolab Hemoglobin A\(_2\) Kit. Hemolysates of the cord blood of carrier B, a normal umbilical cord blood, purified HbF Malta-I and HbA\(_2\), were individually incubated at room temperature in 4 M urea (Fisher Scientific Co., Norcross, Ga.), 10% 2-mercaptoethanol (Eastman Kodak Co., Rochester, N.Y.) and 3% Nonidet P-40 (Particle Data Labs, Elmhurst, Ill.). Separation of the globin chains by isoelectric focusing was carried out on an LKB Multiphor apparatus under the conditions described by Righetti et al. and Comi et al. and described in detail in our previous report. After electrophoresis, the gels were stained with Coomassie brilliant blue G-250 and dried on transparent cellophane sheets. This staining procedure allows quantitative estimation of the synthesized \(\gamma\)-chains.
tation of proteins in acrylamide gel electrophoresis. Densitometric tracings were obtained on a Joyce/Loebl 3-CS microdensitometer.

**Erythropoietic Cell Culture**

Mononuclear cells were separated from the blood of carrier A and the heterozygote for HbC disease using slight modifications of the Ficoll-Isopaque technique described by Boyum. Cell culture was carried out for 14 days using a modification of the methycellulose clonal assay developed by Iscove et al. We used a step III preparation of sheep plasma erythropoietin (Ep) with specific activity of 14 U/mg protein purchased from Connaught Labs, Ltd., Willowdale, Ontario, Canada. One milliliter of culture mixture containing 3 x 10⁶ mononuclear cells, a-medium (Flow Laboratories, Inc., Rockville, Md.), 0.8% methycellulose (Fisher Scientific, Co.), 30% fetal bovine serum (Flow Laboratories, Inc.), 1% deionized bovine serum albumin (Calbiochem, San Diego, Calif.), and 2.0 U Ep was plated in 35-mm Lux standard nontissue culture dishes (Flow Laboratories, Inc.) and incubated at 37°C in a humidified atmosphere flushed with 5% CO₂ in air.

**Globin Chain Biosynthesis in Pooled Erythropoietic Bursts**

Measurement of the biosynthetic rates of globin chains was carried out during the last 2 days of culture as described in our previous reports. Cultures were labeled on day 12 with 2 μCi of uniformly ¹³C-labeled amino acid mixture (NEC-445, New England Nuclear, Boston, Mass.). Two days later, cells were harvested with a Pasteur pipet, washed 3 times with PBS, pelleted in an Eppendorf microcentrifuge tube, and stored overnight at -70°C. Upon thawing at room temperature, samples were lysed by the addition of 0.01 M KCN and Nonidet P-40. The aliquot was mixed with an equal volume of a solution containing 8 M urea, 20% 2-mercaptoethanol, and 6% Nonidet P-40 and incubated for 30 min at room temperature. Separation of globin chains by isoelectric focusing was carried out on an LKB Multiphor apparatus under the conditions described above. Autoradiography on Kodak NS-2T x-ray films (Eastman Kodak Co.) and densitometric tracings were carried out as described previously.

**Globin Chain Biosynthesis in Individual Erythropoietic Bursts**

Globin chain biosynthesis in individual bursts were analyzed by the use of fluorography using a slight modification of the technique described previously. Briefly, on day 14 of incubation, individual bursts were identified using stereomicroscopy and were individually collected into microcentrifuge tubes containing 200 μl PBS and 1 μl of carrier erythrocytes by using a 10-μl Eppendorf pipet. The pellets were then treated with 3 μl solution containing 8 M urea, 3% Nonidet P-40, and 10% 2-mercaptoethanol for 30 min prior to the application of the gels. Separation of globin chains by isoelectric focusing was as described above. After the run, the gels were fixed in 10% trichloroacetic acid in 30% ethanol until the gels became transparent. They were then soaked in ENHANCE (New England Nuclear) for 1 hr and transferred to water. The gels were dried and then exposed for 1 wk at -70°C to Kodak RP "X-Omat" film (Eastman Kodak Co.) that had been previously exposed to a brief flash of light using a photographic unit (Vivitar 283; Vivitar Corp., Santa Monica, Calif.). Quantitation of fluorogram bands was obtained by densitometric tracings.

**RESULTS**

The isoelectric location of Malta-I Gγ globin chain was identified by globin isoelectric focusing of HbF Malta-I obtained from a cord blood sample of carrier B (Fig. 1). HbF Malta-I was prepared by Hb isoelectric focusing. Malta-I Gγ focused slightly cathodal to β-chain. Gamma chains comprised 74.8% of total non-α-chains. Malta-I Gγ, and Aγ chains comprised 21.8%, 57.5%, and 20.7% of the total γ-chains, respectively. Thus, the ratio of Malta-I Gγ/
normal $G_\gamma$ was 0.38. The value for Malta-I $G_\gamma$ matched closely to the proportion of HbF Malta-I reported in the literature.\textsuperscript{14,15} The value for (normal $G_\gamma +$ Malta-I $G_\gamma$)/total $\gamma$ was 0.79, which is within the range of the accepted newborn $G_\gamma:A_\gamma$ ratio.

In the analysis of the globin chains synthesized by pooled erythropoietic bursts from carrier A, the relative proportions of Malta-I $G_\gamma$, normal $G_\gamma$, and $A_\gamma$ chains in the total $\gamma$-chains were 22.1\%, 28.5\%, and 49.4\%, respectively. Therefore, the ratio of Malta-I $G_\gamma$/normal $G_\gamma$ in the labeled $\gamma$-chains was 0.78. The relative proportion of Malta-I $G_\gamma$ in the total $\gamma$-chains (22.1\%) was in agreement with our previous observation on the proportion of HbF Malta-I (24.2\%) in the bursts from the same carrier.\textsuperscript{4} The total $G_\gamma:A_\gamma$ ratio was within the known range of the adult ratio.\textsuperscript{1}

We then analyzed globin chain biosynthesis in 60 separate individual erythropoietic bursts from carrier A. A representative portion of a fluorogram of such analysis is presented in Fig. 2. Variations existed in the proportion of total $\gamma$-chains among individual bursts.\textsuperscript{16} In addition, there were variations in the $G_\gamma:A_\gamma$, Malta-I $G_\gamma:A_\gamma$, and interestingly, Malta-I $G_\gamma$:normal $G_\gamma$ ratios. While almost all bursts revealed biosynthesis of more normal $G_\gamma$ than Malta-I $G_\gamma$, and of more $A_\gamma$ than normal $G_\gamma$, the latter relationship was reversed in some bursts. Densitometric tracings of two such examples are shown in Fig. 3. The mean and

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**Fig. 2.** Portion of fluorogram from cultures of carrier A showing separation by isoelectric focusing of $^{14}$C-labeled globins in individual bursts. $G_\gamma X$ denotes Malta-I $G_\gamma$.

**Fig. 3.** Densitometric tracings of globins from two separate individual bursts varying in Malta-I $G_\gamma:A_\gamma$ ratios.
standard deviation (SD) of Malta-I Gγ/Ag, normal Gγ/Ag, and Malta-I Gγ/normal Gγ were 0.46 ± 0.14, 0.69 ± 0.20, and 0.68 ± 0.10, respectively, and agreed with values for pooled erythropoietic bursts. Distributions of the above ratios in individual bursts are presented in Fig. 4. Comparison of the coefficients of variations of the distributions of the three parameters by F test confirmed that the variation of Malta-I Gγ/normal Gγ ratio is significantly (p < 0.05) lower than those of Malta-I Gγ/Ag and normal Gγ/Ag.

Fig. 4. Distributions of the values for Malta-I Gγ/normal Gγ (GγX/Gγ), normal Gγ/Ag (Gγ/Ag), and Malta-I Gγ/Ag (GγX/Ag) in 60 individual erythropoietic bursts. The bars represent mean and SD of each group. Note that the coefficient of variations (SD/mean) of GγX/Gγ is significantly smaller than Gγ/Ag and GγX/Ag.
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rations. Simultaneous examinations of the ratios of β'f/normal β in 20 separate bursts from the carrier of HbC disease revealed a coefficient of variation of 0.06 for the allelic β-gene expression.

DISCUSSION

Recent invention by Italian investigators of isoelectric focusing separation of globin chains in the presence of 8 M urea and 3% Nonidet P-40 has provided an ideal method for quantitation of small amounts of G7 and Aγ chains. Availability of a cord blood sample from a carrier in the family that we previously studied enabled us to identify the isoelectric location of this mutant Gγ chain. A distinct protein band appeared just cathodal to β-globin chain in the isoelectric focusing of the hemolysate of the carrier B cord blood. While separation by this method is not based solely on the differences in the intrinsic charges of proteins and is due to interactions between globin and Nonidet P-40, the position of Malta-I Gγ agreed with the expected isoelectric point calculated on the basis of the substitution of neutral histidine with more basic amino acid, arginine, in the mutant Gγ chain.

Analysis of globin chains in cultured erythropoietic bursts of carrier A revealed that 22.1% of the γ-chains are Malta-I Gγ. This value is in agreement with our previous observation on the proportion of HbF Malta-I in total HbF in culture of the same carrier. A significant difference was noted between the proportion of normal Gγ and Malta-I Gγ chains in the total γ-chains synthesized. The reduced level of biosynthesis of Malta-I Gγ chains relative to normal Gγ chains corresponds to similar observations in heterozygotes of non-α hemoglobinopathies and may be due to any one of the many speculated mechanisms for reduction of Hb variations; i.e., defects in transcription, processing and transport of mRNA, and subnormal efficiency in association with α-chains, etc.

In the analysis of the cord blood hemolysates, the relative proportion of Malta-I Gγ chain in total γ-chains was 21.8%, which corresponded closely to the reported values for proportions of HbF Malta-I in total HbF. In the same analysis, the ratio of Malta-I Gγ/normal Gγ was 0.38. Altay et al. reported that the proportion of HbF Malta-I in HbF from adult carriers is approximately 4.8%, which indicated an even lower ratio of Malta-I Gγ/normal Gγ than 0.38. Recently, Slightom et al. reported the presence of variations in the base sequences between the intervening sequences (IVS) of allelic Gγ chain genes of each chromosome. The difference in the ratios of Malta-I Gγ/normal Gγ between our data and those of Altay et al. may possibly be due to differences in the structures of IVS of the γ-genes, since our subjects are Black and Altay’s subjects consist of Maltese.

The value (0.38) for the ratio of Malta-I Gγ/normal Gγ in the hemolysate of the cord blood of carrier B was significantly lower than that (0.78) in the synthesized γ-chains of the adult carrier A. It is possible that F cells containing high concentrations of Malta-I Gγ chain have a shorter lifespan than F cells with little Malta-I Gγ in the circulation, or the release of the former from the marrow into the circulation is selectively inhibited. Alternatively, regulation of γ-gene expression by the IVS in culture may differ from that in vivo. Studies of larger numbers of samples from subjects with different ethnic backgrounds are necessary for further clarification of the mechanisms.

We observed variations in the ratios of normal Gγ:Aγ, Malta-I Gγ:Aγ, and Malta-I Gγ: normal Gγ in individual bursts. Coefficients of variations for each ratio were 0.34, 0.28, and 0.14, respectively. The significant variation in the expression of the two Gγ genes is clearly shown in the scattergram of Fig. 4. The coefficient of the variation of the ratios of Malta-I Gγ: normal Gγ (0.14) was much larger than that (0.07) of β-gene expression in individual bursts from a patient with SC disease that we reported previously, and the coefficient of the variation (0.06) in 19 individual bursts from a heterozygote HbC disease that we examined simultaneously. The difference in the coefficients of variations for clonal expression of γ and β alleles may be that the former involves reactivation of physiologically dormant genes in culture, while the latter genes are expressed in vivo. While the coefficients of variations in the ratios of Malta-I Gγ:Aγ and normal Gγ:Aγ are almost twice as large as that for Malta-I Gγ: normal Gγ, variations in the expression of the two allelic Aγ genes may have contributed to the variance. We recently observed that commitment to become F cells continues during burst formation in culture of adult erythropoietic progenitors. Cellular expression of the ratios of four γ-genes may also be determined at variable stages of burst formation.

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REFERENCES


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