STRUCTURAL EVIDENCE FOR γ GLOBIN CHAIN SYNTHESIS IN ADULT BONE MARROW CULTURES

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Summary: In adult human bone marrow cultures increased amounts of a protein with chromatographic characteristics of fetal hemoglobin are synthesized. The structural similarity between this {^{3}H} leucyl labelled protein and fetal Hb was examined. Upon partial sequence of the {^{3}H} labelled protein and carrier γ globin, incorporation of radioactivity was found only at positions normally occupied by leucyl residues in the γ chain. There was complete coincidence of radioactivity and optical density profiles of Sephadex G-50 chromatography of cyanogen bromide fragments of the carrier γ globin and the {^{3}H} labelled protein produced in culture. Specific activities of cyanogen bromide fragments were as expected by their composition in leucyl residues. Thus, by all chromatographic and structural criteria, the labelled protein appears to be human γ chain.

In a previous study we examined the in vitro regulation of fetal hemoglobin synthesis in human adults (1), using a bone marrow culture system which permits selective proliferation of erythroid cells and formation of erythroid colonies each of which derives from a single committed, erythropoietin-sensitive stem cell. Monospecific fluorescent antibodies against Hb F or Hb A were used for detection of types of hemoglobin synthesized in clones of erythroid cells, while globin chain synthesis in culture was estimated with measurements of {^{3}H} leucyl incorporation into hemoglobin and purified globin chains. The data suggested stimulated synthesis of fetal hemoglobin in adult erythroid cells, in vitro (1). Further examination of the radioactive fetal hemoglobin synthesized in culture was undertaken in order to obtain structural evidence for its identity with Hb F and the γ globin chain.

MATERIALS AND METHODS

Adult bone marrow cells were cultured in plasma clots as previously described (1, 2). Cultures were labelled by adding 10 μCi of {^{3}H} leucine (specific activity 80 Ci/mmol) per clot at day 7. Forty-eight hours later the cultures were harvested and the clots were rinsed extensively in large volumes of phosphate buffered saline, pH 7.0. Subsequently, the cells were suspended in a small volume of distilled water. They were disrupted by sonication followed by freeze-thawing and clear hemolysates were received after centrifugation at 23000 x g. After addition of carrier fetal and adult hemoglobins, the dialysed lysates were subjected to CM-Sephadex chromatography (1) for separation of hemoglobin fractions. The globins were further purified on Sephadex G-100 columns equilibrated and developed with 20% (v/v) formic acid, and globin chains were separated on carboxymethyl cellulose columns with a slightly modified 8M urea - 2 mercaptoethanol buffer system (6).

Cleavage at methionyl residues was accomplished by dissolving 8 to 12 mg of the γ chain in 1 ml 70% formic acid, adding 50 mg cyanogen bromide, and incubating at room temperature in the dark overnight (3). The solvent and cyanogen bromide were removed by lyophilization, and peptides were separated on a 2.5 x 90 cm column of Sephadex G-50 (Superfine) equilibrated and eluted with 9% formic acid.

Digests at arginyl residues (3) were performed by dissolving the γ chain (10 mg) in 4 M guanidine hydrochloride (3 ml) and adding 0.3ml citraconic anhydride (re-distilled under vacuum) in 25 μl aliquots under fast stirring over a period of 15 minutes. The pH of the solution was maintained between 8 and 10 by addition of 12 N NaOH. After all the anhydride was added, the solution was desalted in a 2.5 x 30 cm column of Sephadex G-25 equilibrated and eluted with 0.05 M $KHCO_3$, pH 9,0, the protein peak was adjusted to pH 8.0 at 37°C, 0.5 mg of bovine trypsin (Sigma, DCC-treated) was added and the digest was allowed to proceed at 37° for 20 minutes. The digest was stopped by addition of 2 ml 88% formic acid, the solution was stirred for an additional hour to remove the citraconyl groups followed by lyophilization. The peptides were separated on a Sephadex G-50 Superfine column (2.5 x 90 cm) in 9% formic acid.

Sequenator analyses in the Beckman Model 890 C Sequencer followed the procedures of Hermodson et al. (4). Amino acid analyses were performed on a Durrum amino acid analyzer. Radioactivities were determined by standard techniques on a Packard Tri-Carb Model 574 scintillation counter.

RESULTS

The close coincidence of peptide and radioactivity profiles obtained from gel filtration of the cyanogen bromide digests of carrier γ chain and the {^{3}H} leucyl γ chains synthesized in culture is shown in figure 1. Peptide peaks and peaks of radioactivity coincide closely indicating that the labelled protein , upon cyanogen bromide digestion, is cleaved into fragments with molecular sizes similar to those of the carrier γ chain. The coincidence of peptide and radioactivity profiles obtained from gel filtration of the arginyl digests is shown in figure 2.

The peptide peaks from the cyanogen bromide digest were pooled, lyophilized, and dissolved in 3 ml of 33% acetic acid. Samples were withdrawn for amino

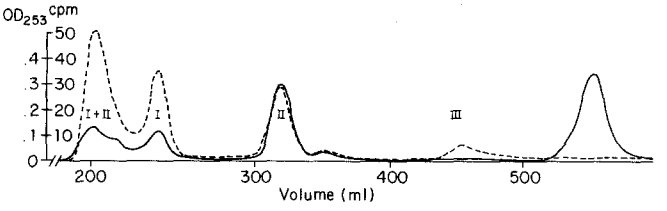

Fig. 1. Sephadex G-50 chromatography of cyanogen bromide peptides of carrier γ globin and the {^{3}H} leucyl labelled protein synthesized in adult bone marrow cultures. Latin numerals denote the cyanogen bromide peptides as identified by amino acid analysis. The dotted line depicts radioactivity, solid line the optical density measurements.

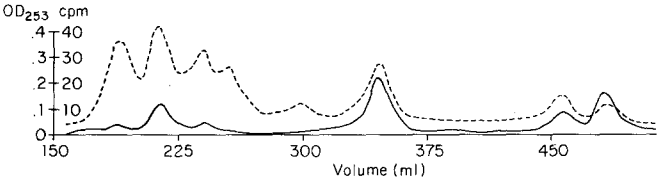

Fig. 2. Sephadex G-50 chromatography of arginyl peptides of carrier γ globin and the {^{3}H} leucyl labelled protein synthesized in culture. Dotted line denotes radioactivity, solid line optical density.

acid analyses and for counting of the radioactivity. The first peak (centered around 200 ml and containing the shoulder) gave an amino acid analysis corresponding to the first 133 residues of γ chain and had a specific activity of 1910 dpm/μmole Leu. The second peak (around 250 ml) was residues 56-133 of the γ chain and had a specific activity of 1410 dmp/μmole Leu. The third peak (around 330 ml) was the amino-terminal 55 residues having a specific activity of 1840 dpm/μmole Leu. The small peak of radioactivity having no optical density (around 470 ml) was the carboxy-terminal 13 residues having a specific activity of 1650 dpm/μmole Leu. Thus the expected three fragments of the γ chain were obtained along with an overlap fragment due to incomplete cleavage at Met_{55}, and the specific activities of the fragments were constant within the sampling errors of the experiment.

To further demonstrate that the labelled protein was human γ chain, two

sequenator degradations were performed. Sequenator degration of 4 mg (250 μ mole) human γ chain containing the synthetic [^{3}H] leucine labelled protein showed only γ chain sequence through 32 cycles. The degradation gave a 95.5% stepwise yield. The fraction of the sequenator products which was not used for gas chromatographic identification of the γ chain sequence was counted in the scintillation counter in an Omnifluor-dioxane cocktail. Only cycles 14 and 15 had significantly higher radioactivity than background levels, cycle 14 having 70 cpm and cycle 15 having 30 cpm, the latter reflecting overlap of Leu_{14} into cycle 15 due to incomplete reaction of the Edman degradation in the previous cycles. The background level of radioactivity rose linearly from 15 cpm in the first cycle to 40 cpm by cycle 30 reflecting the usual rise in amino acid background observed in sequenator analyses. This effect obscured the expected increases in radioactivity due to leucines 28, 31 and 32. Approximately 50 μmoles of the small arginyl fragment (residues 31-40, the peak centered around 455 ml, figure 2) was degraded as above for five cycles. The sequence Leu-Leu-Val-Val-Tyr was observed in good yield. The first two cycles had 23 and 21 cpm respectively while the rest had background levels only. These analyses indicated that the labelled protein did in fact have leucyl residues where the γ chain has leucyl residues, and no other cycle was observed to have significant radioactivity. Any contaminant protein having leucyl residues in positions other than those of the γ chain and being present at a mole ratio of 20% or more of the labelled γ chain would have been detected.

DISCUSSION

This study of γ chains synthesized in culture was necessary in view of the previous evidence for synthesis, in suspension bone marrow cultures, of non-globin proteins co-chromatographing with γ chains on CM-Cellulose columns (5). These contaminants, however, fail to elute with Hb F on ion exchange chromatography or gel filtration (5). The possibility of artifactual findings in our experiments because of such "γ like" proteins is unlikely since the radioactive γ chain derives from a protein which has the chromatographic

behavior and molecular size of fetal hemoglobin (1). The γ chain nature of the radioactive fraction is demonstrated by the data of partial radiosequence and from the cyanogen bromide fractionations, showing complete coincidence of optical density and radioactivity profiles as well as specific activities constant within the sampling error.

In contrast to suspension cultures where there is minimal proliferation of erythroid cells and globin chain synthesis quickly declines, the culture system used permits a selective proliferation of erythroid cells (1, 2). The clonal proliferation of erythroid cell populations provides ideal conditions for cellular and molecular investigations of the globin gene "switch" during development as well as for experiments aimed to stimulate Hb F synthesis in erythroid cells from normal persons as well as persons with β chain hemoglobinopathies. It is of interest now to determine which factors are responsible for the observed stimulation of fetal hemoglobin synthesis in vitro (1), and whether this phenomenon is mediated primarily through induction of γ gene transcription or through stimulation of proliferation of cells predetermined to direct γ globin synthesis in vivo.

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