Monoclonal Antibody to the γ Chain of Human Fetal Hemoglobin Used to Develop an
Enzyme Immunoassay

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A monoclonal antibody (mAb) that recognizes the γ chain of human fetal hemoglobin (Hb F) has been produced by cell hybridization techniques. The mAb reacts with Hb F (α2γ2), Hb Bart’s (γ4), and Hb Kenya (γ-β hybrid), but does not cross-react with Hb A (α2β2) or Hb A2 (α2δ2). We describe a direct enzyme-linked immunoassay (ELISA) for measurement of Hb F, in which hemoglobins from standards or from unknown hemolysates are covalently bound to the wells of microtiter plates. The antigen is quantified by addition of the γ-specific mAb, followed by anti-mouse IgG conjugated with horse radish peroxidase, and incubation with the substrate, tetramethylbenzidine. Absorbances at 630 nm are directly proportional to the amount of Hb F present in the standards or samples. Results for Hb F in 53 hemolysates agreed well with values obtained by "high-performance" liquid chromatography, RIA, alkali denaturation, and magnetic affinity immunoassay. This ELISA can detect a 0.5% proportion of Hb F in 1 h and offers distinct advantages over other techniques currently in use.

Hemoglobin F or fetal hemoglobin (Hb F) is the major component of the erythrocytes in cord blood. At birth, the concentration of Hb F declines considerably, owing to the switch from γ to β globin gene expression (1, 2). In newborns, values of Hb F can be as high as 80%, progressively decreasing throughout the neonatal period to <10% by 20 weeks postpartum (3). Hemolysates from normal adults contain, on average, <1% Hb F (4, 5) and range from <0.1% to as high as 4% (6). Increased proportions of Hb F have been found in patients with sickle cell disease, hereditary persistence of fetal hemoglobin (HPFH), β-thalassemia, and other conditions (7).

At present, Hb F is routinely quantified by use of an alkaline denaturation procedure (FAD), which is not accurate at low and high values for Hb F (8). To overcome this problem, we and others have described the production and application of mono-specific polyclonal antibodies to quantitate Hb F by radioimmunoassay (9, 10). More recently, we have developed a magnetic affinity immunoassay (MAIA) to quantify Hbs F, S, and C (11). This assay, which is specific and rapid, can detect Hb F at 0.05% of total Hb, but like RIA, it has the disadvantages of radioactive components and limited amounts of monospecific antibodies. Monoclonal antibodies for the γ chain of Hb F have been described (12) and used in immunofluorescence studies to detect and quantify fetal cells (13, 14) or, in combination with an mAb for β chain, in the antenatal diagnosis of β-thalassemia (15). It is well recognized that an accurate quantification of Hb F would be valuable in studies of gene therapy in sickle cell disease (16), of Hb expression in experimental models (17, 18), or in conditions associated with high values for Hb F (19). Here we describe the production of a γ-specific mAb and the development of a rapid, specific, and sensitive enzyme-linked immunosorbent assay (ELISA) for quantifying Hb F.

Materials and Methods

Hemoglobin Purification and Blood Samples

Hb A from normal adult blood, Hb A2 from subjects with β-thalassemia trait, Hb F from normal newborns, and Hb Kenya from one heterozygous individual were isolated by column chromatography on diethylaminoethylcellulose (Whatman DE-52) as described by Abraham et al. (20). Hb Bart's was isolated from patients with α-thalassemia trait by column chromatography on carboxymethylcellulose (Whatman CM-52) as described by Schroeder et al. (21). Blood samples were kindly supplied by Dr. A. Kutlar (Sickle Cell Center, Medical College of Georgia), and hemolysates were prepared as described elsewhere (9).

Immunization Schedule

A two-month-old female BALB/cByJ mouse was immunized intraperitoneally with 1 mg of Hb Bart's in complete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO). Two weekly booster injections were given at the same dose and route but in incomplete adjuvant. Five days later the mouse received 0.1 mg of the antigen intravenously for three consecutive days, then was killed. Serum collected from the retroorbital cavity after each immunization was tested against Hb Bart's, Hb F, and Hb A by the ELISA.

Cell Fusion and Cultures

The hybridomas were produced generally as described by Taggart and Samloff (22) and Lane et al. (23). A day after the last intravenous injection, the hyperimmune spleen was aseptically removed and the splenic lymphocytes were fused with SP2/0-Ag14 myeloma cells (24) with use of polyethylene glycol. Hybrids were dispensed into five 96-well macrophage feeder plates and selected in hypoxantine–aminopterin–thymidine medium. Starting 12 days after fusion, we screened the hybridoma cultures for antibody production.

Hybridoma Screening and Ascites Fluid Production

Hybridoma screening was done by ELISA as before (25). The culture fluids were tested individually against Hbs Bart's, F, and A. One of the cultures that reacted with Hb Bart's and Hb F but not with Hb A was cloned by limiting dilution, expanded, and used to generate large quantities of mAb in ascites fluid (25). The mAb was isotype (Mono Ab-ID ELIA, Kit A; Zymed Laboratories, Inc., South San Francisco, CA), characterized, and incorporated in an immunoassay.

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Development of an ELISA for Hb F Quantification

Microtiter plates (Dynatech, Alexandria, VA) were activated for 30 min with glutaraldehyde, 2.5 mL/L in binding buffer (sodium carbonate, 50 mmol/L, pH 9.6). After washing the plates with distilled water, we coated them with Hb F and Hb A at 1, 10, or 100 μg per well in 100-μL aliquots of binding buffer for 1 h. The remaining free binding sites were blocked with Tris-buffered saline (TBS; 20 mmol of Tris hydrochloride and 500 mmol of NaCl per liter, pH 7.5) containing 2 mL of Tween 20 per liter (TBST). After 10-fold dilutions of the mAb (10–3–10–6 in TBST) were incubated for 1 h, the plates were washed with TBST, then incubated for 1 h with goat anti-mouse IgG-horseradish peroxidase (EC 1.11.1.7) conjugate (Bio-Rad Laboratories, Richmond, CA), diluted 1000-fold in TBST. After four washes with TBST and two washes with TBS, we added the substrate tetramethylbenzidine (TMB), prepared as described elsewhere (25), and recorded the absorbance at 630 nm after 5 to 15 min.

To develop a standard curve we diluted Hb F in Hb A (1 mg/mL) to yield various proportions of Hb F, from 0.1% to 50%. Wells were coated and reacted with a 10–4 dilution of the mAb. All other steps were kept constant as above. To quantify Hb F in hemolysates, we coated the wells with 100 μL of 1 mg/mL dilutions of hemolysates and then assayed as described above. To calculate the percentages of Hb F, we extrapolated from the standard curve the absorbances at 630 nm of the unknowns. We used the ELISA to analyze hemolysates of various genotypes (AA, FA, AS, SS, Sβ-thal, Sβ+thals, Aβ-thal, and δβ-thal) and compared the results with those values obtained by "high-performance" liquid chromatography (HPLC), FAD, RIA, and MAIA (11).

To define the shortest time necessary to achieve a rapid assay without interfering with accuracy and sensitivity, we conducted each step of the ELISA for 1, 5, 10, 15, and 30 min and statistically analyzed the resulting absorbances yielded by the standards for linearity, sensitivity, and reproducibility. Finally, we used the new rapid ELISA to study a selected number of hemolysates containing low, intermediate, and high proportions of Hb F, as determined by HPLC (26).

Results

The immune mouse serum was tested on several occasions to ensure specific antibody production against the immunogen. After the third immunization, the antisera showed a strong reactivity with Hb Bart’s and Hb F, but not with Hb A.

Of the original 480 cultures, 200 showed growth and confluence, and were therefore tested for immunoreactivity by ELISA. Of these, 60% reacted equally with Hb Bart’s, F, and A; 39% did not react with either Hb; and only 1% of the cultures were specific for γ chain. One of these clones (γ1-11491) was used to generate ascites fluid. This IgG1,κ mAb also reacted with Hb Kenya, a γ-β hybrid Hb (27), indicating an antigenic site in the amino terminal portion of the γ chain.

ELISA for Hb F Quantification

As previously reported, activation of the wells of microtiter plates with glutaraldehyde is essential, to increase the binding of Hbs. Similarly, TMB is the substrate of choice for a rapid assay (25). Figure 1 shows the sensitivity and specificity of the mAb. No cross-reactivity with Hb A was apparent at any antigen concentration or mAb dilution. A progressive decrease in A630 nm was evident at all antigen concentrations with increasing mAb dilutions. Maximum absorbance (1.998) was obtained with 100 μg of Hb F per well, on using a 10–3 dilution of the mAb. Decreases of 20%, 70%, and 85% were observed with increasing mAb dilutions: 10–4, 10–5, and 10–6, respectively. Corresponding decreases at 10 μg of Hb F per well were 56%, 89%, and 95%; and at 1 μg per well they were 71%, 95%, and 97%.

After finding the optimal amount of Hb F to coat the wells (100 μg) and the optimal mAb dilution (10–4), we developed standard curves for proportions of Hb F ranging from 50% to 0.1% (Figure 2). We observed a linear decrease in absorbance by decreasing amounts of Hb F on the wells (γ = 0.022x + 0.332; r = 0.900). The lowest proportion of standard Hb F that we could detect was 0.5%. This was represented by an average absorbance of 0.24 (n = 43)

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Fig. 1. ELISA titration of the γ-specific mAb against purified Hb F, 100 ( ), 10 (○), and 1 (■) mg per well, or purified Hb A, 100 mg per well (△)

Fig. 2. Direct ELISA standard for Hb F quantification
Each point represents the average of 26 assays ± SD

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compared with a background absorbance of 0.047 (n = 20) yielded by 0% of Hb F, i.e., 100% Hb A (t = 19.75, P < 0.001, Student’s t-test). Reproducibility of the standards was assessed by running 25 assays in duplicate on different days (interassay CV range: 3.1%–17.5%) and one assay performed 24 times in one day (intra-assay CV range: 2.7%–10.0%).

Using the above procedure, we quantified Hb F in 53 hemolysates and compared the results with those obtained by other techniques (Figure 3). Hemolysates were diluted to 1 mg/mL, like the standards, and assayed as above. We analyzed 22 samples by the ELISA (x) and compared the results with those by HPLC (γ) (y = 1.021x + 0.116; r = 0.986), 31 samples were compared with FAD (γ = 1.209x – 0.310; r = 0.977), 12 samples were compared with MAIA (γ = 0.830x + 0.886; r = 0.975), and eight samples were compared with radioimmunoassay (γ = 1.226x – 0.311; r = 0.997). We found a good correlation with each technique, but a shift to the left was seen with FAD at the lowest percentages of Hb F (Figure 3). ELISA values for Hb F in 22 hemolysates from normal adults averaged 0.62% (SD 0.39%) and ranged from 0.1% to 1.9%. Twelve SS hemolysates averaged 14.9% (SD 9.5%) and ranged from 2.4% to 27%. Hb F values for three AS patients were not different from those for normal persons (0.6%, SD 0.52%). Increased percentages of Hb F were found in two SS patients (11.6%, SD 0.14%), three Sβ-thal (8.2%, SD 0.85%), four Aβ-thal (2.2%, SD 1.7%), and one δβ-thal (98%). Hb F values for five hemolysates from normal newborns averaged 36.2% (SD 15.7%) and ranged from 15% to 53%.

In an effort to speed the assay, we performed each step of the ELISA for different periods of time, ranging from 1 to 30 min. The following minimum time course was established: activation 5 min; Hb costing 15 min; blocking 1 min; mAb reaction 15 min; second Ab 15 min; and development with substrate 5 to 15 min. Further decreases in time resulted in significant decreases in reactivity and sensitivity (Table 1).

We then evaluated this 1-h assay for reproducibility. Intra-assay CVs ranged from 2.9% to 16.8% (one assay run 24 times) and the interassay CV ranged from 3.6% to 16.1% (20 assays run in duplicate). We studied the applicability of this fast ELISA, using hemolysates containing known proportions of Hb F as determined by HPLC. The ELISA gave values of 50% (SD 3.1%) for three normal cord hemolysates, 18% (SD 5%) for three SS patients, 5.0% (SD 1.5%) for two Sβ-thalassemia, and 0.3% (SD 0.09%) for three normal adults. Corresponding values obtained by HPLC were: 55.2% (SD 4.8%), 20.1% (SD 3.2%), 4.4% (SD 2.4%), and 0.48% (SD 0.1%), respectively.

### Table 1. Effect of Varying the Incubation Time on ELISA Results for the Standards

<table>
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<tr>
<th>Time, min*</th>
<th>60</th>
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<th>5</th>
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<td>0.205</td>
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<td>5</td>
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<tr>
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<td>2.9–16.1</td>
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</table>

* Standards were coated to the plate and reacted with the anti-Hb and second antibody for the indicated period of time. Mean absorbance at 630 nm.

### Discussion

We thus produced an mAb specific for the γ chain of human Hb from a mouse immunized with Hb Bart’s (γγ). The mAb did not specifically recognize Hb Bart’s, as indicated by its strong reactivity with Hb F (αγγ). The epitope involved in this reactivity is present in the amino-terminal portion of the γ chain, as indicated by the reaction of the mAb with Hb Kenya, which has a non-α chain composed of the amino-terminal portion of a γ chain and the carboxy terminal portion of a β chain (27). Gamma-specific mAbs have been produced by using Hb F (12–14) or γ-globin chain (15) as immunogens. These mAbs recognized epitopes within the cyanogen bromide fragment 1, residues 1–55 (12), or fragment 2, residues 56–133 (13), and the former mAb reacted with Hb Bart’s (12). These mAbs have been used in immunofluorescence studies of fetal cells (13, 14) or in combination with a β-specific mAb for the antenatal diagnosis of β-thalassemia (15), and in studies of Hb expression in a human erythroleukemia cell line (17) and in erythroid clones produced from human burst-forming units (18).

It is well recognized that an accurate, simple assay to quantify Hb F in the range of 1% to 40% would be valuable in studies of gene therapy in sickle cell disease or β-thalassemia (16), Hb expression in experimental models (17, 18), or in conditions associated with above-normal values for Hb F (19). Currently, Hb F is quantified by the tedious FAD technique (8, 28) or by time-consuming column chromatography (29). Radioimmunoassays (9, 10) and, more recently, an MAIA (11) have been shown to be specific and faster but have the inconvenience of radioactive compounds and limited amounts of polyclonal antibodies. We have therefore incorporated a γ-specific mAb in a quantitative ELISA for Hb F. The results (Figure 1) make it clear that the Hb is quantitatively bound to the wells and that the reaction with the mAb is proportional to the amount of Hb bound to the wells. Using a saturating...
concentration of Hb (100 μg per well), we prepared standards containing Hb F in the range of 0.1% to 50%, which we reacted with a saturating mAb dilution (10⁻⁴) and developed with the indicator system, peroxidase-conjugated second antibody and TMB (Figure 2). The increase in color yield was proportional to the logarithmic increase in antigen. The assay becomes nonlinear when the proportion of Hb F is >50% or <0.5%. The validity of the ELISA was established by its good correlation with other techniques (Figure 3). The reproducibility of the assay is demonstrated by the relatively low CVs of the standards (Table 1). The assay was facilitated by use of shorter incubations. The reaction with glutaraldehyde and the blocking step are essential but not time-dependent (same reactivity on using 60-min or 1-min incubation). On the other hand, both the coating step and the reaction with the mAb and second antibody seemed to be time-dependent and saturable. No differences in reactivity were observed when incubation times were shortened from 1 h to 15 min. Further decreases in time resulted in significant decreases of reactivity affecting the sensitivity, linearity, and reproducibility of the assay (Table 1). A comparison of the standards by ELISAs carried out for 4 h or 1 h showed two non-superimposable curves, owing to small differences in reactivity, but with similar slopes, coefficients of correlation, and CVs (Table 1). In both cases, the sensitivity was 0.5%. This rapid assay has the advantages of relative simplicity, ability to process multiple samples within 1 h, and potential for automation.

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References