A Novel Monoclonal Antibody Based Diagnostic Test for α-Thalassemia-1 Carriers Due to the (−SEA/) Deletion

By Hong-yuan Luo, Bryan J. Clarke, Jack Gauldie, Margaret Patterson, Shuen-Kuei Liao, and David H.K. Chui

The presence of minute amounts of embryonic ζ-globin chains in adult hemolysates is a marker for carriers of α-thalassemia-1 resulting from (−SEA/) deletion. Recently, we developed a murine monoclonal anti-embryonic ζ-globin chain antibody, 8E8. By using this antibody, we have now established a slot-blot immunobinding assay for the rapid detection of ζ-globin chains in adult hemolysates. ζ-globin chains were found to be present in 30 blood samples obtained from individuals who were carriers of α-thalassemia-1. In another 30 blood samples from individuals who were not carriers of the (−SEA/) deletion, ζ-globin chains were not detected. This simple diagnostic test can be used in appropriate populations to identify those couples at risk of conceiving fetuses afflicted with the Hb Bart’s hydrops fetalis syndrome due to homozygous α-thalassemia.

HUMAN EMBRYONIC ζ-globin chain is an α-globin–like chain present during the first trimester of gestation as components of Hb Gower 1 (ζ²ζ/ε²) and Hb Portland 1 (ζ²ε²). In normal fetuses, a minute amount of ζ-globin chains persists until near birth. In fetuses with the Hb Bart’s hydrops fetalis syndrome due to the deletion of all four α-globin genes, significant amounts of ζ-globin chains are synthesized even during the third trimester of gestation. Recently, a highly sensitive and specific radioimmunoassay for human ζ-globin chains was developed by using rabbit monospecific antihuman ζ-globin chain antibodies. When using this assay, minute amounts of ζ-globin chains are found to be present in hemolysates from adult individuals who are carriers of α-thalassemia-1 due to the (−SEA/) deletion.

In this investigation, a stable murine hybridoma cell line has been established that secretes murine antihuman embryonic ζ-globin chain monoclonal antibody (MoAb). By using this antibody, we have devised a simple and specific slot-blot immunobinding assay (SBIA) for the detection of ζ-globin chains in adult hemolysates. Evidence is provided that indicates the specificity of this immunoassay for the identification of individuals who are carriers of α-thalassemia-1 resulting from the (−SEA/) deletion.

MATERIALS AND METHODS

Preparation of ζ-globin chains. The hemoglobin fractions of Hb Bart’s hydrops fetalis hemolysate were isolated by diethylaminoethyl (DEAE)-cellulose column chromatography. Different batches of hemoglobin fraction I containing ζ-, γ-, η-, and β-globin chains were pooled and concentrated by using Amicon Diaflow ultrafiltration membrane PM10 (Amicon Corp, Danvers, MA) and stored at −70°C. This fraction I stock solution, used as the standard in all subsequent SBIAs, was found to contain 46% embryonic ζ-globin chains as determined by Triton X-100/acid urea/polyacrylamide gel electrophoresis.

For immunization purposes, the gel containing the ζ-globin band was cut out, placed in phosphate-buffered saline (PBS), pH 7.2, for 30 minutes, and emulsified with an equal volume of either complete or incomplete Freund’s adjuvants (GIBCO, Grand Island, NY). Hybridoma construction. Each BALB/c mouse was initially administered an intraperitoneal injection of an emulsion containing 10 to 40 µg of ζ-globin chains in polyacrylamide gel and complete Freund’s adjuvant. This was repeated once a week for 2 successive weeks except that the emulsion contained incomplete Freund’s adjuvant. Three days after the third injection, serum samples were obtained and tested for anti-ζ-globin activities by using solid-phase radioimmunoassay. The mouse showing the highest immune response against ζ-globin chains received an additional intraperitoneal injection of 10 µg of ζ-globin chains in polyacrylamide gel. The spleen of this mouse was used for the generation of hybridoma cell lines.

The cellular fusion between mouse myeloma cells and disaggregated splenocytes was performed as previously described. A quantity of 10⁶ spleen cells was mixed with 10¹ P3-NS-1/1-Ag-4 mouse myeloma cells, and one-half mL of 40% polyethylene glycol (molecular weight, 1,000; Baker Chemical Co, Phillipsburg, NJ) was added to the cell pellet to induce cell fusion. The cells were later resuspended in Iscove’s modified Dulbecco’s medium (IMDM) containing 10% heat-inactivated fetal calf serum (HyClone Sterile Systems, Logan, UT) and hypoxanthine/aminopterin/thymidine (HAT).

After ten to 14 days in culture at 37°C, cellular colonies were observed. The culture supernatant was examined for anti-ζ-globin activity by solid-phase radioimmunoassay. Those colonies found to produce anti-ζ-globin antibodies were expanded in culture. The hybridomas that showed vigorous growth and high titer of anti-ζ-globin antibody production were clones by limited dilution in culture medium containing hypoxanthine-thymidine. Two clones showing the most vigorous growth and high levels of anti-ζ-globin antibody production were selected, cloned once more, and cultured in IMDM containing 5% fetal calf serum.

For ascites production, BALB/c mice were each primed with 0.5 mL pristane by intraperitoneal injection. Approximately 1 week later, 2 × 10⁷ hybridoma cells in 0.2 mL PBS were injected intraperitoneally. After another 2 weeks, ascitic fluid would accumulate to a sufficient amount for repeated harvesting by aspiration. Purification of MoAbs. While constantly stirring, ammonium sulfate was added to the hybridoma ascitic fluid or the supernatant of hybridoma tissue culture medium until 50% saturation was achieved.

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reached. The precipitated protein was recovered by centrifugation and dissolved in a small volume of 0.01 mol/L PBS, pH 8.0. Antibodies were further purified by affinity chromatography using an Affi-Gel protein A MAPS II kit (Bio-Rad Laboratories No. 153-6159, Richmond, CA).

**Solid-phase radioimmunoassay and enzyme-linked immunosorbent assay.** Two hundred nanograms of either fraction I containing \( \alpha_2 \), \( \beta \)-, and \( \gamma \)-globin chains or adult or cord blood hemolysates in 50 \( \mu \)L bicarbonate coating buffer containing 15 mmol/L Na\(_2\)CO\(_3\), 35 mmol/L NaHCO\(_3\), and 0.02\% Na\(_2\)SO\(_4\), pH 9.6, was added to Immunol 2 Removawell strips (Dynatech Laboratories, Alexandria, VA) overnight at 4\(^\circ\)C. After washing, free protein binding sites in the Immulon 2 Removawell were blocked by incubation with 2\% bovine serum albumin (BSA; Sigma Chemical Co, St Louis) in PBS, pH 7.4, and 0.05\% Tween 20 for 2 hours at room temperature. After further washing, 50 \( \mu \)L of antibody preparations was added and incubated for two hours at room temperature; this was followed by 50 \( \mu \)L of rabbit antimonutis IgG antibodies (Cedarlane Laboratories, Hornby, Canada), at a 1:500 dilution in the same buffer. Then 125\%I-protein A (New England Nuclear, Boston; specific activity, 9.8 Ci/g of protein A) in the amount of 20,000 cpm was added and the bound radioactivity was determined in a Beckman 300 gamma counter (Beckman Instruments, Inc, Fullerton, CA).

Purified MoAb 8E8 was conjugated with alkaline phosphatase as previously described and added to the Removawells previously coated with various hemolysates. Color was developed by the addition of substrate p-nitrophenyl phosphate disodium (Sigma) and then incubated with MoAb anti-\( \gamma \) conjugated with alkaline phosphatase. Another membrane was similarly treated except that both rabbit anti-Hb fraction I antiserum and affinity chromatography-purified rabbit polyclonal anti-\( \gamma \)-globin antibodies were used to react with the blotted globins on the membrane. After reaction with these antibodies, the membrane was exposed to goat antirabbit IgG antibodies conjugated with alkaline phosphatase (Zymed Laboratories, San Francisco). Color was developed by nitroblue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma).

**Hematologic and gene mapping data.** Human venous blood was drawn and anticoagulated with EDTA. Hematologic parameters were determined by standard laboratory procedures. Gene mapping of the \( \gamma \)-globin gene cluster was performed by Southern blot analysis as previously described. One microgram of hemolysate in 20 \( \mu \)L of Tris-buffered saline (TBS; 10 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl) was blotted by means of a Minifold II Slot-Blotter (Schleicher & Schuell, Keene, NH) to a pretreated nitrocellulose filter (Schleicher & Schuell). Afterwards, the membrane was allowed to air dry completely.

The membrane was then wetted briefly in TBS and subsequently immersed in TBS with 1 \( \% \) Tween 20 (Sigma) for 30 minutes at room temperature. The membrane was then incubated for 30 minutes at room temperature with gentle agitation in 50 to 100 mL MoAb 8E8 conjugated with alkaline phosphatase at 1:100 to 1:200 dilution in TBS containing 1 \( \% \) BSA and 0.1 \( \% \) Tween 20. Afterward, the membrane was washed three times, each for 15 minutes, in TBS containing 1 \( \% \) Tween 20 for the first washing and 0.3 \( \% \) Tween 20 for the second and third washings. Color was developed by reacting with approximately 10 mL of substrate solution at 37\(^\circ\)C. The substrate solution was prepared just before use by mixing 10 mL of buffer (100 mmol/L Tris-HCl, pH 9.5, 100 mmol/L NaCl, 5 mmol/L MgCl\(_2\)), 66 \( \mu \)L nitroblue tetrazolium (50 mg/mL in 70% dimethylformamide, Sigma), and 33 \( \mu \)L 5-bromo-4-chloro-3-indolyl phosphate (50 mg/mL in dimethylformamide, Sigma). The reaction was allowed to proceed for about five to ten minutes until the desired color intensity was achieved and was stopped by rinsing the membrane in deionized water for several minutes. The membrane was air dried and stored by sealing in a plastic bag.

The color intensity of each slot was scanned by a Model 620 video densitometer (Bio-Rad), and the data were recorded by an HP 3392A recording integrator. The peak heights in absorbance units were used for quantitation.

The procedure for liquid-phase radioimmunoassay using rabbit monospecific anti-\( \gamma \)-globin antibodies has been previously published.

**RESULTS**

**Construction of hybridoma cell lines.** Five BALB/c mice were immunized, and after three weekly injections, two of the five mice showed immune responses to \( \gamma \)-globin chains as demonstrated by reactivity in the solid-phase radioimmunoassay to fraction I and not to adult or cord blood hemolysates. Spleenic lymphocytes from the mouse showing a high anti-\( \gamma \)-globin immune response were fused with P3-NS-1/1-Ag-4 mouse myeloma cells by the polyethylene glycol

Blot cell (Bio-Rad) with the electrodes set 8 cm apart in 25 mmol/L Tris and 192 mmol/L glycine buffer at 0.15 \( \% \) for 18 hours. After transfer, the membrane was exposed to 1 \( \% \) Tween 20 for 30 minutes and then incubated with MoAb anti-\( \gamma \) conjugated with alkaline phosphatase. Another membrane was similarly treated except that both rabbit anti-Hb fraction I antiserum and affinity chromatography-purified rabbit polyclonal anti-\( \gamma \)-globin antibodies were used to react with the blotted globins on the membrane. After reaction with these antibodies, the membrane was exposed to goat antirabbit IgG antibodies conjugated with alkaline phosphatase (Zymed Laboratories, San Francisco). Color was developed by nitroblue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma).
In one fusion experiment, colonial growths were observed in 383 wells in a total of nine 96-well plates. The culture supernatants in these wells were screened for the presence of anti-ε-globin antibodies by solid-phase radioimmunomassay, of which 88 were positive. Eight positive clones were subcloned. From these, two different clones, designated 7E10 and 8E8, showed the most vigorous growth and the highest titers of antibodies. These two clones were expanded by spinner cultures and by ascites tumor production in BALB/c mice. The concentration of MoAb was 17 μg/mL in tissue culture supernatant and 1 to 2.7 mg/mL in ascitic fluid. One hybridoma, 7E10, was rather unstable in terms of antibody production and cell growth after 20 passages. Thus, the rest of this investigation was performed on the 8E8 hybridoma cell line.

Monospecificity of the MoAb 8E8. The MoAb 8E8 is of the IgG1 subtype as revealed by the Ouchterlony double-diffusion technique (data not shown). The specificity of the antibody against human embryonic ε-globin chains was determined by ELISA. The antibody was conjugated with alkaline phosphatase. The conjugated MoAb was found to react only with fraction I containing ε, αγ, and β-globin chains but not with adult or cord blood hemolysates containing αγ, δ, and α-globin chains (Figs 1 and 2). Moreover, as little as 3 ng of ε-globin chains could be detected positively by solid-phase ELISA using 6 ng of the MoAb 8E8 conjugated to alkaline phosphatase (Fig 1).

Western blotting shows that antibody 8E8 reacts with embryonic ε-globin chains but not with ε, δ, and β-globin chains (Fig 3). Similarly, the immunoprecipitation experiment demonstrates that the MoAb 8E8 reacts against only ε-globin chains and not with δ and ε-globin chains (Fig 4).

SBIA for ε-globin chains. Figure 5 illustrates the SBIA for human embryonic ε-globin chains. In each assay, eight...
standards ranging from 0% to 2.8% \( \xi \)-globin chains in adult hemolysate were included. The assay can detect with confidence as little as 0.04% of \( \xi \)-globin chains.

The proportion of \( \xi \)-globin present in unknown hemolysate samples can be estimated by visually matching the color intensity of the test hemolysate bands with the standards. Alternatively, the bands can be scanned and quantitated by densitometry. The within-assay precision using densitometry was assessed by running standard samples six times in one day. The coefficient of variation ranged from 17% for the samples containing 0.04% \( \xi \)-globin to 7% for the samples containing 0.7% \( \xi \)-globin. The between-assay precision was measured by assaying duplicate standard samples in seven runs on separate days. The coefficient of variation ranged from 52% for the samples containing 0.04% \( \xi \)-globin to 9% for the samples containing 0.7% \( \xi \)-globin.

Preliminary studies have shown that results by visual estimation are almost identical to those obtained by densitometry (data not shown). In subsequent experiments, data were obtained by the visual estimation method by two independent observers. Figure 6 illustrates the correlation between the results obtained by SBIA as compared with those obtained by the liquid-phase radioimmunoassay using rabbit polyclonal anti-\( \xi \)-globin antibodies. In 131 adult and cord blood hemolysate samples analyzed by both methods, the correlation coefficient was 0.96. However, there appears to be a slight overestimation of \( \xi \)-globin chains by SBIA as compared with the liquid-phase radioimmunoassay.

Detection of \( \alpha \)-thalassemia-I carriers due to the \( (\_\text{SEA}^+) \) deletion. Hemolysates from 60 individuals were analyzed by SBIA. The results were estimated visually by two independent observers without prior knowledge of the \( \xi \)-\( \alpha \)-globin genotypes of these individuals. Table I illustrates that, in 30 individual documents by gene mapping to be carriers of the \( (\_\text{SEA}^+) \) deletion, \( \xi \)-globin chains were present in their hemolysates as assessed by SBIA. On the other hand, in 30 other individuals whose genotypes were either normal...
DIAGNOSTIC TEST FOR α-THALASSEMIA-1

Table 1. β′-Globin in α-Thalassemias

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of Individuals Examined</th>
<th>Mean Value of β′-Globin in Hemolysates by ELISA + SD (%)</th>
<th>Range of β′-Globin in Hemolysates by SBIA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>aa/aa</td>
<td>14</td>
<td>Undetectable</td>
<td>Undetectable</td>
</tr>
<tr>
<td>−α−α/αα</td>
<td>10</td>
<td>Undetectable</td>
<td>Undetectable</td>
</tr>
<tr>
<td>−α−α/αα−α</td>
<td>6</td>
<td>Undetectable</td>
<td>Undetectable</td>
</tr>
<tr>
<td>αα/−−α/SEA</td>
<td>20</td>
<td>0.20 ± 0.09</td>
<td>0.06–0.43</td>
</tr>
<tr>
<td>αα/−/−SEA</td>
<td>4</td>
<td>0.31 ± 0.23</td>
<td>0.19–0.66</td>
</tr>
<tr>
<td>α−/−−/−SEA</td>
<td>6</td>
<td>0.27 ± 0.05</td>
<td>0.19–0.33</td>
</tr>
</tbody>
</table>

* −α−α/aa, Deletion of one α-globin gene from one chromosome, the so-called rightward deletion.
† −α−α/−−α−α, Deletion of two α-globin genes, one from each of the two homologous chromosomes.
§ −−α/−SEA, α Constant Spring gene with point mutation at the termination codon of an α-globin gene.

(aα/aa), heterozygous for the one rightward α-globin gene deletion (−α−α/aa), or homozygous for the right α-globin gene deletion (−α−α/−−α−α), β′-globin chains were not detected in their hemolysates. The presence or absence of β′-globin chains in these 60 hemolysate samples was confirmed by Triton X-100/acid urea/polyacrylamide gel electrophoresis (data not shown).

DISCUSSION

We have constructed a murine hybridoma cell line, 8E8, that secretes antihuman embryonic β′-globin chain MoAb. This antibody is of IgG1 subtype, and it does not react with human ε, δ, γ, γ', β, and α-globin chains as demonstrated by ELISA, Western blotting, and immunoprecipitation. The cell line has been found to be stable over many passages and produces copious amounts of anti-β′-globin antibody.

By using murine antihuman β′-globin chain MoAb 8E8, we have established a simple and specific SBIA for the detection of β′-globin chains in adult hemolysates. By conjugating alkaline phosphatase directly to the antibody, the need for a second antibody as a detector is not necessary, thus avoiding the possibility of cross-reactivity due to the second antibody and false-positive test results. The SBIA is relatively simple and does not require the use of radioactivity or sophisticated instrumentation, and the results can be semiquantitated by visually comparing the color intensities of different hemolysate bands with the known standards.

Infants with hemoglobin Bart’s hydrops fetalis syndrome due to homozygous α-thalassemia-1 with deletion of all four α-globin genes are stillborn or die shortly after birth or during the third trimester of gestation. Prenancies involving Hb Bart’s hydrops fetalis syndrome are associated with an increased risk of maternal complications such as hydramnios, preeclampsia, antepartum or postpartum hemorrhage, and difficult vaginal delivery.15,16 There is also considerable emotional strain for the mothers and their immediate family members. Prenatal diagnosis for Hb Bart’s hydrops fetalis syndrome is possible by means of Southern blotting, slot blotting, or polymerase chain reaction analysis of DNA extracted from fetal cell samples obtained by either chorionic villi biopsy or amniocentesis.17-20 However, the identification of couples at risk of conceiving fetuses afflicted with Hb Bart’s hydrops fetalis syndrome is problematic and is presently dependent on the history of birth of a previous hydropic fetus.

We have reported that the presence of minute amounts of β′-globin chains in adult hemolysates, detected by a liquid-phase radioimmunoassay using rabbit polyclonal antihuman β′-globin chain antibodies, can serve as a marker for carriers of α-thalassemia-1 due to the (−−SEA/) deletion.4 In this investigation, we have demonstrated that a much simpler immunoassay using the murine anti-β′-globin MoAb 8E8 can similarly detect β′-globin chains in adult hemolysates and can be used as a diagnostic test to detect carriers of α-thalassemia-1 due to the (−−SEA/) deletion.

Extensive deletions involving the complete β′-α-globin gene cluster have been described in Southeast Asia.21 Individuals who are heterozygous for these deletions can also be at risk of having offspring with the Hb Bart’s hydrops fetalis syndrome. In three individuals heterozygous for these large deletions, β′-globin chains were not detected in their hemolysates.22 The gene frequency of these large deletions in Southeast Asia is currently unknown.

The gene frequency of the (−−SEA/) deletion in Southeast Asia and southern China is approximately 3%.15,23 Homozygous α-thalassemia due to the (−−SEA/) deletion is by far the major cause of hydrops fetalis syndrome in that part of the world. The World Health Organization estimated in 1983 that there were probably 20,000 infants born annually who are afflicted with homozygous α-thalassemia.24 The simple immunoassay described here is suitable for screening large populations for carriers of the (−−SEA/) deletion. The sensitivity and specificity of this test does, however, need to be defined under field conditions. The general application of this simple screening test for carriers of α-thalassemia-1 due to the (−−SEA/) deletion in Southeast Asia and southern China may well help to improve the genetic counseling and the quality of obstetric care provided to those women at risk of conceiving infants with Hb Bart’s hydrops fetalis syndrome.

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A novel monoclonal antibody based diagnostic test for alpha-thalassemia-1 carriers due to the (-SEA/) deletion

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