Sickle Hemoglobin: A Specific Radioimmunoassay

By Peter T. Rowley, Richard A. Doherty, Cheryl Rosecrans, and Elsa Cernichiari

For the quantitation of hemoglobin S, a radioimmunoassay has been developed which is specific and highly sensitive. Hemoglobin S was purified by column chromatography and injected with complete Freund’s adjuvant into goats. Each goat serum was tested for reactivity against hemoglobins A and S by immunodiffusion and by quantitative precipitation. Hemoglobin A reactivity was removed by absorption with hemoglobin A. One serum so treated was specific for hemoglobin S; it reacted negligibly with hemoglobins A or F. Hemoglobin S was labeled with $^{125}$I by the chloramine T method. In the radioimmunoassay, complete precipitation of the antigen–antibody complex was insured by the addition of rabbit antigoat gamma globulin. This assay offers reliable and specific quantitation of as little as 1 ng of hemoglobin S. Assuming that 10% of the hemoglobin in fetal blood at 16 wk gestation is of adult type, this assay is capable of detecting the amount of hemoglobin S in $10^{-7}$ ml of homozgyous hemoglobin S blood. The prenatal diagnosis of sickle cell anemia by radioimmunoassay will require, in addition, a method for demonstrating the absence of hemoglobin A and a safe method for obtaining fetal erythrocytes without significant contamination by maternal erythrocytes.

The current investigation was prompted by the possibility of the prenatal diagnosis of sickle cell anemia. The quantitation of hemoglobin S in fetal blood requires an analytic method which is specific and highly sensitive. A radioimmunoassay has been developed to take advantage of the specificity characteristic of immunologic reactions and the sensitivity possible with isotopic measurement. This assay represents only the first step of several toward the prenatal diagnosis of sickle cell anemia by radioimmunoassay.

Boerma and Huisman showed that rabbit anti-hemoglobin S serum can be made specific by absorption with hemoglobin A. Goodman and Campbell found that chicken antisera to hemoglobins A or S are different by quantitative precipitation. Reichlin, Hay, and Levine showed that rabbit anti-hemoglobin A distinguishes hemoglobins S and C from hemoglobin A by microcomplement fixation, but not by quantitative precipitation. Reichlin et al. found anti-beta A-chain antibodies to be 66% as effective in fixing complement with hemo-
globin S as with hemoglobin A. Noble, Reichlin, and Schreiber described the preparation of a fraction of antibodies from goat anti-hemoglobin S serum which would react specifically with hemoglobin S and not with hemoglobin A. Hemoglobin S-specific antibodies make feasible the development of an assay for hemoglobin S in the presence of hemoglobin A.

MATERIAL AND METHODS

$^{125}$I was obtained as the carrier-free iodide from Amersham and Searle, Arlington Heights, Ill. Chloramine T was obtained from Eastman Kodak Co., Rochester, N.Y. Carboxymethylcellulose Whatman CM 52 was obtained from Reeve Angel, Clifton, N.J. Hemoglobins S, A, and F were purified by carboxymethyl-Sephadex column chromatography from the blood of a sickle cell anemia patient, a normal adult, and a normal newborn, respectively. Purity was verified by isoelectric focusing in polyacrylamide gels.

Four goats less than 1 yr of age were injected with 5 mg of hemoglobin S in complete Freund's adjuvant twice 10 days apart. Serum obtained on the 21st day after the first immunization was tested for anti-hemoglobin S antibodies by double immunodiffusion in agar plates. Only one of the sera showed a strong precipitin band. The reactivity of this serum with hemoglobin A was eliminated by exposure to hemoglobin A bound to carboxymethylcellulose. Carboxymethylcellulose, which had been equilibrated with 10 mM sodium phosphate, pH 6.5, was mixed with an excess of chromatographically purified hemoglobin A in the same buffer and stirred for 5 min at 4°C. The hemoglobin A-carboxymethylcellulose was collected by centrifugation. One milliliter of the above serum was adjusted to pH 6.5 with 1 M HCl and added to 0.17 g of hemoglobin A-carboxymethylcellulose which contained about 27 mg hemoglobin A. The mixture was stirred at 25°C for 16 hr, and the resin removed by centrifugation.

A larger proportion of anti-hemoglobin antibody is of the nonprecipitative type. To insure complete precipitation of antigen-antibody complexes in the radioimmunoassay, rabbit anti-goat gamma globulin was prepared. Goat gamma G2 was prepared by ammonium sulfate precipitation followed by DEAE-cellulose column chromatography.

Purified hemoglobins S, A, and F were labeled with $^{125}$I by the chloramine T procedure of Greenwood et al., except that the reaction contained 0.6 mCi of $^{125}$I and 10 μg of hemoglobin in a total volume of 0.135 ml, and the reaction was permitted to proceed for 12 sec before addition of 0.24 mg sodium metabisulfite and 0.05 ml of human serum. After Sephadex G-25 filtration, 2 volumes of 5% bovine serum albumin was added and the labeled hemoglobin stored at 4°C. Of the hemoglobin thus labeled, 64% was precipitable by anti-hemoglobin S antisemur. Presumably the remaining labeled hemoglobin S was not precipitable because it had been denatured in the labeling process. The iodine monochloride labeling method was less satisfactory in this regard.

For radioimmunoassay of hemoglobin S, 0.01–0.05 ml of the unknown solution of hemoglobin S containing 1–30 ng was added to a series of tubes. To each was added 50 μl of $^{125}$I-hemoglobin S containing 16,000 cpm and approximately 4 ng of hemoglobin. Standards of known concentration and control tubes containing serum diluent or wash buffer (see next paragraph) in place of the unknown solution were included. To each tube was added the absorbed hemoglobin S serum diluted 1:256 with serum diluent; this concentration had previously been determined to precipitate 50% of the maximally precipitable labeled hemoglobin S in the absence of unlabeled hemoglobin S (50% × 64% = 32%) and insured that antibody was present in a limiting amount, a requirement for competition between labeled and unlabeled antigen. After 36 hr at 4°C, 0.2 ml of rabbit anti-goat gamma globulin serum, diluted 1:4 with 1% albumin, was added to insure precipitation of all of the antigen-antibody complexes. After an additional 16 hr at 4°C, the tubes were centrifuged in a swinging bucket rotor for 30 min at 2000 g in an International Centrifuge Model PR2. After three washings with wash buffer, the precipitates were counted in a Nuclear-Chicago Model 4230 gamma counter. All determinations were made in duplicate.

Wash buffer contained, per liter, 3.2 g Na$_2$HPO$_4$, 9 g NaCl, 0.1 g Na azide, 2 g bovine serum albumin Fraction V, 1.9 g Na$_2$-EDTA (pH 7.2). Dilutions of hemoglobin, labeled or unlabeled, were made with 1% bovine serum albumin in wash buffer. Serum diluent was pre-immunization goat serum diluted 1:150 with the same 1% albumin.
RESULTS

Figure 1 depicts the results of the radioimmunoassay for known amounts of unlabeled hemoglobin S. The counts precipitated are inversely proportional to the log of the amount of unlabeled hemoglobin S. The useful range of the assay with this serum is approximately 1–30 ng of hemoglobin S.

The assay is specific for hemoglobin S, since hemoglobins A and F are not precipitated. The results shown in Fig. 1 for hemoglobin S are unaltered when an equal quantity of hemoglobin A is also present.

The labeled hemoglobin S is as stable as many protein hormones similarly labeled; approximately a 10% decrease in counts precipitable is noted after 1 mo. The only previously described radioimmunoassay used 125I rather than 131I; 131I-hemoglobin S has the disadvantage of requiring frequent preparation because of the more rapid decay of the isotope and a greater instability of the protein subjected to this more energetic isotope.

DISCUSSION

The radioimmunoassay described represents an advance in the quantitation of hemoglobin S. The assay is specific, since reaction with hemoglobins A and F are negligible. It is also highly sensitive, permitting the quantitation of amounts as small as 1 ng.

The application of this radioimmunoassay that has principally concerned us is its possible contribution to the prenatal diagnosis of sickle cell anemia. For the prenatal diagnosis of sickle cell anemia, a method must demonstrate that (1) fetus has hemoglobin S, (2) the fetus lacks hemoglobin A, and (3) maternal hemoglobins, if present in the sample, are not confounding the analysis. The second requirement, that the fetus lacks hemoglobin A, is necessary since hemoglobin S will be found in a fetus heterozygous, as well as one homozygous, for the sickle gene. The third requirement is necessary because, in most cases where prenatal diagnosis is indicated, the mother will have sickle trait and thus have both hemoglobins S and A.

The radioimmunoassay described in this paper relates principally to the first requirement. This assay method has the sensitivity needed for the detection of sickle hemoglobin in fetal blood. During the second trimester of pregnancy, 5%–10% of the hemoglobin is adult in type, according to studies by a number of authors and also by us. Assuming that the hemoglobin concentration is at least 10 g/100 ml at this time, the radioimmunoassay described should be
able to detect the amount of hemoglobin S present in $10^{-7}$ ml of blood from a homozygous hemoglobin S fetus of this age.

Regarding the second requirement, the prenatal diagnosis of sickle cell anemia by radioimmunoassay will require, in addition, a method for demonstrating the absence of hemoglobin A. For the radioimmunoassay of hemoglobin A, we are trying to prepare a serum which recognizes hemoglobin A, but not hemoglobin S, but have not yet succeeded. Since goat hemoglobin more closely resembles hemoglobin A than hemoglobin S, a goat antiserum which recognizes hemoglobin A and not hemoglobin S may be more difficult to obtain than the reverse. For this purpose we are investigating other species as well.

The third requirement, that maternal hemoglobins not confound the analysis, requires consideration of the nature of the sample available. For simplicity of analysis, a pure sample of fetal blood is obviously the most desirable. The best chance of obtaining such a sample during pregnancy would be afforded by sampling the fetus under direct vision. A fetoscope with blood sampling capacity is under development in several laboratories including our own. Whether such a sampling device will provide fetal blood uncontaminated by maternal blood remains to be seen.

If the fetal sample is only slightly contaminated by maternal blood, the radioimmunoassay approach may still be adequate. The degree of maternal contamination can be estimated by determining the distribution of hemoglobin F throughout the cell population by the Kleihauer-Betke method. Maternal contamination of 1% or less will not be a serious impediment to analysis by radioimmunoassay, since the adult hemoglobins of the fetus will be present in five to ten times greater amounts than the hemoglobins of maternal origin.

If greater degrees of maternal contamination are present, specific antiserum to hemoglobins S and A may still be of value as preparative reagents. Others have advocated that a mixture of fetal and maternal cells be incubated with radioactive amino acids (since most of the hemoglobin-synthesizing cells will be fetal in origin) and that the lysate be chromatographed or electrophoresed to determine which hemoglobins are radioactive. Alternatively, the analysis of such a radioactive lysate could be performed by precipitation with specific hemoglobin S or hemoglobin A antiserum. The latter method requires much less sample material than other methods and permits a larger number of analyses.

In summary, the radioimmunoassay for hemoglobin S we have developed, when combined with one for hemoglobin A to be developed, should provide the fetal genotype, given a sample of pure or minimally contaminated fetal blood.

Specific hemoglobin radioimmunoassays provide powerful tools for microanalysis. Their application may include not only the prenatal diagnosis of hemoglobinopathies, but also the elucidation of hemoglobin development at very early stages in the normal fetus and the microanalysis of blood samples for medicolegal purposes.

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