Immunologic Studies of Hemoglobins

II. Quantitative Precipitin Test Using Anti Fetal Hemoglobin Sera

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In the previous paper, data were presented which suggest that an anti fetal hemoglobin serum of high titer can be obtained by the immunization of rabbits with purified fetal hemoglobin incorporated into Freund's adjuvant mixtures. Since preliminary studies with this serum indicated the presence of fetal hemoglobin not only in blood obtained from the newborn infant but also in specimens from patients with sickle cell anemia and a number of other syndromes where an alkali resistant, fetal-like, hemoglobin fraction has been demonstrated by the denaturation procedure, it was felt advisable to study the nature of this antiserum more extensively. Furthermore, hemoglobin specimens from many hematologically normal adult subjects were found to give weak positive reactions with the serum. By developing a quantitative precipitin test based upon the relationship between the time of reaction and the concentration of fetal hemoglobin, it has been possible to measure accurately the amount of the embryonic pigment in any blood specimen. A comparison between the values for the quantity of alkali resistant hemoglobin in a given sample determined chemically and of fetal hemoglobin determined immunologically suggests that the resistant, fetal-like pigment found under various circumstances is probably identical with the normally occurring hemoglobin of the fetus.

Methods

Preparation of Antisera

Three samples of anti F hemoglobin sera were prepared by the methods previously outlined. Throughout these studies, a 1:1 dilution of antiserum in saline was employed.

Preparation of Hemoglobin Solutions

Hemoglobin solutions were prepared from erythrocytes washed three times with normal saline and hemolyzed by the addition of 2 to 3 volumes of distilled H2O and one-half volume of toluene. Following centrifugation, the clear red hemoglobin layer was accurately adjusted to a concentration of 0.1 Gm. hemoglobin per 100 ml. solution by the addition of normal saline.

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This investigation was started during a Research Fellowship in Medicine of the American College of Physicians, 1951-1952 and completed during tenure of a special United States Public Health Service Fellowship, 1952-1953.
Precipitin Tests

Precipitin tests were carried out at 20°C. in chemically clean, uniform bore tubes of 3 mm. internal diameter and approximately 30 mm. length. Approximately 0.05 ml. of the 0.1 Gm. per cent hemoglobin solution was layered over an equal volume of a 1:1 dilution of anti F hemoglobin serum. The time required for the precipitin ring to become visible was noted in each test. By proper shielding of the microprecipitin tube from the light source, the appearance time of the ring could be accurately determined with relative ease. Checks by independent observers showed little difference in this time, and repeated observations on the same specimen revealed minimal variations.

Technic for Standardization of the Anti F Sera

The fetal hemoglobin content of five cord blood hemoglobin specimens was accurately determined by the alkali denaturation procedure as described in previous publications.2 Aliquots of these cord blood specimens were accurately adjusted to 0.1 Gm. per cent hemoglobin concentrations by the addition of normal saline. Similarly, a specimen of hemoglobin, prepared from the red cells of a normal adult subject, which failed to react with the anti F serum even after 24 hours, was prepared at exactly 0.1 Gm. per cent hemoglobin concentration. Using the latter as a diluent, the cord blood hemoglobin solution (of known fetal hemoglobin content) was progressively diluted by half through a series of twelve tubes, so that the final mixture contained 1 part cord blood hemoglobin to 4095 parts normal hemoglobin. The concentration of F hemoglobin in each of these dilutions was then calculated. Micro-precipitin tests were carried out in a water bath at 20°C. and the appearance time of the precipitin ring in each tube carefully noted. When the logarithm of the time of reaction in minutes as the abscissa was plotted against the logarithm of the per cent of fetal hemoglobin in the specimens as the ordinate, a straight line relationship was obtained. Anti F serum from each animal was thus standardized against all five cord blood specimens and their twelve dilutions so that each standard curve consisted of sixty-five points. Mathematical analysis of the graphs constructed revealed that all were of the type \( y = bx^n \) where \( b \) and \( n \) are constants, \( y = \) per cent of fetal hemoglobin and \( x = \) the time of reaction in minutes. Expressed as a logarithmic function these formulae assume the form of \( \log y = \log b + n \log x \), where \( b \) and \( n \) may be calculated from the standardization curves.*

Alkali Denaturation Test for the Determination of Fetal Hemoglobin

The procedure has been adequately described in previous publications.2 In each instance where the immunologic technic indicated the presence of 1.5 per cent F hemoglobin or more, the results were checked by the chemical determination of the embryonic pigment. Since the latter method is inadequate below about 2 per cent fetal hemoglobin,2 values less than 1.5 per cent were rarely checked by the chemical procedure.

Results

Standardization Curves of Anti F Hemoglobin Sera

A typical standardization curve is presented in figure 1. With concentrations of fetal hemoglobin of 10 per cent or higher, the reaction took place very rapidly, with some sera in approximately 1 to 2 minutes. The error in timing such rapidly occurring reactions is probably considerable, and in the range above 10 per cent fetal hemoglobin, the points tended to be slightly off the curve. From 10 per

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* Determination of \( b \) and \( n \) may be done as follows: To determine \( b \): when \( \log x = 0 \), i.e., where line crosses ordinate, \( \log y = \log b \) or \( y = b \). Since \( y \) may be determined from the graph, \( b \) is also known. To determine \( n \): when \( \log y = 0 \), i.e., where line crosses abscissa, \( 0 = \log b + n \log x \). Since \( x \) may be calculated from the graph and \( b \) has been determined, \( n = - \frac{\log b}{\log x} \).
cent to approximately 0.5 per cent fetal hemoglobin (using hemoglobin specimens with known amounts of fetal hemoglobin) the values were noted to fall on a remarkably straight line, the precipitin ring appearing as a sharp, white precipitate at the interface. The variation in repeated readings was ±1 to 2 minutes. With concentrations of less than 0.5 per cent fetal hemoglobin, the reaction took place after a relatively prolonged interval and errors in reading the time of reaction were again probably considerable. However, checks of within five minutes were usually noted and errors in this range were found to represent but little difference in the percentage of fetal hemoglobin. Furthermore, with values below 0.05 per cent fetal hemoglobin in the standard solutions, the appearance time of the precipitin rings could not be accurately determined because the precipitate had a much fuzzier character. Variations of ±10 to 15 minutes, however, did not alter the curve appreciably. In some cases, it was possible to carry out the curves to as little as 0.02 per cent fetal hemoglobin, the reaction taking place with different sera in from 4 to 6 hours. The normal hemoglobin specimen used as the diluent failed to give a positive precipitin test with the anti F hemoglobin sera during the 24 hours of observation.

Although there is an increasing error in the timing of the reaction with pro-
gressively more dilute solutions of fetal hemoglobin, this factor plays a relatively
minor role between 10 per cent and 0.5 per cent F hemoglobin, the range of
greatest interest. Because of the nature of the log graph employed, large errors
in time with more dilute solutions do not affect the results significantly. This
becomes evident when one reads values from the standardization curve for time
intervals of, for example, 210 minutes and 240 minutes. These would represent
centers of 0.04 per cent and 0.03 per cent fetal hemoglobin, respectively
(fig. 1). On the other hand, as the per cent of fetal hemoglobin becomes greater,
determination of the time of appearance becomes more accurate. Only when
concentrations of greater than 10 per cent fetal hemoglobin are encountered,
does the reaction take place so rapidly that variations of less than 1 minute in
the timing introduce serious errors.

The relationship between the concentration of fetal hemoglobin and the time
of reaction has been noted with each of the three specimens of sera so examined.
It should, however, be emphasized that every serum must be independently
standardized and that the values for b and n in the equation \( y = bx^n \) will differ
for each curve. Consequently, although a given blood specimen may react at
different times with each of these antisera, the concentration of fetal hemo-
globin remains constant in every case. Furthermore, hemoglobin specimens pre-
pared from the erythrocytes of normal adults which do not react with one of
the sera, fail to react with the others.

**Concentration of Fetal Hemoglobin in Normal Adults and in Patients with Various
Hematologic Disorders**

**Fetal hemoglobin in normal adults.** Hemoglobin solutions from the red cells of
one hundred normal human adults were studied for the presence of fetal hemo-
globin by the quantitative immunologic technic. The distribution of the values
found for fetal hemoglobin is presented in figure 2. In fifty-nine of the speci-
mens, values of less than 0.05 per cent fetal hemoglobin were noted, and in
approximately half of these (thirty of fifty-nine) no fetal compound was de-
monstrable after 4 to 6 hours of reaction (equivalent to less than about 0.03
per cent fetal hemoglobin). Many of the latter were followed for up to twelve
hours, without a positive precipitin test for hemoglobin being observed. Of the
remainder, forty were noted to have from 0.05 per cent to 0.5 per cent fetal
hemoglobin and only one a value higher than 0.5 per cent. Repeat determina-
tions over a period of eight to twelve weeks in fifteen normal subjects revealed
little variation in the level of fetal hemoglobin, the maximum range usually
being of the order of ±0.1 per cent fetal hemoglobin. In the instance of the
one individual with a fetal hemoglobin concentration of greater than 0.5 per
cent, however, values of 0.6, 0.67, 0.82, 0.98, 1.2 and 1.47 per cent were ob-
tained. These results suggest that in normal adult individuals, a level of 1.0
per cent F hemoglobin approximates the upper limit of normal.

**Sickle cell anemia.** Sixteen patients with unequivocal diagnoses of sickle cell
anemia were studied. In thirteen of these, the quantity of fetal hemoglobin was
greater than 2.0 per cent. Values for fetal hemoglobin in these individuals were
determined on the same samples by the alkali denaturation procedure (table 2).
Except in the one instance of a patient with 13.5 per cent F hemoglobin by the
immunologic procedure, agreement between the two technics was remarkably good. As has been pointed out, however, minimal errors in reading the appearance times of the precipitin ring with values of over 10 per cent F hemoglobin introduce marked variations in the per cent of fetal hemoglobin. Three of the sixteen patients had less than 1 per cent fetal hemoglobin, none having been transfused for from eight to twelve weeks prior to study. Determination of the fetal compound by the chemical test also failed to reveal abnormal quantities of fetal hemoglobin. Inasmuch as values below 2.0 per cent cannot be evaluated by the latter procedure, however, no comparison between the two technics in this range is justified.

Sickle cell trait. Twelve individuals with sickle cell trait without associated anemia of any type showed the following distribution of fetal hemoglobin values:

<table>
<thead>
<tr>
<th>Fetal Hemoglobin</th>
<th>Number of Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Less than 0.05%</td>
<td>4</td>
</tr>
<tr>
<td>0.05% to 0.5%</td>
<td>7</td>
</tr>
<tr>
<td>0.5% to 1.0%</td>
<td>1</td>
</tr>
</tbody>
</table>

Mediterranean anemia. Blood specimens from three individuals with moderately severe Mediterranean anemia had fetal hemoglobin concentrations of 6.3 per cent, 4.0 per cent, and 1.3 per cent, the latter sample having been obtained from a patient heavily transfused prior to testing. One patient with thalassemia
minor had 0.75 and 1.2 per cent F hemoglobin on two occasions. Again, comparative values by the alkali denaturation test were in good agreement.

Hereditary spherocytosis. Nine patients with hereditary spherocytosis were studied; six of these were members of the same family. In five of the six related individuals more than 2.0 per cent fetal hemoglobin was present by both immunologic and chemical determinations. The remaining member of this group had only 0.7 per cent F hemoglobin, while for the three unrelated patients, values of 0.82, 0.38, and 0.1 per cent F hemoglobin were detected.

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Number of individuals with:</th>
<th>Less than 0.5% F hemoglobin</th>
<th>0.5%-1.0% F hemoglobin</th>
<th>More than 1.0% F hemoglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal adults</td>
<td>99</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Sickle cell anemia</td>
<td>0</td>
<td>3</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Sickle cell trait</td>
<td>10</td>
<td>2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Hereditary spherocytosis</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Mediterranean syndrome</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Acute leukemia</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Chronic leukemia</td>
<td>9</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Hodgkins disease</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Carcinoma</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Pernicious anemia</td>
<td>16</td>
<td>0</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Polycythemia</td>
<td>13</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Fe deficiency</td>
<td>19</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Refractory anemia</td>
<td>7</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Pancytopenia</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Pure red cell anemia</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Thrombocytopenic purpura</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Agranulocytosis</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Acquired hemolytic anemia</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Miscellaneous hematologic syndromes. Values obtained in over one hundred miscellaneous conditions are presented in table 1. With values greater than 2.0 per cent F hemoglobin, checks with the chemical technic were done and are found in table 2. These results corroborate previous studies which indicated the presence of fetal or fetal-like hemoglobin in certain acquired hematologic disorders in which the marrow appeared to be under significant stress (leukemia, metastatic carcinoma, multiple myeloma, etc.). Of the individuals whose erythrocytes were found to have over 1.0 per cent fetal hemoglobin, one had chronic leukemia, one metastatic carcinoma to the marrow, four had untreated pernicious anemia, one polycythemia vera, one iron deficiency anemia and three had pancytopenia of varying etiology (one drug induced, two chronic idiopathic). Of particular interest are two brothers with so-called chronic aregenerative anemia (pure red cell anemia or erythrocytogenesis imperfecta) each of whom had abnormal quantities of fetal hemoglobin. Finally one unexplained value of 2.0 per cent F hemoglobin was detected in a normal pregnant woman. Whether
this represents placental transfer of fetal red cells cannot be ascertained at this
time, but postpartum studies may yield the answer. In twelve other pregnant
women values below 0.85 per cent fetal hemoglobin were noted.

<table>
<thead>
<tr>
<th>Specimen from patient with:</th>
<th>% F hemoglobin</th>
<th>Specimen from patient with:</th>
<th>% F hemoglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Precipitin test</td>
<td>Alkali denaturation</td>
<td></td>
</tr>
<tr>
<td>Sickle cell anemia</td>
<td>2.1</td>
<td>2.3</td>
<td>Hereditary spherocytosis</td>
</tr>
<tr>
<td>Sickle cell anemia</td>
<td>2.0</td>
<td>2.5</td>
<td>Hereditary spherocytosis</td>
</tr>
<tr>
<td>Sickle cell anemia</td>
<td>6.0</td>
<td>7.6</td>
<td>Hereditary spherocytosis</td>
</tr>
<tr>
<td>Sickle cell anemia</td>
<td>3.2</td>
<td>4.2</td>
<td>Hereditary spherocytosis</td>
</tr>
<tr>
<td>Sickle cell anemia</td>
<td>15.5</td>
<td>11.0</td>
<td>Hereditary spherocytosis</td>
</tr>
<tr>
<td>Sickle cell anemia</td>
<td>4.0</td>
<td>4.0</td>
<td>Mediterranean anemia</td>
</tr>
<tr>
<td>Sickle cell anemia</td>
<td>0.89</td>
<td>1.0</td>
<td>Mediterranean anemia</td>
</tr>
<tr>
<td>Sickle cell anemia</td>
<td>4.0</td>
<td>3.3</td>
<td>Mediterranean anemia</td>
</tr>
<tr>
<td>Sickle cell anemia</td>
<td>3.5</td>
<td>3.2</td>
<td>Chronic myeloctic leukemia</td>
</tr>
<tr>
<td>Sickel cell anemia</td>
<td>3.8</td>
<td>2.9</td>
<td>Carcinoma with metastases</td>
</tr>
<tr>
<td>Sickel cell trait</td>
<td>0.05</td>
<td>0.8</td>
<td>Untreated pernicious anemia</td>
</tr>
<tr>
<td>Sickel cell trait</td>
<td>0.055</td>
<td>0.7</td>
<td>Untreated pernicious anemia</td>
</tr>
<tr>
<td>Sickel cell trait</td>
<td>0.064</td>
<td>0.1</td>
<td>Untreated pernicious anemia</td>
</tr>
<tr>
<td>Sickel cell trait</td>
<td>0.142</td>
<td>0.9</td>
<td>Pure red cell anemia</td>
</tr>
<tr>
<td>Hereditary spherocytosis</td>
<td>0.38</td>
<td>1.0</td>
<td>Pure red cell anemia</td>
</tr>
<tr>
<td>Hereditary spherocytosis</td>
<td>0.64</td>
<td>1.6</td>
<td>Hypoplastic anemia</td>
</tr>
<tr>
<td>Hereditary spherocytosis</td>
<td>0.7</td>
<td>1.4</td>
<td>Pan cytopenia</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Although no direct proof exists that the anti fetal hemoglobin sera employed
in this study are specific for fetal hemoglobin, several types of indirect evidence
strongly support this conclusion. Perhaps the most important is furnished by
the straight line relationship noted between the logarithm of the time of reac-
tion and the logarithm of the concentration of fetal hemoglobin in standard
solutions. It would be surprising if this phenomenon would occur in the absence
of specificity of the sera for fetal hemoglobin. Although an explanation for this
particular relationship in terms of the postulated concepts of antibody-antigen
reactions is not evident, these observations are by no means unique, since some-
what similar quantitative phenomena have been noted by Hooker and Boyd6, 7
using a number of immunologic systems. Indeed, the determination of the zone
of optimal proportions by either the Dean and Webb 6 or the Ramon 6 technics
also involves a consideration of the time of flocculation in relationship to various
concentrations of antigen and antibody. Except for the work of Hooker and
Boyd, 6 little has been written concerning the mechanics and usefulness of this
reaction. As Boyd 6 points out, however, the relationship between the velocity
of reaction and concentration of antigen permits a rapid and accurate estimation
of extremely small amounts of antigen. Our own experience would tend to
confirm this observation. However, there exists one important difference be-
between the studies of Hooker and Boyd and those reported in this paper. The former investigators noted a direct straight line relationship between the time of flocculation and the concentration of antigen, working in the zone of antibody excess. This was observed in a number of immunologic systems including a hemoglobin-antihemoglobin mixture. The present experiments indicate a logarithmic relationship between the two variables, probably also in the zone of antibody excess, although no determinations of antibody nitrogen were carried out to prove this point. Whether this results from the basic difference in the manner in which the two studies were carried out (diffuse versus local flocculation), or whether the characteristics of the sera as prepared by the respective investigators are different, is not clear at present.

Further support for the specificity of the anti F hemoglobin sera is provided by the observation that approximately 30 per cent of normal adult blood specimens fail to give positive reactions with the anti F hemoglobin sera after prolonged periods of observation. It would be reasonable to assume that high titered sera would react with all normal hemoglobin specimens if the reaction were a nonspecific one between the antibody and any type of human hemoglobin. Furthermore, the close correlation between percentages of fetal hemoglobin as determined immunologically and those obtained by chemical analysis strongly supports the concept of specificity.

With the available evidence suggesting that the anti fetal hemoglobin serum is specific for the embryonic pigment, it becomes possible to evaluate the nature of the alkali-resistant hemoglobin fractions observed in the hereditary hemolytic syndromes and in some acquired hematologic diseases, as leukemia, pernicious anemia, metastatic carcinoma, etc., more adequately. Previous studies, employing the method of fractional denaturation, in which the rates of reaction of the alkali resistant fractions were determined, suggested that, at least in some instances, the resistant hemoglobin component differed from the fetal compound. Because of the inherent difficulty of the technic, however, the data were felt to be of an inconclusive nature. In view of the fact that each of these resistant hemoglobin fractions react with the specific anti fetal hemoglobin serum it is likely that they are identical with the normally occurring embryonic pigment. This conclusion is even more strongly suggested by the fact that from a quantitative standpoint alkali-resistant hemoglobins give positive immunologic tests in a manner identical with that of fetal hemoglobin. The excellent agreement between the amounts of fetal or fetal-like hemoglobin as determined chemically and immunologically, in the range where the tests are reliable, constitutes a strong argument in favor of the identity of the two (table 2). One should probably, therefore, speak of “abnormal quantities” of fetal hemoglobin occurring in the diseases in question rather than of abnormal fetal-like hemoglobin fractions.

The interesting observations encountered in studying hemoglobin specimens from normal adults deserve further comment. The presence of fetal hemoglobin in small amounts has been postulated by a number of investigators although quantitative data have, in general, been lacking. These studies have been reviewed in the previous paper. Perhaps the most thorough investigations have been carried out by Roche and Darrien who report that three varieties of atypical hemoglobin may be detected by solubility studies in amounts of up to
10 per cent of the total. Whether these represent fetal hemoglobin variants or forms of adult hemoglobin undergoing some chemical changes (such as methemoglobin or sulfhemoglobin formation) cannot be ascertained from their data. Our own data strongly support the view that fetal hemoglobin is present in the circulation of at least 70 per cent of the adult population, but in extremely small amounts, a value of 1.0 per cent approximating the upper limit of normal.

Despite the fact that fetal hemoglobin seems to be present in the red cells of a large proportion of patients with the hereditary hemolytic syndromes, it has been impossible to correlate the amounts of the embryonic pigment with the clinical severity of the disease, the hematologic picture, or the age of the individual. This was especially well brought out in the study of patients with sickle cell anemia, inasmuch as the three individuals lacking abnormal amounts of fetal hemoglobin all suffered from severe manifestations of the disease.

SUMMARY

1. A quantitative immunologic technic for the determination of fetal hemoglobin has been developed using a specific anti fetal hemoglobin serum.

2. Fetal hemoglobin occurs in many normal adult individuals in extremely small quantities: values of up to 1.0 per cent of the total hemoglobin may be in the form of the embryonic compound.

3. The alkali-resistant hemoglobin fractions detected by chemical procedures in a number of hematologic diseases are, in all probability, identical with the normally occurring fetal hemoglobin of the red cells of newborn infants. It is suggested, therefore, that these diseases are characterized by abnormal quantities of fetal hemoglobin rather than by an abnormal fetal-like component.

REFERENCES


Immunologic Studies of Hemoglobins: II. Quantitative Precipitin Test Using Anti Fetal Hemoglobin Sera

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