

Carbonic Anhydrase and Fetal Hemoglobin in Thyrotoxicosis

By LIE-INJO LUAN ENG, L. HOLLANDER AND H. HUGH FUDENBERG

IN A RECENT REPORT Lie-Injo and Tarail¹ described a condition characterized by a deficiency of both types I and II² (also designated B and C)³ of carbonic anhydrase. This deficiency, though associated with a high level of fetal hemoglobin, was not due to hemoglobinopathy, hereditary persistence of fetal hemoglobin, or any of the known types of thalassemia. Because of lack of proof of hereditary basis and uncertainty whether the changes in the patient were caused by a specific etiology, the condition was designated as a new syndrome rather than as a specific disease entity, as such a combination of findings had not been described before.

These findings prompted us to study erythrocyte carbonic anhydrase levels in different disease states, especially those with unexplained increase of fetal hemoglobin. We now report the occurrence of carbonic anhydrase deficiency in patients with thyrotoxicosis, several of whom had an increase of HbF level in the blood.

MATERIALS AND METHODS

Hemoglobin electrophoresis was performed in starch gel⁴ and in agar gel.⁵ Alkali denaturation of hemoglobin was done by the method of Singer et al.,⁶ and 2 per cent of the total amount of hemoglobin was taken as the upper limit of normal. Quantitative analysis of hemoglobin components was carried out on diethylaminoethyl (DEAE) cellulose column chromatography by the method of Huisman and Dozy.⁷

Carbonic anhydrase activity in the red blood cells was estimated by the quantitative method of Wilbur and Anderson,⁸ which is based on that of Roughton and Booth.⁹ By this method the rate of change of pH is measured when CO₂ in solution in H₂O is converted to H₂CO₃ in the presence of the enzyme. Enzyme activity in hemolysates prepared from packed red blood cells was measured in two different concentrations and expressed in arbitrary enzyme units per milligram Hb. A unit of activity is defined as that amount of enzyme required to reduce the blank reaction time under the stated conditions to half its value and is given by the formula:⁹

From the G. W. Hooper Foundation and the Section of Hematology and Immunology, Department of Medicine, University of California Medical Center, San Francisco, California, and the Permanente Medical Group, Oakland, California.

This study was supported by Research Grant R01 HE 10486-10 GEN from the NIH, USPHS; by the University of California International Center for Medical Research and Training (Hooper Foundation, San Francisco School of Medicine) with Research Grant TW 00144 from the Office of International Research, NIH, USPHS; by a grant from the University of California School of Medicine Committee on Research; and by a Hematology Training Grant, HE 05677, NIH, USPHS.

First submitted Feb. 29, 1967; accepted for publication April 7, 1967.

LIE-INJO LUAN ENG, M.D., PH.D., D.T.M. & H.: Associate Research Hematologist, The G. W. Hooper Foundation, University of California San Francisco Medical Center, San Francisco, Calif. L. HOLLANDER, M.D.: Chief, Department of Internal Medicine, Permanente Medical Group, Oakland, Calif. H. HUGH FUDENBERG, M.D.: Professor of Medicine, Section of Hematology and Immunology, Department of Medicine, University of California San Francisco Medical Center, San Francisco, Calif.

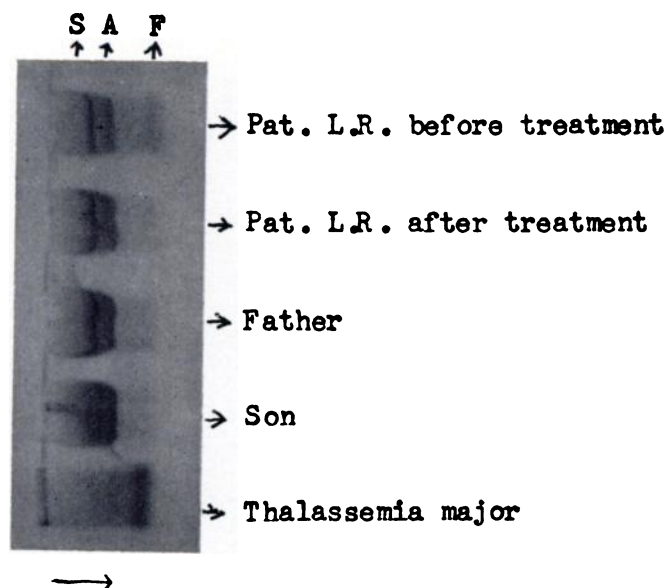


Fig. 1.—Agar gel electrophoresis pH 6.2 of the hemolysate of patient L.R., showing significant amount of Hb F before and strongly reduced amount of Hb F after treatment.

$$\text{Activity} = \frac{R - R_0}{R_0} \quad \text{units}$$

R and R_0 = the rates in the presence and absence of enzyme.

Carbonic anhydrase was qualitatively visualized by starch-gel electrophoresis using the buffer pH 9.5 of Haut et al.¹⁰ and at pH 8.6 with Tris-HCl-boric acid-EDTA buffer, after which the gel was stained with amido black. Specific staining for carbonic anhydrase followed Häusler's method.¹¹

CASES STUDIED

Mrs. L.R., a 44-year-old Negro woman, complained of loss of weight, fatigue, irritability, and nervousness of long duration. She had a past history of anemia one year previously, for which she was treated with iron and vitamin B₁₂ injections. When examined on January 17, 1966, she was found nonanemic and without jaundice. Her thyroid was moderately and diffusely enlarged, and she had clinical signs of thyrotoxicosis.

Laboratory findings: Hb 12.6 Gm./100 ml., WBC and platelet counts normal. Sickling test of the red blood cells was positive. Alkali-resistant hemoglobin was 17 per cent of total. On electrophoresis in cellulose acetate, Hb S was 30 per cent of the total hemoglobin. No other abnormal hemoglobin was detected. Bone marrow was normal with normal amount of iron. Protein-bound iodine was 10.2 μ g. per cent. Serum iron was normal. Findings in the first half of February, 1966 were: Hb 13.9 Gm./100 ml.; PCV 41.5 per cent; WBC and platelet counts normal. Differential count of the white blood cells normal. Hemoglobin analysis: Hb F 19.0 per cent, Hb S 31.9 per cent, Hb A 46.0 per cent, and Hb A₂ 3.1 per cent. The presence of Hb S was confirmed by a positive sickling test, by starch-gel and agar-gel electrophoresis; the presence of Hb F was shown by alkali denaturation and by agar-gel electrophoresis (see Fig. 1). The acid-elution technic of Kleihauer and Betke¹² showed that the fetal hemoglobin was heterogeneously distributed in the red blood cells. Hemoglobin analysis repeated on two different days gave essentially the same pattern, with Hb F ranging from 19 to 20 per cent of the total hemoglobin.

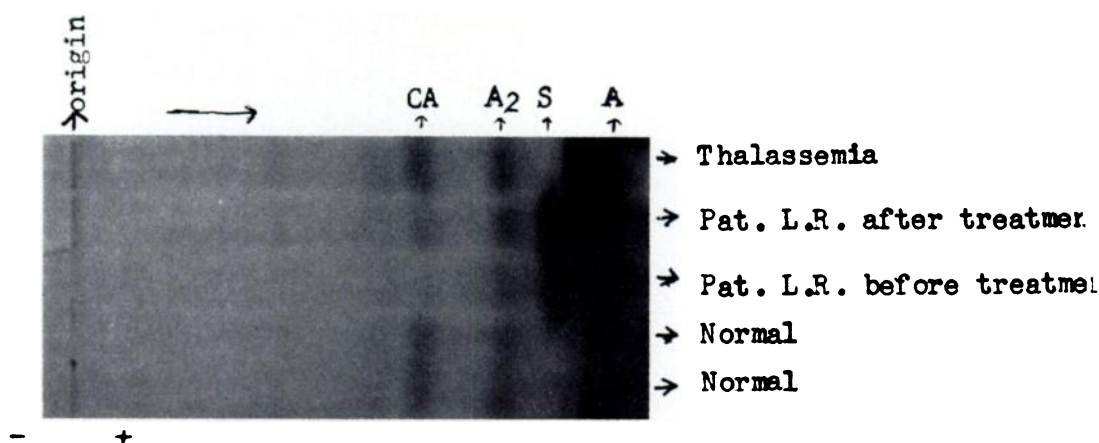


Fig. 2.—Starch gel electrophoresis pH 9.5 stained with amido black, showing carbonic anhydrase before and after treatment. The blood of the normal controls was drawn on the same dates as those of the patient.

This hemoglobin pattern was distinctly unusual, and it did not fit into any of the known patterns of thalassemia, hemoglobinopathy, or their combinations. Therefore, we thought the patient would be suitable for the study of carbonic anhydrase; indeed carbonic anhydrase in her red blood cells was definitely deficient. While in 70 normal controls a level of 38.4 to 70.9 units/mg. Hb was found, the patient's erythrocyte carbonic anhydrase was only 20 units/mg. Hb. The activity of the two different carbonic anhydrase types, B and C (I and II) was not estimated separately; however, from the electrophoretic pattern, it seems that although type B was primarily decreased, type C might also have been diminished.

Since the patient had thyrotoxicosis, this condition might possibly have led to carbonic anhydrase deficiency and, directly or indirectly, to an increase of fetal hemoglobin. To test this possibility, we treated the patient for hyperthyroidism and carefully measured her red cell carbonic anhydrase and fetal hemoglobin during and after treatment. On February 16, 1966, she received an oral dose of 8 mc. of I-131. On March 14, she was still obviously thyrotoxic, hyperkinetic with tachycardia, and had lost more weight. PBI, 14.5 μ g. per cent, was still increased. Hemoglobin analysis was essentially unchanged with an increase of Hb F; Hb S was definitely below half the total amount of hemoglobin; Hb A₂ was normal. The carbonic anhydrase level, although slightly higher, was still deficient. The patient was then treated with Lugol's solution and showed clinical improvement when seen on March 31. On April 28 she appeared euthyroid. Hb F was 11 per cent and Hb S was 35 per cent of the total amount of hemoglobin. On July 27, erythrocyte carbonic anhydrase level of 43.1 units/mg. Hb was normal (see Fig. 2), and the level of Hb F had dropped further to 6.6 per cent. On August 30, Hb F was 4.4 per cent, but carbonic anhydrase level was normal, 62.9 units/mg. Hb. Because of signs of hypothyroidism the patient was then given 1 grain of thyroid per day and was still on this daily dosage when we saw her again on January 7, 1967. Her carbonic anhydrase level was normal, but the Hb F was still slightly elevated, 6.0 per cent of the total hemoglobin.

The family history showed a strong tendency to thyroid disease. The patient's paternal grandfather and a paternal aunt each had died several years previously after an operation for goiter. A paternal uncle had been hospitalized because of respiratory difficulty due to an enlarged thyroid. Another paternal aunt had a large thyroid but no clinical symptoms. We examined the parents, a son, and two paternal uncles of the patient. The mother was physically normal without enlargement of the thyroid but her Hb level and PCV were at the lower limits of normal. The father, who appeared physically normal without clear

Table 1.—Erythrocyte Carbonic Anhydrase Activity and Hb F Levels in Thyrotoxicosis

Case No.	Sex	Age	Clinical symptoms when studied	* CA units/mg. Hb	Hb F
1	F	44	+	20.0	19.5
2	M	24	+	17.0	5.1
3	F	23	+	30.3	0.7
4	M	32	+	30.6	1.6
5	M	27	+	27.1	1.1
6	M	36	+	31.8	0.5
7	F	14	—	50.0	1.2
8	M	14	+	16.5	1.8
9	M	50	+	24.1	0.7
10	F	63	+	59.5	0.8
11	F	71	—	47.7	2.9
12	F	37	±	31.9	0.8
13	F	17	+	29.5	1.6

* CA = Carbonic Anhydrase

Carbonic anhydrase activity in 70 normal healthy persons ranges from 38.4 to 70.9 units per mg. Hb.

evidence of thyroid enlargement, was a carrier of Hb S trait, and his Hb S was definitely below 50 per cent. Fetal hemoglobin was 2.4 per cent, representing a slight unexplained increase. The son was physically normal, had no Hb S, and his hemoglobin level and PCV were at the lower limits of normal. One paternal uncle was a carrier of the Hb S trait but was otherwise entirely normal. The other paternal uncle, who had been hospitalized for difficult breathing due to an enlarged thyroid, was also a carrier of the Hb S trait, but his Hb F level was normal. By the time we saw him, his complaints had disappeared in response to iodine therapy. In all the patient's relatives whom we examined, the carbonic anhydrase levels in the red blood cells were normal and the results of thyroid function tests were within normal limits.

We postulated from findings in Case 1 that thyrotoxicosis may lead to carbonic anhydrase deficiency and, directly or indirectly, to an increase of fetal hemoglobin. Twelve other patients who were not related to this family were therefore examined for erythrocyte carbonic anhydrase and Hb F levels. In most cases opportunities for such studies were available only after treatment had been instituted. Nine had a definite deficiency of erythrocyte carbonic anhydrase three had normal enzyme levels. In seven of the nine patients with low enzyme levels thyroid function tests were carried out. Tests in six showed a definite increase of thyroid function; in one the results were borderline. The other two with low enzyme levels were not studied when blood for carbonic anhydrase estimation was drawn. In the three patients with normal enzyme levels, thyroid function tests gave normal results in two; in the third the PBI test was within normal limits, but radioactive iodine uptake remained elevated. Fetal hemoglobin in one patient with enzyme deficiency was increased to 5.1 per cent; in one with normal enzyme level it was increased to 2.9 per cent. This latter patient was the one whose PBI was normal but the test showed a high uptake of radioactive iodine.

DISCUSSION

The hemoglobin pattern in the first patient was unusual—consisting of an Hb

S level of less than 50 per cent, a high Hb F level of about 19 per cent, a normal Hb A₂ level, and the rest Hb A—and did not follow any of the known patterns found in hemoglobinopathies, thalassemia or their combinations. In homozygous sickle-cell anemia, one would expect the hemoglobin to be almost all Hb S, with a small amount of Hb F and no Hb A. In Hb S-beta thalassemia one would expect an Hb S level of more than 50 per cent and an increase of Hb A₂; also, no evidence of beta thalassemia was found in either parent of this patient. In Hb S-alpha thalassemia there is no increase of Hb F. Also in Hb S associated with hereditary persistence of fetal hemoglobin, Hb S usually exceeds 50 per cent and the distribution of Hb F in the red blood cells is homogeneous, while in this patient the distribution was heterogeneous. The unexplained high level of fetal hemoglobin in Case 1 (19.5 per cent) before treatment of the concomitant thyrotoxicosis, the decrease to 4.4 per cent after treatment, and the changes in erythrocyte carbonic anhydrase level, deficient before and normal after treatment, all led to the conclusion that thyrotoxicosis probably caused the carbonic anhydrase deficiency and the increase of fetal hemoglobin in the erythrocytes. The finding of an erythrocyte carbonic anhydrase deficiency in nine other cases of hyperthyroidism also supports the assumption that thyrotoxicosis leads to carbonic anhydrase deficiency. Of the three cases of thyrotoxicosis with normal carbonic anhydrase level, one patient had thyrotoxicosis from childhood but had been treated continuously. At the time we estimated her carbonic anhydrase and fetal Hb, she was free from clinical symptoms and thyroid function tests were normal, but she had an unexplained hepatosplenomegaly. In the three patients with normal erythrocyte carbonic anhydrase level, the thyroid function tests gave normal results for two and borderline results for one. In this last one Hb F was slightly elevated. The presence or absence of antithyroglobulin (agglutination method), antithyroid microsomal antibody (fluorescent antibody method), and LATS activity (bioassay) showed no correlation with carbonic anhydrase or Hb F levels.

Several explanations relating to the mechanism of the deficient carbonic anhydrase activity in the red cells are possible: (1) Thyrotoxic substances may inhibit carbonic anhydrase activity. (2) An increase of metabolism in thyrotoxicosis may require disposal of an increased amount of CO₂. This is accomplished with the help of the enzyme carbonic anhydrase in the red blood cells. Increased demand for carbonic anhydrase may lead to depletion of the available enzyme. (3) Thyrotoxic substances may depress the production of carbonic anhydrase.

The increase of fetal hemoglobin in the first patient and in two others, one with an enzyme deficiency and one without, is hard to explain. It is tempting to postulate that the increase of fetal hemoglobin is a compensatory mechanism caused by chronic carbonic anhydrase deficiency. Since the lifespan of erythrocytes is 100 to 120 days, Hb F level may still be present when the carbonic anhydrase level becomes normal. An Hb F level of 4.4 per cent in Case 1, however, was reached after the patient had been treated for about 5 months, long after her carbonic anhydrase level had returned to normal. The slight elevation of her Hb F level while she was taking the thyroid drug and while the enzyme level was normal points more to a direct influence of the thyroid

hormone on the increase of Hb F. Also, the slight increase of Hb F in the father, who was not deficient in erythrocyte carbonic anhydrase, does not fit into the theory that deficiency of the enzyme was the cause of the increase. Therefore, the possibility that thyrotoxic substances lead to an increase of fetal hemoglobin production, even without carbonic anhydrase deficiency, warrants further study. Furthermore, the possibility must be kept in mind that the persistent slight elevation of Hb F, together with the father's slight elevation of fetal hemoglobin may represent some type of genetic defect directly involving Hb F production and independent of thyrotoxicosis.

What part, if any, carbonic anhydrase deficiency plays in producing clinical symptoms in thyrotoxicosis remains uncertain. Nevertheless, since the disease is a condition associated with an increased metabolic rate, the disposal of increased amounts of CO₂ from the tissues may be necessary. If so, further studies of the role of carbonic anhydrase deficiency in thyrotoxicosis appear clearly warranted.

Addendum: Dr. D. J. Weatherall has informed us that the patient, whose type B carbonic anhydrase deficiency he and Dr. R. F. Rieder¹³ described, had hyperthyroidism. Dr. Weatherall believes that type B carbonic anhydrase is depressed by the thyroid hormones.

SUMMARY

An adult patient with thyrotoxicosis had erythrocyte carbonic anhydrase deficiency and an abnormally high level of fetal hemoglobin. After treatment of the hyperthyroidism, the erythrocyte carbonic anhydrase level became normal and the level of Hb F dropped. Twelve other patients with hyperthyroidism and not related to this index case were studied. Most of them were already under treatment. Nine had erythrocyte carbonic anhydrase deficiency; two had an increase of fetal hemoglobin. The possible relationship of increase of fetal hemoglobin, erythrocyte carbonic anhydrase deficiency and thyrotoxicosis is discussed.

SUMMARIO IN INTERLINGUA

Un patiente adulte con thyrotoxicosis habeva carente nivellos erythrocytic de anhydrase carbonic e anormalmente alte nivellos de hemoglobina fetal. Post le tractamento del hyperthyroidismo, le nivello erythrocytic de anhydrase carbonic deveniva normal, e le nivello de hemoglobina fetal descendeva. Dece-duo altere patientes con hyperthyroidismo esseva similmente studiate. Nulle de illes esseva consanguineo del probando. Le majoritate del 12 esseva jam sub tractamento. Novem manifestava carentia erythrocytic de anhydrase carbonic, e duo habeva un augmento de hemoglobina fetal. Es commentate le relation possibile inter augmento de hemoglobina fetal, carentia erythrocytic de anhydrase carbonic, e thyrotoxicosis.

ACKNOWLEDGMENT

We wish to thank all colleagues who provided blood samples from thyrotoxicosis patients, especially Dr. A. Blum of the Thyroid Clinic, University of California San Francisco Medical Center, who also carried out the LATS activity (bioassay) studies, and Dr. K. Wuepper of the Section of Hematology and Immunology, who carried out the thyroid antibody studies.

REFERENCES

1. Lie-Injo Luan Eng and Tarail, R.: new syndrome. *Nature* (London) 211:47, 1966.
Carbonic anhydrase deficiency with persistence of foetal haemoglobin: A
2. Tashian, R. E., and Shaw, M. W.: In-

- heritance of an erythrocyte acetyl-esterase variant in man. *Amer. J. Hum. Genet.* 14:295, 1962.
3. Rickli, E. E., Chazanfar, S. A. S., Gibbons, B. H., and Edsall, J. T.: Carbonic anhydrases from human erythrocytes: Preparation and properties of two enzymes. *J. Biol. Chem.* 239: 1065, 1964.
 4. Smithies, O.: An improved procedure for starch-gel electrophoresis: further variation in the serum proteins of normal individuals. *Biochem. J.* 71: 585, 1959.
 5. Robinson, A. R., Robson, M., Harrison, A. P., and Zuelzer, W. W.: A new technique for differentiation of hemoglobin. *J. Lab. Clin. Med.* 50:745, 1957.
 6. Singer, K., Chernoff, A. I., and Singer, L.: Studies on abnormal hemoglobins. I. Their demonstration in sickle-cell anemia and other hematologic disorders by means of alkali denaturation. *Blood* 6:413, 1951.
 7. Huisman, T. H. J., and Dozy, A. M.: Studies on the heterogeneity of hemoglobin. IX. The use of tris (hydroxymethyl) aminomethane-HCL buffers in the anion-exchange chromatography of hemoglobins. *J. Chromatogr.* 19:160, 1965.
 8. Wilbur, K. M., and Anderson, N. G.: Electrometric and colorimetric determination of carbonic anhydrase. *J. Biol. Chem.*: 176:147, 1948.
 9. Roughton, F. J. W., and Booth, V. H.: The effect of substrate concentration, pH and other factors upon the activity of carbonic anhydrase. *Biochem. J.* 40:319, 1946.
 10. Haut, A., Tudhope, G. R., Cartwright, G. E., and Wintrobe, M. M.: The nonhemoglobin erythrocytic proteins, studied by electrophoresis on starch gel. *J. Clin. Invest.* 41:579, 1962.
 11. Häusler, G.: Zur Technik und Spezifität des histochemischen Carboanhydrasenachweises im Modellversuch und in Gewebsschnitten von Rattenieren. *Histochemie* 1:29, 1958. *Histochemie* 1:29, 1958.
 12. Kleihauer, E., and Betke, K. I.: Praktische Anwendung des Nachweises von Hb F-haltigen Zellen in fixierten Blutaussstrichen. *Internist (Berlin)* 1: 292, 1960.
 13. Rieder, R. F., and Weatherall, D. J.: A variation in the electrophoretic pattern of human erythrocyte carbonic anhydrase. *Nature* 203:1364, 1964.