The Peroxide-Erythrocyte Hemolysis Test

Experiences in Patients with Cirrhosis, Jaundice, and Polyneuritis

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SERUM tocopherol concentrations lower than normal have been described in patients with malnutrition and malabsorption of fat,[1,2] cirrhosis,[3,4] hyperthyroidism,[5] and in newborn infants.[6] Nevertheless, no human counterpart to vitamin E deficiency in animals has been demonstrated.

The role of vitamin E in preventing hemorrhagic necrosis of the liver induced by deficient diet in animals is well recognized,[7,8] and hepatic abnormalities have been described in animals on deficient diets prior to the development of massive necrosis.[9] These may include karyolysis, karyorrhexis, mitosis, hemorrhage, and the development of refractile eosinophilic cytoplasmic granules. These lesions in some ways are like the "alcoholic hyalin" and necrosis described in the livers of alcoholics with acute hepatic decompensation,[10,11] suggesting that the state of vitamin E nutrition of these patients should be investigated. This was done with a red blood cell hemolysis test, using dilute hydrogen peroxide, which has been correlated with serum α-tocopherol levels in animals,[12] infants and children,[13] and patients with malabsorption of fat.[13,14]

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MATERIALS

Hemolysis tests were performed on blood from 52 patients hospitalized on the wards of the Boston City Hospital. This group included 42 patients with alcoholic cirrhosis, one with "postnecrotic" cirrhosis, four without clinical evidence of liver disease but with symptoms of acute alcohol withdrawal, three with severe malnutrition, one with hyperthyroid myopathy, and one with diabetic polyneuritis and hepatomegaly. Diagnosis of the liver disease was substantiated by autopsy in seven instances and by liver biopsy in nine. Twenty-three patients had clinical icterus (bilirubin above 2.0 mg per 100 ml or icterus index above 20). The ages ranged from 27 to 72 years among the 17 females and 35 males. The patients, selected from those seen in consultation, were chosen to represent chronic and acute cirrhosis, undernutrition, and peripheral neuritis. Three patients with increased hemolysis were followed with serial tests.

METHODS

Erythrocyte sensitivity to hemolysis by dilute hydrogen peroxide was tested using the modification of Horwitt *et al.*,[15] which is similar to the method of Gordon *et al.*[13] and of Rose and György.[12] Variations from this modification included the use of 50 ml boiling flasks in a Seligson-type rotator to agitate the red cells consistently during incubation at 37°C for

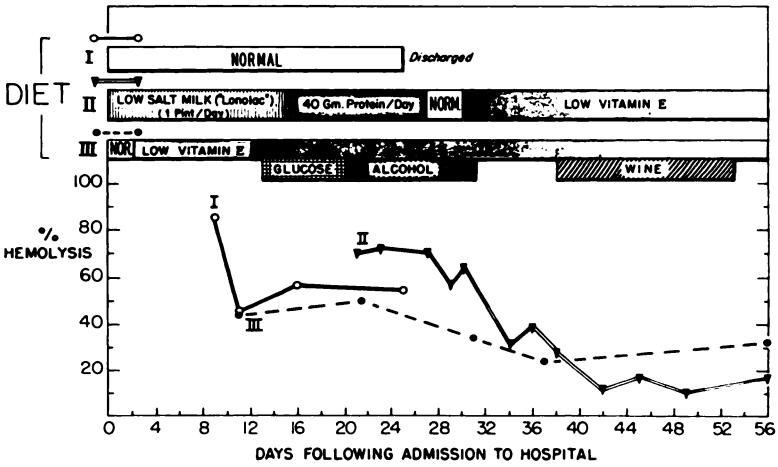

Fig. 1. Erythrocyte hemolysis test following feeding in three alcoholic patients with cirrhosis.

three hours, and the use of 1.0 ml of blood rather than 0.2 ml. Tests were done in triplicate, with error of duplicate samples usually less than 5 per cent for values over 10 per cent hemolysis. Hemolysis of 50 per cent or more has been considered significantly positive when correlated with tocopherol concentration,[13,15] and we also have used this criteria. Eight tests performed initially on ambulatory normal individuals uniformly gave results of less than 5 per cent hemolysis, while umbilical cord blood obtained at the time of delivery from a 16-year-old Negro with a normal, full-term, spontaneous delivery had 34 per cent hemolysis. Cells from normal individuals used concurrently with patients' cells consistently revealed less than 5 per cent hemolysis.

Two patients were given measured diets on which the α-tocopherol intake was calculated using standard tables.[16] Creatine excretion in the urine was measured in one of these patients using the method of Folin.[17] Fat absorption was measured in two patients using oleic acid labeled with I^{131} as described below.[18]

RESULTS

Two of 52 patients had a positive test; that is, red blood cell hemolysis greater than 50 per cent (Table I). There was, however, no definite correlation of erythrocyte hemolysis with the presence or absence of jaundice. The four patients with alcoholic withdrawal symptoms, and three of four with severe malnutrition without cirrhosis, showed less than 10 per cent hemolysis.

Specimens of liver from 16 patients obtained at autopsy or biopsy and stained with hematoxylin-eosin and with phyloxine-methylene blue were reviewed. They were evaluated for the presence or absence of hyalin in liver cells together with necrosis and polymorphonuclear infiltration and have been correlated with the results of the hemolysis test (see Table I). No clear differentiation is evident. The lack of

TABLE I
Results of Erythrocyte Hemolysis Test in 52 Patients

Diagnosis	Hemolysis test: Under 10%	10%–50%	Over 50%
Clinical			
Cirrhosis			
Nonjaundiced	12	7	0
Jaundiced	10	12	2
Alcohol withdrawal	4	0	0
Undernutrition alone	3	1	0
Diabetes, hepatomegaly	0	1	0
TOTAL	29	21	2
Pathologic[a]			
Cirrhosis			
No "alcoholic hyalin" and necrosis	4	3	0
With "alcoholic hyalin" and necrosis	3	3	1
No cirrhosis	2	0	0
TOTAL	9	6	1

[a] *Pathologic* includes the 16 patients in whom microscopic examination of the liver was possible.

correlation is exemplified by patients with livers showing large amounts of hyalin, extensive necrosis, and fibrosis, whose blood was found to have less than 10 per cent hemolysis. In contrast, the patient whose liver had no hyalin, minimal fibrosis, and severe fatty infiltration had 43 per cent erythrocyte hemolysis. The only liver biopsy in a patient with greater than 50 per cent hemolysis did reveal hyalin, fat, slight necrosis, and cirrhosis.

The presence of severe fatty metamorphosis was not always associated with a positive hemolysis test. One patient with an acute fatal illness suggestive of vitamin E deficiency as seen in animals had extensive hepatic fat with massive central lobular hemorrhagic necrosis, masses of hyalin material in liver cells, and intra-alveolar pulmonary hemorrhage; however, the hemolysis test was less than 10 per cent. Thus, we were unable to demonstrate a correlation between the presence in the liver of "alcoholic hyalin" and necrosis or fatty change and a positive hemolysis test.

The three patients with hemolysis tests above 45 per cent were followed with serial tests. Patient 1 was offered a normal diet, but the amount consumed was not recorded. The Boston City Hospital normal diet was estimated to contain about 25 mg total tocopherol and 17 mg α-tocopherol. Patients 2 and 3 each had a short period of three to four days when a normal diet was offered and then received a measured diet estimated to contain 40 g protein, 6 to 8 mg of total tocopherol, and 4 to 5 mg of α-tocopherol daily (Fig. 1). Patient 3 in addition received a Dexin*-grapefruit juice drink for which was substituted an alcohol grapefruit juice drink (33 g of alcohol every six hours) in isocaloric amounts after a suitable control period. Later, port wine equivalent to 33 g alcohol every six hours was substituted. It can be seen from Figure 1 that these measures had no effect in preventing the return of the hemolysis test toward normal. Urine creatine excretion measured in Patient 2 revealed between 10 and 25 mg creatine excreted on the fourth, fifth, and seventh days after receiving a house diet for 4 days. Thereafter, there was no creatinuria on repeated determinations. Fat absorption was estimated by measuring serum radioactivity four hours after oral ingestion of approximately 25 μc of I^{131}-labeled triolein given in a mixture of peanut oil, water, and Tween 80.[18] In Patients 1 and 2 there was 16.7 and 8.75 per cent, respectively, of administered radioactivity in the calculated plasma volume at four hours, compared to the normal value of 10 to 15 per cent. There was no evidence of fluid retention at this time.

In reviewing the records of Patients 1 and 2, who had 85 and 70 per cent hemolysis, respectively, certain similarities were noted. Both were severely alcoholic males with long histories of excessive whiskey and beer intake and poor diets. Each had suffered progressive muscular weakness of nine months to one year's duration, with increasing severity in the one to two weeks prior to admission which had confined each to bed rest at home. Delirium on admission with memory loss and history of weight loss were present in both. On examination they were extremely undernourished and dehydrated and had mild jaundice (serum bilirubin 2.9 and 3.4 mg per 100 ml, respectively), slightly enlarged livers, and ankle edema. There were signs of probable vitamin deficiencies in each. Patient 1 had nystagmus prior to thiamine administration, and Patient 2 had a smooth red tongue and perifollicular hemorrhages and corkscrew hairs over the thigh suggesting scurvy. Mild congestive heart failure was present in Patient 1 with a blood pressure of 78/62 mm Hg, while Patient 2 had severe congestive heart failure with a blood pressure of 80/65 mm Hg and was suspected of having beriberi heart disease, although this was not definitely established. Severe muscular wasting and signs compatible with marked nutritional polyneuropathy were present in both, while Patient 2 had evidence suggesting spinal cord involvement as well. Laboratory evidence of acute hepatic dysfunction was present, and blood studies revealed hematocrits of 45 and 36 per cent, respectively, with moderately hypochromic, normocytic cells, some macrocytes, marked

* Dexin, a partially hydrolyzed starch, produced by Burroughs Wellcome Co., Tuckahoe, New York.

anisocytosis, and target cells. The course was one of gradual improvement following intensified and diverse therapies; however, no α-tocopherol other than in food, as described earlier, was given. Improvement in the polyneuropathy and muscular weakness took several months.

Patient 3, with an erythrocyte hemolysis test of 44 per cent, was a chronic alcoholic who had continued to eat moderately well and had retained his job. His history was of fatigue, weakness, and weight loss of several months' duration. On admission he had clinical and laboratory evidence suggestive of severe folic acid deficiency: hemoglobin 6.2 g per 100 ml, hematocrit 20 per cent, erythrocyte count 130 million per cu mm, mean cell volume 155, mean corpuscular hemoglobin concentration 31, reticulocytes 1.8 per cent, serum vitamin B_{12} content 1900 $\mu\mu$g per ml, leukocyte count 6,800 per cu mm (71 per cent polymorphonuclear); bone marrow was hyperplastic with megaloblasts and atypical white blood cell precursors. There was jaundice (bilirubin 3.4 mg per 100 ml) with an enlarged liver, a palpable spleen tip, and slight but definite ascites. He was obese and did not appear as severely undernourished as the other two patients. Neurologic examination revealed depression of deep tendon reflexes bilaterally with some evidence of peripheral neuritis, but not as severe as in Patients 1 and 2. He improved on a low-folic acid, low-vitamin E diet; however, he received a normal diet for a three-day period prior to the measured diet (see Fig. 1). One month after admission, laboratory data were: hemoglobin 13.3 g per 100 ml, hematocrit 44.3 per cent, erythrocyte count 4.01 million per cu mm, reticulocytes 0.3 per cent, mean cell volume 111, mean corpuscular hemoglobin concentration 30, leukocyte count 8,000 per cu mm (43 per cent polymorphonuclear).

In view of the similarity of these cases, other cases of nutritional polyneuropathy were sought and tested. The results are shown in Table II and indicate a somewhat higher degree of hemolysis than in Table I, although patients in Table II are also included in Table I. There was no direct correlation between clinical estimate of severity of peripheral neuropathy and results of the hemolysis test. Two nonalcoholic patients were studied, one of which, with hyperthyroid myopathy, had less than 10 per cent hemolysis, while the other, with diabetic polyneuropathy, had 28 per cent hemolysis. No tests were performed to determine whether myopathy existed in addition to neuropathy in these patients.

TABLE II

Results of Erythrocyte Hemolysis Test in 14 Patients[a] Who Had Clinical Evidence of Polyneuropathy

Polyneuropathy	Hemolysis test		
	Under 10%	10%–50%	Over 50%
Cirrhosis			
Nonjaundiced	1	4	0
Jaundiced	2	3	2
Other	1	1	0
TOTAL	4	8	2

[a] These patients are included in Table I.

DISCUSSION

The results presented here indicate no definite abnormality of the erythrocyte hemolysis test in patients with cirrhosis. The slight abnormality seen in some patients with cirrhosis may be due to deficient fat absorption,[18] as low serum tocopherol levels with positive hemolysis tests have been described in patients with malabsorption of fat.[1,14,19] Klatskin,[20] however, has reported normal α-tocopherol absorption in a group of 11 hospitalized patients with cirrhosis and suggests that decreased intake may account for low α-tocopherol levels. Measurement of fat absorption in the two patients with highest hemolysis test suggested a mild defect in one patient only.

No correlation of erythrocyte hemolysis was found with the presence or absence of jaundice or with pathologic evidence of "alcoholic hyalin" and necrosis of the liver. This is consistent with previous studies of cirrhosis which have indicated lower than normal mean blood tocopherol levels in cirrhosis and no correlation with degree of hepatic dysfunction, type of liver disease, serum bilirubin levels, or serum cholesterol levels.[3,4]

The unexpected positive hemolysis tests (over 50 per cent hemolysis) found in two patients with only mild cirrhosis and with similar

illnesses manifesting delirium, muscular wasting, severe polyneuropathy, and cardiac failure suggested vitamin E deficiency. Naturally occurring vitamin E deficiency in swine may be associated with sudden onset of coma and death, hepatic necrosis, voluntary muscle degeneration, pulmonary edema and pulmonary hemorrhage.[21] Vitamin E-deficient diets or diets containing vitamin E antagonists* in swine and rats may produce severe liver necrosis, muscular dystrophy, ceroid pigment in the liver cells, and demyelinization of peripheral and spinal nerves.[22–26] Monkeys deficient in vitamin E have been observed to have abnormal cardiac function with electrocardiographic abnormalities similar to those found in thiamine-deficient monkeys.[27] The positive hemolysis tests in these two patients and the presence of similar lesions in vitamin E-deficient animals only suggests but does not prove a deficiency of α-tocopherol in the etiology of their illness.

The gradual return of the hemolysis tests to normal following feeding minimal amounts of α-tocopherol in food indicates either that only small amounts of vitamin E are needed to produce a normal test or that other factors may affect the test. Horwitt and associates[15] fed 15 mg tocopherol a day to a subject previously depleted by a 2 mg tocopherol diet and noted a gradual rise in serum tocopherol with a fall in hemolysis test over several months. Thus, it would seem that between 2 and 15 mg daily of α-tocopherol were needed for the presence of a normal hemolysis test. Our results suggest that three to four days of normal diet followed by only 5 mg of α-tocopherol daily may reverse a positive hemolysis test.

It should be emphasized that the hemolysis test may not be specific for α-tocopherol and that it does not completely parallel blood α-tocopherol concentrations. Other factors such as red cell-catalase content may affect the test.[28] It is possible that extreme undernutrition may affect the hemolysis test in a nonspecific manner; however, our patients with severe undernutrition alone did not show marked abnormalities. Although there is no direct correlation, serum α-tocopherol levels of less than 0.5 mg per 100 ml have been associated with positive hemolysis tests.[15,29] Also, humans on experimental 2 mg α-tocopherol diets develop a gradual decrease in serum α-tocopherol with an increase in the hemolysis test.[15] Recently, a case of biliary cirrhosis has been described with low blood α-tocopherol levels, a positive hemolysis test and creatinuria.[30] These laboratory abnormalities improved with administration of large amounts of α-tocopherol. There have been other reports suggestive of human vitamin E deficiency;[14,22,31,32] however, in none of these reports is there evidence of a definite etiologic relationship between lack of vitamin E and a clinical syndrome.

Alcoholic peripheral polyneuritis has been associated with vitamin B deficiency for many years.[33,34] The results reported here in the two patients with marked abnormalities of hemolysis test and the tendency toward increased hemolysis in patients with nutritional polyneuropathy would suggest further investigation of vitamin E in this circumstance.

SUMMARY

Red blood cell sensitivity to hemolysis with hydrogen peroxide has been determined in 52 patients, 49 of whom had cirrhosis.

No definite correlation was found with the severity of cirrhosis, the presence of jaundice, or the presence of "alcoholic hyalin" and necrosis of the liver.

Only two patients had strongly positive tests (greater than 50 per cent hemolysis), and both had multivitamin deficiency with mild cirrhosis and severe polyneuropathy suggesting but not proving vitamin E deficiency in these two patients.

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* Substances reported to antagonize vitamin E include tri-*o*-cresyl phosphate (ginger paralysis), carbon tetrachloride, sulfonamides, pyridine, sodium sulfite, and unsaturated fatty acids.[22]

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Science is always wrong. It never solves a problem without creating ten more.—George Bernard Shaw