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Abstract

”Free” iron, a potentially radical-generating low mass iron, and not found in normal human blood, was increased in the serum of blood-transfused thalassemia major patients seen in the Yangon General Hospital, Yangon, Myanmar (Burma). The low mass iron was detected by the bleomycin assay. Fifty-one blood samples were analyzed (from 28 males and 23 females). High “free” iron was detected in 47 sera samples from thalassemia patients. Serum ferritin, which reflects the body store iron, was higher than the normal range (10-200 ng/ml) in 49 patients. On the other hand, serum iron of 39 sera samples fell within the normal range (50-150 micrograms/dl). Four were less than 50 micrograms/dl and eight were more than 150 micrograms/dl. Almost all the patients' sera of normal or higher serum iron level contained ”free” iron. Thus, almost all the sera from thalassemic patients from Myanmar contain bleomycin-detectable iron, even when serum iron is within the normal range. In developing countries where undernutrition is prevalent (serum albumin in these patients was 3.6 +/- 0.4 g/dl, $P < 0.0001$ vs. control value of 4.0 - 4.8 g/dl), normal serum iron does not preclude the presence of free iron in the serum.

KEYWORDS: thalassemia, free iron, hemochromatosis, iron overload, serum iron, bleomycin-detectable iron, free radical

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Serum Bleomycin-Detectable Iron in Patients with Thalassemia Major with Normal Range of Serum Iron

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"Free" iron, a potentially radical-generating low mass iron, and not found in normal human blood, was increased in the serum of blood-transfused thalassemia major patients seen in the Yangon General Hospital, Yangon, Myanmar (Burma). The low mass iron was detected by the bleomycin assay. Fifty-one blood samples were analyzed (from 28 males and 23 females). High "free" iron was detected in 47 sera samples from thalassemia patients. Serum ferritin, which reflects the body store iron, was higher than the normal range (10 − 200 ng/ml) in 49 patients. On the other hand, serum iron of 39 sera samples fell within the normal range (50 − 150 μg/dl). Four were less than 50 μg/dl and eight were more than 150 μg/dl. Almost all the patients' sera of normal or higher serum iron level contained "free" iron. Thus, almost all the sera from thalassemic patients from Myanmar contain bleomycin-detectable iron, even when serum iron is within the normal range. In developing countries where undernutrition is prevalent (serum albumin in these patients was 3.6 ± 0.4 g/dl, $P < 0.0001$ *vs.* control value of 4.0 − 4.8 g/dl), normal serum iron does not preclude the presence of free iron in the serum.

Key words: thalassemia, free iron, hemochromatosis, iron overload, serum iron, bleomycin-detectable iron, free radical

In primary hemochromatosis and transfusion-dependent anemias, iron overload causes widespread organ dysfunction and leads to early death. Thalassemia major shows features due to transfusional and absorptive iron overload. Many patients with thalassemia major succumb before puberty because of iron overload. Iron deposits in heart muscle may cause dysfunction and ultimately lead to heart failure. Other features of secondary hemochromatosis are common. Therefore the aim of treatment is usually to prevent or delay hemochromatosis.

The pathological effects of iron overload are generally thought to be due to the ability of non-transferrin bound iron (NTBI) to catalyze free radical oxidation (1). Although the precise nature of NTBI is uncertain, there are several studies which reported NTBI by various methods in idiopathic hemochromatosis or in transfusion-dependent congenital anemias like thalassemia major or sickle cell anemia (2-12). However, information on the prevalence of NTBI before chelation therapy is limited because only a small number of patients were evaluated in the previous reports. In this study, we could measure a relatively large number of sera samples from thalassemic patients without chelation therapy in Myanmar, and we showed almost all the sera from thalassemic patients from Myanmar contained the NTBI detected by the bleomycin assay (5, 8, 13, 14), even in the patients whose serum iron was within the normal range.

Subjects and Methods

Assays. The non-protein-bound iron content of serum samples was measured by the bleomycin assay of Gutteridge *et al.* (5, 8, 13, 14). The result is expressed as absorbance at 532 nm. The serum albumin and iron was assayed using a Hitachi 7150 Autoanalyser (Hitachi, Chiba, Japan). Serum ferritin was estimated by RIA Kit (New England Nuclear, US.). Statistical analysis for serum albumin was done by the test for the mean, and the two-sample Wilcoxon test (Mann-Whitney test) was used for other data.

Patients. Serum samples were collected from

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Table I Patients' background data

Sex	No.	Age distribution (Median) (year)	Transfusion period (Median) (year)	Total blood transfused (ml)
Male	28	1-12 (7)	0-8.5 (2.5)	5500 ± 5200 (n = 21)
Female	23	0-13 (7)	0.5-10.5 (4)	4820 ± 3340 (n = 20)

thalassemia major patients seen in the Yangon General Hospital, Yangon, Myanmar (Burma). Serum samples were also obtained from 10 normal volunteers (eight female and one male Barmars, and one Japanese, 29.3 ± 8.8 years). To avoid contamination ,from external iron, the blood samples were collected with new plastic disposable syringes, and the sera were separated immediately. They were stored at −20°C until assay. A total of 77 serum samples were collected, but high GPT (over 150 IU/l), high bilirubin (over 3 mg/dl) sera were omitted from the study to exclude the patients with hyperferritinemia caused by liver damage.

Fifty-one samples were included in the final analysis (28 males and 23 females). The patients consisted mostly of Barmars. A few patients of Shan, Kachin, Kayin, and India were also included. The background data of the patients are summarized in Table 1. The ages of the patients ranged from 8 months to 13 years old with transfusion history starting from 2 months to 8 years after birth. The amount of transfused blood ranged from 200 to 600 ml a month. None of them were receiving deferroxamin or other chelation therapy. Their serum albumin was 3.6 ± 0.4 g/dl (n=51, $P < 0.0001$ *vs.* control values of 4.0 − 4.8 g/dl). No data were available regarding possible complications secondary to iron overload. All the samples were analyzed irrespective of the background variation.

Results

All the sera samples from 10 healthy volunteers showed from 0.03 to 0.08 absorbance (0.04 ± 0.02) at 532 nm in the bleomycin tests. Therefore, we defined the absorbance above 0.1 as positive for bleomycin-detectable iron (or simply free iron) since, as reported previously, sera from healthy men of different ages contain no bleomycin detectable iron (5, 8, 13, 14). Only four sera samples from patients with thalassemia showed absorbance of less than 0.1. Serum ferritin, which reflects the body store iron, was high in 49 patients. High values were not necessarily associated with the duration of transfusion history, the amount of transfused blood, the age of the patients, or the age of onset.

Fig. 1 shows the relation between serum ferritin (normal range: 10 − 200 ng/ml) and free iron. A normal level of free iron was seen only in 4 patients. Two of them (20 month old boy who just started transfusion, and one year old boy who had received 100 ml of blood) had normal levels of ferritin. In one female patient, free iron was normal even though the level of ferritin was high. However, she had low serum iron of 38 μg/dl. She was 7 years old, and started transfusions at 10 month after birth. A total of 5,100 ml of blood was transfused at the time of examination. There was mild elevation of serum GPT (109 IU/l) and LDH (591 IU/l). The cause of hypoferrinemia was not known. The free iron level between normal and thalassemic sera was significantly different ($P < 0.001$), and the free iron level between the normal ferritin group and the high ferritin group was also significantly different ($P < 0.001$). There was no relation between serum albumin and the ferritin level of the

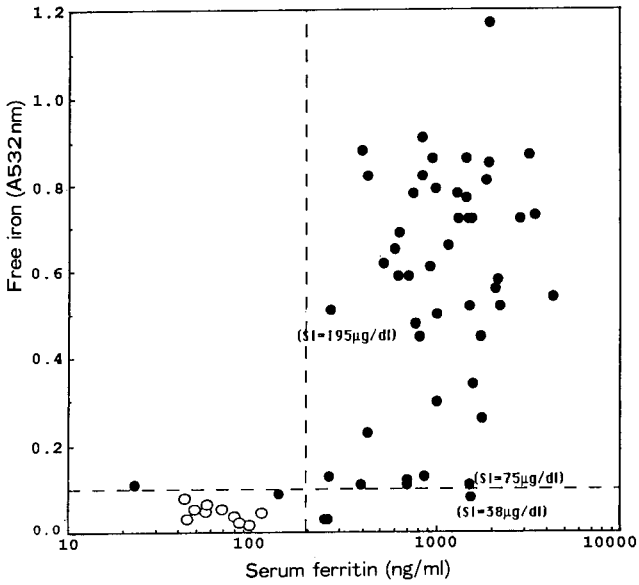

Fig. 1 Correlation between serum ferritin and free iron in 51 thalassemia patients. All except two showed increased serum ferritin. Hyperferritinemia is associated with free iron in the serum (except one patient with low serum iron). Closed circles: Thalassemia patients. Open circles: Normal healthy controls. Vertical dotted line: Normal range (10 to 200 ng/ml). Horizontal dotted line: Upper normal limit of absorbance for free iron (0.1 absorbance).

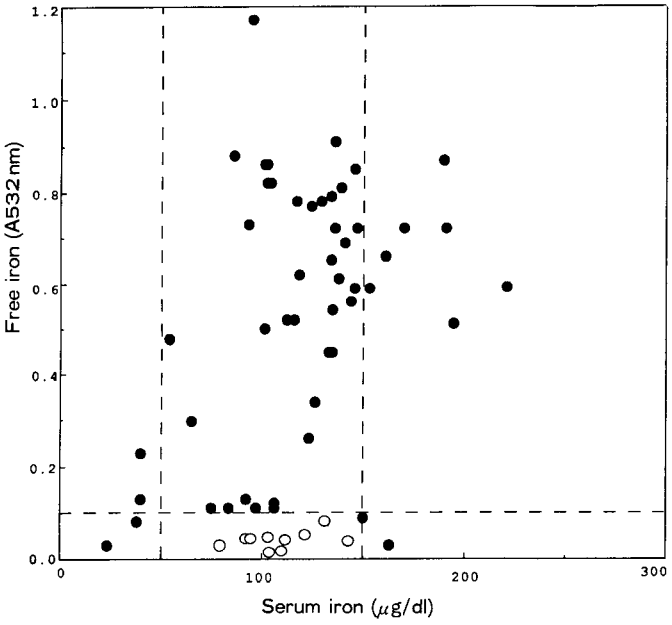

Fig. 2 Correlation between free iron and serum iron. Forty-three patients had normal or slightly lower serum iron. Four patients had less than 0.1 absorbance for free iron. Closed circles: Thalassemia patients. Open circles: Normal healthy controls. Vertical dotted line: Reference value for serum iron (50 to 150 μg/dl). Horizontal dotted line: Upper normal limit of absorbance for free iron (0.1 absorbance).

patients.

Serum iron from 39 sera samples fell within the normal range of 50 to 150 μg/dl. Four were less than 50 μg/dl and eight were more than 150 μg/dl (Fig. 2). All except two of the sera samples showing a normal to high serum iron level had a borderline to high level of free iron ($P < 0.001$ *vs.* healthy sera); those two samples were from the patients described above with a short history of transfusion and a low serum ferritin level. The free iron level between groups of normal serum iron level and high serum iron level was not significantly different ($P > 0.5$). The serum iron level between normal and thalassemic patients was not significantly different ($P > 0.5$).

Discussion

Non-transferrin-bound iron (NTBI) might facilitate the production of free radicals that promote damage to lipids, protein, and DNA (15). The existence of NTBI in iron-overload patients was reported repeatedly using various methods (2–12). Some methods are currently in use. Gutteridge *et al.* developed an assay method for "free" iron (5, 8, 13, 14). The assay depends on the fact that degradation of DNA by the antibiotic bleomycin requires trace levels of iron salts. Iron bound to proteins such as ferritin, transferrin or hemoglobin is not detected. Application of the assay to human serum or plasma shows that no "free" iron is present in normal volunteers (8, 9).

Another method was developed recently by Singh *et al.* (16). This method is a direct measurement of non-protein- bound iron using a large excess of nitrilotriacetic acid to remove and to complex all low-molecular-mass iron and iron nonspecifically bound to serum proteins. A modification of Singh's method was used by Al-Refaie to measure serum non-transferrin-bound iron in 52 beta-thalassemia major patients (12). We used the bleomycin-detectable iron method because it is a sensitive and specific assay for iron, and bleomycin-detectable iron is shown to accelerate free radical reactions (8), and also one of the chelaters used in Singh's method was not available commercially. We recently simplified Singh's method so that commercially available chemicals can be used (17). It has been claimed that the reproducibility of the bleomycin-detectable iron method is rather poor (16). Therefore, all of the assays were done by one person, and normal sera was included among the assay tubes to make sure that the reading of normal sera was always below 0.1.

As to the nature of NTBI, Hershko *et al.* suggested it is an iron complex loosely bound to albumin (2, 3), while others postulated it to be an iron-binding polypeptide (18). The suggestion that NTBI is present in serum as iron citrate (10) has been criticized because of the limited solubility of iron (5×10^{-9} M at pH 7.4) at the physiologi-

cal concentration of free citrate (10^{-5}M) (16).

Al-Refaie *et al.*, using modified Singh's method (12), reported that the level of NTBI correlated significantly with the logarithm of the serum ferritin concentration, total serum iron concentration and transferrin saturation. In our present data, there was no correlation between serum ferritin/iron concentration and "free" iron. The reasons for these discrepacies are not clear, but there are several possible explanations: (a) difference in the method of detecting "free iron", (b) differences in the patients' background, espcially in terms of nutrition, prevalence of liver diseases that affect iron status, (c) history of chelation therapy. In any events, the presence of high serum ferritin concentration was associated with the existence of "free" iron in both studies. Of the 51 patients in the present study, 39 had free iron in their sera even though their serum iron concentration was normal (Fig. 2). This suggests that the serum iron level is not a good index of the NTBI level.

In the population with a low serum albumin level (an index of inadequate nutrition or liver cell damage) iron binding capacity was also expected to be low, although we could not determine the total iron binding capacity due to a technical problem. However, with normal serum iron, it is unlikely that serum transferrin is 100 % saturated. This means free iron could be present in the serum even though the iron-binding capacity is unsaturated. The existence of bleomycin detectable iron in the presence of remaining iron binding capacity was reported before (4, 8, 9, 19), and slow iron transfer from low-molecular-mass iron to transferrin was suggested as the reason (9). Animal studies showed that low-molecular-mass iron was taken up efficiently by the liver (20). If this is applicable to humans, the liver of thalassemic patients, already loaded with iron as shown by high serum ferritin (21), might have a decreased capacity for clearing low-molecular-mass iron. Conversely, low-molecular-mass iron might be secreted, although this is unlikely (22), by the iron-loaded liver. At least some iron comes from damaged liver cells by rupture, because iron-mediated lipid peroxidation is high in iron overload (23, 24).

Serum with an extremely low iron content, or a normal ferritin level did not have NTBI, even if multiple transfusions had been performed. It is speculated that transient hypoferrinemia might have increased the iron-binding capacity of the serum, thus lowering free iron.

Free iron is capable of stimulating the peroxidation of lipids, and is a powerful mediator of oxygen-derived free radicals. The oxygen-derived radical reactions stimulated by free iron is important in the pathology of thalassemia major because free iron is more readily taken up by tissues than transferrin-bound iron (20). So far, desferrioxamine is the only agent that decrease iron overload (25). It not only helps excrete iron from the body, but also interferes with radical reactions by sequestrating iron from the free radical reactions. Studies on other iron-chelating agents for clinical use are now in progress (25).

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References

1. Halliday J and Powell L: Hemochromatosis and other diseases associated with iron overload: in Iron and Human Disease, Lauffer R ed. CRC Press, Inc., Boca Raton (1992) pp 131-160.
2. Hershko C and Rachmilewitz EA: Non-transferrin plasma iron in patients with transfusional iron overload; in Proteins of Iron Storage and Transport in Biochemistry and Medicine, Crichton RR ed. North-Holland Publishing Company, Amsterdam (1975) pp 427-432.
3. Hershko C, Graham G, Bates GW and Rachmilewitz EA: Non-specific serum iron in thalassaemia: An abnormal serum iron fraction of potential toxicity. Br. J. Haematol. (1978) **40**, 255-263.
4. Batey RG, Fong LC, Shamir S and Sherlock S: A non-transferrin-bound serum iron in idiopathic hemochromatosis. Dig Dis Sci (1980) **25**, 340-346.
5. Gutteridge JMC, Rowley DA, Griffiths E and Halliwell B: Low-molecular-weight iron complexes and oxygen radical reaction in idiopathic haemochromatosis. Clin Sci (1985) **68**, 463-467.
6. Peters SW, Jones BM, Jacobs A and Wagstaff M: 'Free' iron and lipid peroxidation in the plasma of patients with iron overload; in Proteins of Iron Storage and Transport. Spik G, Montreul J, Crichton RR, JM ed, Elsevier Science Publishers, Amsterdam (1985) pp 321-324.
7. Wang WC, Ahmed N and Hanna M: Non-transferrin-bound iron in long-term transfusion in children with congenital anemias. J Pediatr (1986) **108**, 552-557.
8. Gutteridge JMC and Halliwell B: Radical-promoting loosely-bound iron in biological fluids and the bleomycin assay. Life Chem Rep (1987) **4**, 113-142.
9. Aruoma OI, Bomford A, Polson RJ and Halliwell B: Nontransferrin-bound iron in plasma from hemochromatosis patients: Effect of phlebotomy therapy. Blood (1988) **72**, 1416-1419.
10. Grootveld M, Bell JD, Halliwell B, Aruoma OI, Bomford A and Sadler PJ: Non-transferrin-bound iron in plasma or serum from patients with idiopathic hemochromatosis: Characterization by high performance liquid chromatography and nuclear magnetic resonance spectroscopy. J Biol Chem (1989) **264**, 4417-4422.
11. Cantinieaux B, Boelaert JR, Demeuleneire J, Kerrels V and Fondu P: Neutrophils from patients with secondary haemosiderosis contain excessive amount of autotoxic iron. Eur J Haematol (1993) **51**, 161-165.
12. Al-Refaie FN, Wickens DG, Wonke B, Kontoghiorghes GJ and Hoffbrand AV: Serum non-transferrin-bound iron in beta-thalassaemia major patients treated with desferrioxamine and L1. Br J Haematol

(1992) **82**, 431–436.
13. Gutteridge JMC, Rowley DA and Halliwell B: Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts: Detection of 'free' iron in biological systems by using bleomycin-dependent degradation of DNA. Biochem J (1981) **199**, 263–265.
14. Gutteridge JMC and Hou Y: Iron complexes and their reactivity in the bleomycin assay for radical-promoting loosely-bound iron. Free Rad Res Commun (1986) **2**, 143–151.
15. Halliwell B and Gutteridge JMC: Free Radicals in Biology and Medicine, Second Ed.,. Oxford University Press, Oxford (1989) pp 126–130.
16. Singh S, Hider RC and Porter JB: A direct method for quantification of non-transferrin-bound iron. Anal Biochem (1990) **186**, 320–323.
17. Zhang D, Okada S, Kawabata T and Yasuda T: An improved simple colorimetric method for quantitation of non-transferrin-bound iron in serum. Biochem Mol Biol Int (1995) **35**, 635–641.
18. Stojkovski S, Goumakos W and Sarkar B: Iron(III)-binding polypeptide in human cord and adult serum: Isolation, purification and partial characterization. Biochim Biophys Acta (1992) **1137**, 155–161.
19. Halliwell B, Aruoma OI, Mufti G and Bomford A: Bleomycin-detectable iron in serum from leukaemic patients before and after chemotherapy. FEBS (Fed Eur Biochem Soc) Lett (1988) **241**, 202–204.
20. Craven CM, Alexander J, Eldridge M, Kushner JP, Bernstein S and Kapalan J: Tissue distribution and clearance kinetics of non-transferrin-bound iron in the hypotransferrinemic mouse: A rodent model for haemochromatosis. Proc Natl Acad Sci USA. (1987) **84**, 3457–3461.
21. Brittenham GM, Cohen AR, Mclaren CE, Martin MB, Griffith PM, Nienhuis AW, Young NS, Allen CJ, Farrell DE and Harris JW: Hepatic iron stores and plasma ferritin concentration in patients with sickle cell anemia and thalassemia major. Am J Hematol (1993) **42**, 81–85.
22. Hultcrantz R and Glaumann H: Studies on the rat liver following iron overload: Biochemical studies after iron mobilization. Lab Invest (1982) **46**, 383–392.
23. Bacon BR, Tavill AS, Brittenham GM, Park CH and Recknagel RO: Hepatic lipid peroxidation *in vivo* in rats with chronic iron overload. J Clin Invest (1983) **71**, 429–439.
24. Younes M, Eberhardt I and Lemoine R: Effect of iron overload on spontaneous and xenobiotic-induced lipid peroxidation *in vivo*. J Appl Toxicol (1989) **9**, 103–108.
25. Brittenham GM: Development of iron-chelating agents for clinical use. Blood (1992) **80**, 569–574.

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