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Binding of Serum Ferritin to Concanavalin A: Patients with Homozygous β Thalassaemia and Transfusional Iron Overload

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SUMMARY. Serum ferritin concentrations have been measured in 124 patients with homozygous β thalassaemia who were between 2 and 21 years old, had received 11-504 units of blood but had not undergone splenectomy. There were highly significant correlations between serum ferritin concentration and both the amount of blood transfused and alanine amino-transferase (ALT) activity. However, multivariate analysis showed that units of blood and ALT activity together only accounted for about 30% of the variation in serum ferritin concentration. Little of the remaining variation could be explained by other variables related to iron metabolism or liver damage. The concentration of concanavalin A binding ferritin increased rapidly with the number of units of blood up to 100 units but thereafter showed no further increase with number of transfusions. The concentration of non-binding ferritin was more closely related to transfusion load. These results suggest that the secretion of glycosylated ferritin from reticuloendothelial cells reaches a maximum with increasing iron accumulation, perhaps reflecting a maximum rate of synthesis. Ferritinaemia in patients with transfusional iron overload therefore seems to be the result of the combined effects of increased ferritin synthesis and the release of intracellular ferritin from damaged cells. A simple relationship between serum ferritin and iron stores cannot be assumed when ferritin concentrations exceed 4000 $\mu\text{g/l}$ or in patients who have received more than 100 units of transfused blood.

In normal subjects serum ferritin concentrations are closely related to storage iron levels (Walters *et al*, 1973). Serum ferritin is almost invariably low in patients with iron deficiency anaemia and increased in patients with iron overload (Worwood, 1979). However, iron overload also causes tissue damage and the relationship between serum ferritin and iron stores may be complicated by the pathological release of ferritin, particularly from damaged liver parenchymal cells (Prieto *et al*, 1975).

It has recently been shown that much of the ferritin in normal serum binds to concanavalin

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A (con A) whereas ferritin from human heart, liver and spleen shows little binding (Worwood *et al*, 1979). Furthermore, much of the micro-heterogeneity of serum ferritin preparations from patients with idiopathic haemochromatosis is abolished by incubation with neuraminidase although the same treatment has no effect on the pI of ferritin from heart, liver and spleen (Cragg *et al*, 1980). These studies suggest that part of the serum ferritin is glycosylated. Animal studies have shown that the clearance of ferritin from the plasma is very rapid with a half-life of about 10 min (Siimes & Dallman, 1974; Unger & Hershko, 1974; Pollack *et al*, 1978; Halliday *et al*, 1979) making it unlikely that ferritin is glycosylated in plasma. In addition, most of the ferritin in serum from patients with massive hepatic necrosis, in whom direct release of ferritin from liver cell cytosol might be expected, does not bind to con A (Worwood *et al*, 1979). These findings suggest that some of the circulating ferritin enters the plasma by secretion rather than leakage across damaged membranes and that glycosylation takes place intracellularly. The assay of 'con A binding ferritin' might therefore be a more useful way of assessing iron stores in patients with liver damage. We have examined this possibility in patients with iron overload due to repeated blood transfusion. For simplicity of writing we will refer to 'con A binding ferritin' as glycosylated ferritin, although failure to bind to concanavalin A does not imply that a protein does not contain carbohydrate residues.

PATIENTS

Serum samples were obtained from patients with homozygous thalassaemia who were receiving regular blood transfusions and were under the care of the Hellenic Red Cross Thalassaemia Clinic in Athens. Patients who had undergone splenectomy, showed evidence of blood loss or had a positive test for HB_sAg were not included in the study. One patient with a serum ALT activity greater than nine standard deviations from the mean was also excluded. Ferritin determinations were carried out on 124 serum samples from 124 patients. Most samples of serum showed the presence of HB_s antibody indicating previous hepatitis. Some of the patients had been treated with intramuscular desferrioxamine with varying degrees of regularity; supplementary ascorbic acid was not administered.

METHODS

Serum ferritin concentrations and binding to con A were measured as described by Worwood *et al* (1979). Transferrin saturation was calculated from the serum iron concentration and total iron binding capacity which were measured using a modification of the method of Young & Hicks (1965). Alanine aminotransferase (ALT) and γ glutamyl transferase (γ -GT) activities were measured at the Hellenic Red Cross Laboratory, Athens. The influence on ferritin levels of variables 1–8 in Table I was analysed by stepwise multiple regression (Dixon, 1975).

RESULTS

The relationship between serum ferritin concentration and variables 1, 2 and 4–8 in Table I was first examined. The 'total' serum ferritin concentration was related to the number of units of blood received (Fig 1a; $r=0.38$, $P<0.001$), ALT activity (Fig 1b; $r=0.41$, $P<0.001$), age

TABLE I. Clinical and laboratory data in 124 patients with homozygous β thalassaemia

Variable	All patients (mean \pm SD)	26 patients receiving less than 100 units of blood	98 patients receiving more than 100 units of blood
1. Age (years)	10.9 \pm 4.6	5.5 \pm 1.9	12.4 \pm 3.9
2. No. of units of blood (500 ml) received	198 \pm 110	54 \pm 28	236 \pm 90
3. No. of units of blood per kg body weight	6 \pm 3	2 \pm 2	7 \pm 2
4. Serum iron concn (μ mol/l)	31.7 \pm 6.6	30.7 \pm 7.1	31.9 \pm 6.4
5. Total iron-binding capacity (μ mol/l)	38.2 \pm 6.6	37.1 \pm 5.2	38.5 \pm 6.9
6. ALT activity (u/l)	75.4 \pm 47.7	56.0 \pm 40.8	80.5 \pm 48.3
7. γ GT activity (u/l)	19.7 \pm 16.6	11.6 \pm 6.3	21.9 \pm 17.7
8. Total amount of desferrioxamine received (g)	59 \pm 165	7 \pm 16	73 \pm 183
9. Serum ferritin concn (μ g/l)	5697 \pm 2471	3776 \pm 2062	6207 \pm 2322
10. Glycosylated ferritin concn (μ g/l)	2260 \pm 987	1635 \pm 843	2426 \pm 958
11. Non-glycosylated ferritin concn (μ g/l)	3434 \pm 1791	2133 \pm 1315	3780 \pm 1746
12. % glycosylated ferritin	42 \pm 11	46 \pm 11	40 \pm 11

($r=0.39$, $P<0.001$) and γ GT activity ($r=0.24$, $P<0.01$). The distribution of γ GT activities was uneven with most of the values within the normal range. Stepwise regression analysis showed that only 35% of the variation in serum ferritin concentration was explained by the independent variables considered and that only ALT activity and the number of units of blood were significant correlated variables. However, there was a much closer relationship between serum ferritin concentration and units of blood transfused in patients receiving up to 100 units of blood ($r=0.73$, $P<0.001$). 53% of the variation in serum ferritin concentration was explained by units of blood transfused and another 19% by ALT activity (for serum ferritin concentration and ALT activity, $r=0.52$, $P<0.01$). Above 100 units of blood there was no correlation with the number of units of blood transfused ($r=0.12$, $P>0.2$) but there was a correlation with

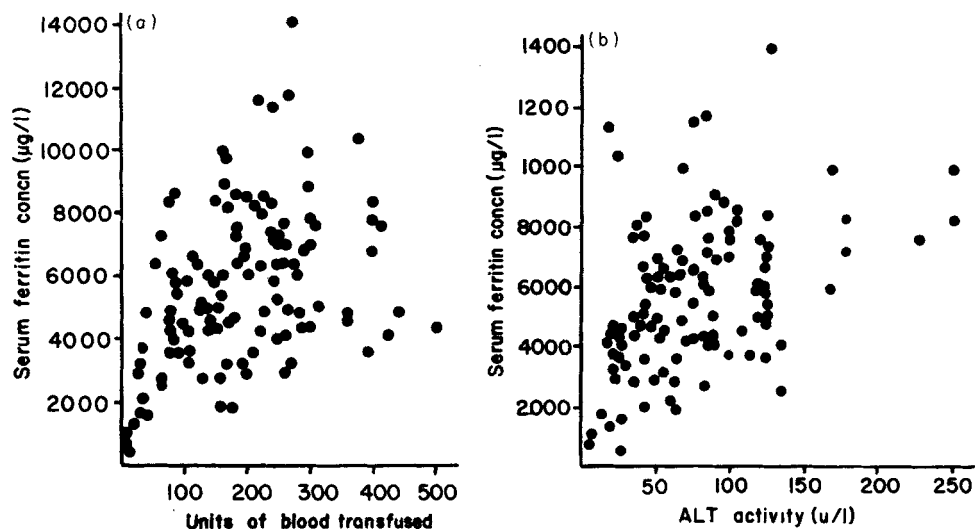


FIG 1. The relationship between serum ferritin concentration and (a) number of units of blood transfused and (b) ALT activity.

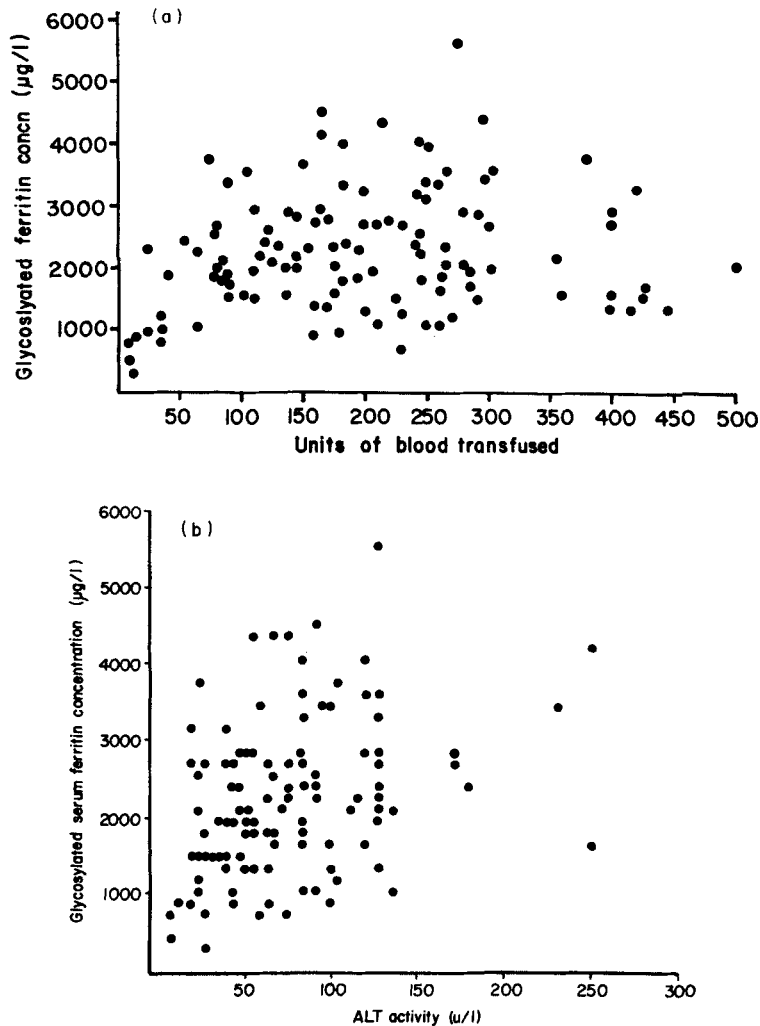


Fig 2. The relationship between glycosylated serum ferritin concentration and (a) number of units of blood transfused and (b) ALT activity.

ALT activity ($r=0.34$, $P<0.01$). ALT activity was not related to the number of units of blood transfused above or below 100 units ($r=0.08$ for all patients).

The glycosylated ferritin concentration was related to the number of units of blood transfused (Fig 2a; $r=0.23$, $P<0.01$) and ALT activity (Fig 2b; $r=0.37$, $P<0.001$). However, although there was a sharp increase in the concentration of glycosylated ferritin up to a transfusion load of 100 units of blood ($r=0.73$, $P<0.001$) there was no significant relationship above this level ($r=-0.03$). For the 26 patients who had received up to 100 units of blood there was a significant relationship with ALT activity ($r=0.40$, $P<0.05$). There was also a significant relationship above 100 units ($r=0.32$, $P<0.005$).

There was a better correlation between non-glycosylated ferritin concentration and units of blood transfused (Fig 3a; $r=0.39$, $P<0.001$) and also a significant correlation with ALT

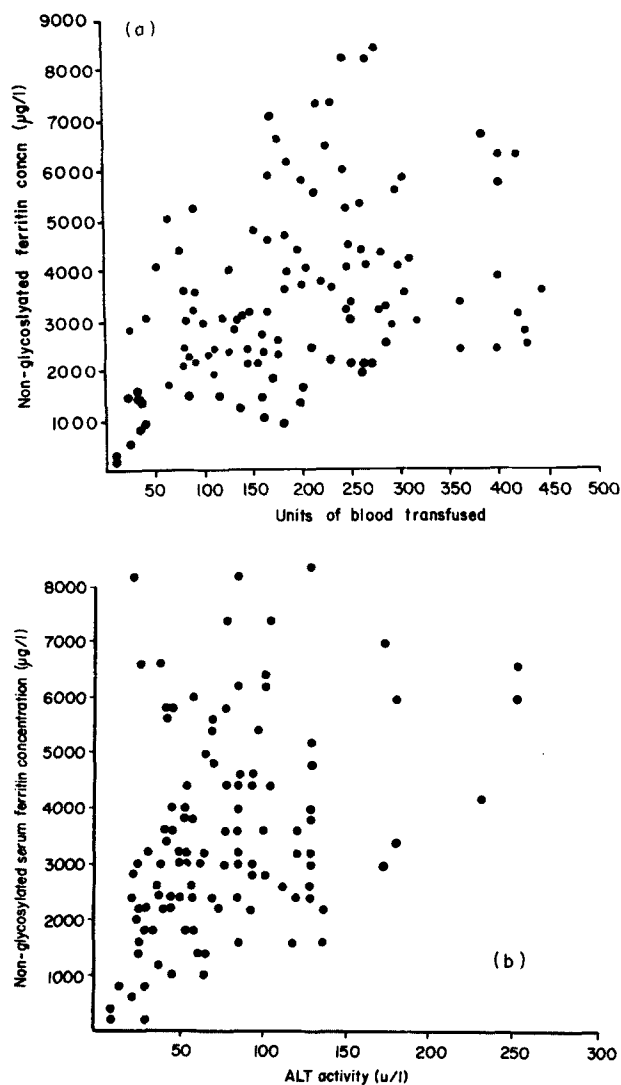


FIG 3. The relationship between non-glycosylated serum ferritin concentration and (a) number of units of blood transfused and (b) ALT activity.

activity (Fig 3b; $r=0.36$, $P<0.001$). For patients receiving up to 100 units of blood there was a significant correlation between non-glycosylated serum ferritin concentration and number of units of blood transfused ($r=0.68$, $P<0.001$) and ALT activity ($r=0.54$, $P<0.01$). Above 100 units the correlation coefficients were $r=0.18$ ($0.1>P>0.05$) and $r=0.27$ ($P<0.01$) respectively.

For all 124 patients the percentage of serum ferritin bound to concanavalin A decreased with units of blood transfused ($r=-0.34$, $P<0.001$) and serum ferritin concentration ($r=-0.39$, $P<0.001$). There was a relationship between percentage of serum ferritin bound to concanavalin A and serum ferritin concentration in patients receiving up to 100 units of blood ($r=-0.47$, $P<0.02$) and in those receiving more than 100 units of blood ($r=-0.31$, $P<0.01$).

It was possible that units of blood given/kg body weight would provide a better index of storage iron levels than transfusion load alone. However, similar correlations were obtained between serum ferritin concentration and either units of blood or units/kg body weight. There was a highly significant correlation between units of blood given and blood/kg body weight ($r=0.88$, $P<0.001$). The correlation between 'total' serum ferritin and units of blood/kg body weight was slightly higher than that with units of blood ($r=0.41$ and 0.38 respectively). For glycosylated ferritin the corresponding values of r were 0.25 and 0.23 and for non-glycosylated ferritin 0.42 and 0.39 .

Prieto *et al* (1975) found that the serum ferritin:aspartate transaminase ratio was closely related to liver iron concentration in patients with liver disease (including primary and secondary haemochromatosis). However, in this group of 124 patients the serum ferritin:ALT ratio did not correlate with units blood/kg body weight ($r=0.14$, $P>0.1$) or with transfusion load ($r=0.17$, $P>0.05$).

DISCUSSION

In this group of patients both storage iron and liver damage were important factors influencing serum ferritin concentrations. An interesting finding was the relationship between glycosylated ferritin and units of blood transfused. There was a good correlation between these two measurements up to a load of 100 units of blood but no correlation was seen above this level. It seems that the level of glycosylated ferritin reaches a maximum after transfusion of 100 units of blood. If ferritin is secreted from cells after glycosylation this may reflect a limit to the rate of ferritin synthesis in response to iron overload. Experimental work with iron loaded rabbits (Sturgeon & Shoden, 1964), cultured HeLa cells (Chu & Fineberg, 1969) and Chang liver cells (Bailey-Wood *et al*, 1975) has led to the suggestion that there is a maximum rate of apoferritin synthesis in response to iron. It therefore seems likely that the correlation of serum ferritin concentration with iron stores in grossly overloaded subjects is a fortuitous addition of the effect of iron levels on ferritin synthesis and the effect of cell damage on ferritin release from the liver. In an earlier study of serum ferritin concentration in patients with thalassaemia a decline in rate of increase with increasing transfusion load was also noted (Letsky *et al*, 1974). In addition there was some reduction in the rate of increase of liver iron concentration after about 200 units of blood have been given (Barry *et al*, 1974). This may be related to age and therefore to iron content per unit body weight or to an increase in the rate of iron excretion. However, the apparent limit to glycosylated ferritin concentration is striking, and appears to precede these changes.

If glycosylated ferritin is secreted from cells then it might be expected that serum levels would not be related to ALT activity. However, there was a correlation between the two. This is probably because the liver is responsible for the clearance of ferritin from the plasma (Siimes *et al*, 1974; Unger & Hershko, 1974; Halliday *et al*, 1979). Thus liver damage will cause increased serum ferritin concentrations by reducing the rate of removal of ferritin from the plasma as well as increasing the input of ferritin.

Despite the correlation between serum ferritin concentration and both storage iron and liver damage much of the variation in serum ferritin remained unexplained. To what extent was this due to inadequate methods of measuring storage iron and liver damage? Accurate assessment

of storage iron levels requires either quantitative phlebotomy or analysis of biopsy samples. Neither method was possible in this study and there are obvious limitations in using the number of units of blood transfused as an index of iron stores. However, expressing the transfused iron load as units of blood/kg body weight made little difference to the results. Additional factors which may affect the level of stores are iron absorption and iron loss. These patients were on a 'high transfusion regime', maintaining haemoglobin concentrations at above 9 g/dl, and iron absorption from food would have been in the normal range (up to 2 mg/d; Heinrich *et al*, 1973). Iron losses would be of the same order, so that in a patient receiving 17 units (8.5 litres) of blood each year (about 4 g iron) iron absorption would contribute relatively little compared with transfused iron. The patients had received relatively little chelation therapy and regression analysis showed that the amount of desferrioxamine given had little effect on serum ferritin levels. Thus the transfusion load should provide a reasonable approximation to the level of iron stores in these patients.

The measurement of ALT activity will not necessarily correlate well with ferritin release from the liver, even if the influence of varying levels of storage iron could be abolished, as the clearance of ferritin from the circulation is much more rapid than ALT. The biological half-life for ferritin in plasma is approximately 10 min (see above) and for alanine transaminase 6 d (Wilkinson, 1976). Thus raised ALT levels may possibly be found some days after an episode of necrosis when ferritin levels have already fallen.

It is therefore likely that much of the unexplained variability of serum ferritin concentration in patients with iron overload is due to the inadequate estimation of levels of iron stores and of liver cell damage. Variation due to the method of measurement of serum ferritin concentration (Dawkins *et al*, 1979) and to the ascorbic acid status of the patients may also be important. Many iron overloaded patients have low levels of leucocyte ascorbic acid (Wapnick *et al*, 1970). When supplementation with ascorbic acid is carried out it leads to increased desferrioxamine induced iron excretion (Propper *et al*, 1977). In addition it has been shown experimentally that ascorbic acid depletion in guinea-pigs increases serum ferritin concentration (Roeser *et al*, 1980). The patients studied here did not receive regular supplementation with ascorbic acid.

The present study provides further evidence of variation in the amount of serum ferritin binding to con A in patients with transfusional iron overload but shows that the assay of con A binding ferritin is not of practical value in assessing storage iron levels in such patients. In this series glycosylated ferritin rarely exceeded a concentration of 4000 $\mu\text{g/l}$. There is no simple relationship between serum ferritin concentrations and iron stores when ferritin concentrations exceed 4000 $\mu\text{g/l}$ or in patients who have received more than 100 units of transfused blood.

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