Severe malnutrition is associated with decreased levels of plasma transferrin receptor

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Sixty children aged 1–3 years with protein malnutrition were involved in the present study. Measurements were made of plasma transferrin receptor (TfR), haemoglobin (Hb), ferritin, transferrin and Fe in comparison with twenty apparently healthy age- and sex-matched reference children in Nigeria. Plasma TfR was measured by an immunofluorometric assay. The mean plasma concentrations of both albumin (37 g/l) and transferrin (1·88 g/l) were within reference ranges in the 'healthy' children. The malnourished children had severe protein deficiency as indicated by their significantly lower mean plasma albumin (24·4–28·2 g/l; $P < 0.0001$) and transferrin (1·24–1·53 g/l; $P < 0.0001$) concentrations in comparison with the reference children. In the reference children, the traditional indicators of Fe nutrition (plasma Fe, transferrin Fe saturation and Hb) were within the reference ranges, but ferritin values were raised, indicating acute or chronic infection and/or inflammation. The mean concentrations of plasma TfR (4·2–5·2 mg/l) in the malnourished group were significantly lower than the mean (6·1 mg/l) of the reference children ($P = 0.0009$). In the children with severe malnutrition, none of the indicators of Fe status except Hb (81·5–86·7 g/l; $P < 0.0001$) showed Fe deficiency, including the serum concentration of TfR and the TfR : ferritin ratio, although the Fe status was lower than in the reference children (for Fe $P = 0.009$; and ferritin $P = 0.0004$). In the absence of haemodilution, the low Hb values are a clear indication that the malnourished children were Fe deficient; none of the other indices was indicative of Fe deficiency. This is the first report of TfR levels in malnourished and healthy African children.

Transferrin: Ferritin: Iron: Malnutrition

Despite our extensive knowledge of Fe metabolism, diagnosis of Fe deficiency often remains a difficult problem. Mild Fe deficiency is difficult to diagnose, whatever methods are used. Moreover, even the diagnosis of severe Fe deficiency is complicated, especially in patients with protein malnutrition. Evaluation of Fe stores would be of clinical benefit; however, reliable results can be obtained only by invasive techniques (Skikne *et al.* 1990). Serum transferrin receptor (TfR) is a new measure that reflects the Fe status and the rate of erythropoiesis in adults (Kohgo *et al.* 1986, 1987; Flowers *et al.* 1989; Huebers *et al.* 1990; Skikne *et al.* 1990; Beguin, 1993). According to Skikne *et al.* (1990), tissue Fe requirements are more important determinants of the serum TfR level than increases in erythropoiesis. The amount of the receptor increases soon after signs of Fe deficiency appear, the rise reflecting the paucity of available tissue Fe. In advanced Fe deficiency, the mean

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serum TfR concentration increases to approximately three times the normal mean (Ferguson *et al.* 1992).

The serum TfR concentration has been measured in healthy children and adults (Flowers *et al.* 1989; Carriaga *et al.* 1991; Kivivuori *et al.* 1993; Kuvibidila *et al.* 1994). When erythropoiesis was stimulated in very-low-birth-weight infants and in children with acute lymphoblastic leukaemia, there was a rise in serum TfR level (Kivivuori *et al.* 1994*a,b*). In these studies, the rise could be attributed to either stimulated erythropoiesis or lack of Fe. Measurements of the concentration of serum TfR may have several advantages over other tests used for the assessment of Fe nutrition. Although individuals with low serum ferritin concentrations have a higher risk of Fe deficiency (Hallberg *et al.* 1993) and those with high serum ferritin concentrations probably have sufficient Fe stores, most healthy subjects have intermediate values.

Unlike serum ferritin concentrations, serum TfR concentrations are thought not to be influenced by infections or chronic inflammation, which may be a great advantage (Ferguson *et al.* 1992; Nielsen *et al.* 1994; Petterson *et al.* 1994; Punnonen *et al.* 1994).

It has been suggested that anaemic patients with acute or chronic inflammatory diseases can be distinguished from those with Fe-deficiency anaemia by their low or normal serum TfR concentrations (Ferguson *et al.* 1992).

The aim of the present study was to evaluate the plasma concentrations of TfR in malnourished Nigerian children, who are usually at a greater risk of Fe deficiency than their healthy counterparts.

SUBJECTS AND METHODS

Study population

The procedures followed were in accord with the standards of the Ethical Committee of the University of Calabar, Nigeria, for human experimentation. Informed consent was obtained from the parents of the children. The malnourished children had clinical signs and symptoms of anaemia such as pallor of the skin and conjunctiva, fatigue and lack of appetite. The initial study population consisted of 206 malnourished and 200 reference children aged 1–3 years. After screening for antibodies to human immunodeficiency virus (HIV) and hepatitis B virus surface antigen (HBsAg), sixty patients were enrolled in the study, twenty from each category of malnutrition, and twenty apparently healthy age- and sex-matched Nigerian children from the HIV- and HBsAg-negative population. This selection was necessary both for laboratory safety and affordable cost of specimen analysis. The eighty subjects were randomly picked from the HIV- and HBsAg-negative population. The reference group had a mean age of 2·3 (SD 0·6) years and mean weight of 12·4 (SD 1·0) kg. The malnourished consisted of kwashiorkor (*n* 20, mean age 2·2 (SD 0·6) years, mean weight 8·0 (SD 0·9) kg), marasmic kwashiorkor (*n* 20, mean age 2·2 (SD 0·6) years, mean weight 7·1 (SD 0·6) kg), and marasmus (*n* 20, mean age 2·1 (SD 0·7) years, mean weight 6·9 (SD 0·7) kg. Protein–energy malnutrition (PEM) was diagnosed and subgrouped by the Wellcome classification (Editorial, 1970). We attempted to define severity of malnutrition by plasma albumin values using 30 g/l as cut-off, and plasma ferritin values ≥ 100 µg/l for inflammation. Using these values, we arrived at the following distribution: plasma albumin ≥ 30 g/l, ferritin < 100 µg/l (*n* 12); albumin < 30 g/l, ferritin < 100 µg/l (*n* 43); albumin < 30 g/l, ferritin > 100 µg/l (*n* 4) and albumin ≥ 30 g/l, ferritin > 100 µg/l (*n* 1).

Specimen collection

The samples were collected from Calabar in the southeast, Warri in the Niger Delta, and Ibadan in the Southwestern area, all in the southern part of Nigeria. Blood (2·5–3·0 ml) was collected by venipuncture from each of the subjects in a non-fasting state. The sample was put into a citrated bijou bottle prepared by allowing 50 μl of sodium citrate (280 g/l) to dry. Plasma samples were prepared by centrifuging the blood at about 500 g for 5 min. Samples were stored at $-20°$ until they were transported to Finland in solid CO_2.

Transferrin receptor assay

TfR was assayed by the two-step sandwich-type time-resolved immunofluorimetric technique developed by one of us (Kivivuori *et al.* 1993). All the materials and techniques employed in this assay were the same as previously published (Kivivuori *et al.* 1993). Briefly, into wells in polystyrene plates coated with monoclonal antibody, 100 μl of sample or standard was dispensed in duplicate, diluted in Delfia assay buffer solution (LKB Wallac, Turku, Finland) containing 65 g bovine serum albumin/l (Winlab, Leicester) to simulate the viscosity of serum; 100 μl of assay buffer solution was added and the wells were incubated for 3 h at room temperature with continuous gentle shaking (Wallac Plate Shake 1296-001, LKB Wallac). The wells were washed three times with the washing solution and 100 ng europium-labelled immunoglobulin G (IgG) in 200 μl of the assay buffer was added. After further incubation for 2·5 h at room temperature with continuous gentle shaking, the wells were washed and 200 μl Delfia enhancement solution (LKB Wallac) was added. The wells were shaken gently and after 60 min the fluorescence was measured with a 1230 Arcus Fluorimeter (LKB Wallac) using a counting time of 1 s and a time delay of 400 μs (Soini & Kojola, 1983). The concentration of TfR was read by comparison from a standard curve, constructed from values for a series of dilutions of a standardized serum pool with assay buffer containing 65 g bovine serum albumin/l. As controls we used two dilutions (1 : 50 and 1 : 100) of a serum pool containing 6·4 mg TfR/l. The inter-assay CV were 12·8 and 16·9 % (n 6) and the intra-assay CV were 11·4 and 8·2 % (n 20) with 1 : 100 and 1 : 50 dilutions of the control pool respectively. When known amounts of TfR were added to the serum samples, a mean of 96·8 % of the calculated value was recovered (CV 15·5 %, n 33).

Other methods

Plasma albumin was estimated spectrophotometrically, using a dye-binding technique (bromo cresol green). The plasma Fe (Feren method, Orion Diagnostica, Espoo, Finland) and blood haemoglobin (Hb) (cyanmethaemoglobin technique) concentrations were measured colorimetrically, and plasma transferrin by an immunoturbidimetric method (Anttila & Siimes, 1996). Plasma ferritin was measured with a radioimmunoassay, using commercial reagents (Ferritin RIA Kit, Kodak Clinical Diagnostics, Amersham, Bucks.). For plasma ferritin assay, four controls with concentrations of 13, 55, 163 and 360 μg/l (obtained from Ciba-Corning Diagnostics Corp., Medfield, MA, USA) were used. These were standardized against the World Health Organization reference standard (WHO IRP 80/602). The intra-assay CV were 2·8, 2·6, 2·7 and 3·6 % respectively, while the inter-assay CV were 5·0, 6·1, 4·7, and 5·1 % respectively. The reference range for Fe deficiency (ferritin) is 1–35 μg/l. The transferrin Fe saturation (TIS) was calculated as the ratio of plasma Fe in μmol/l (multiplied by 100) to plasma transferrin in g/l (multiplied by 20). The

samples were screened for HIV antibodies (Enzygnost Kit, Behringwerke AG, Marburg, Germany), and for HBsAg (Murex Diagnostics, Dartford, Kent).

Classification of malnutrition

Within the spectrum of PEM most investigators have recognized three clinical types of severe malnutrition: kwashiorkor, marasmus and marasmic kwashiorkor. The criteria for distinguishing between these three conditions are still based mainly on clinical findings. This means that classification tends to be qualitative. We used the Wellcome classification, which is based on the presence or absence of oedema and the deficit in body weight. The 50th percentile Harvard Boston value was used as weight-for-age standard (Editorial, 1970).

(1) Kwashiorkor. These are children with oedema who weigh between 60 and 80 % of their expected weight for age. It is also recognized, however, that there are some children with oedema and all the clinical features of kwashiorkor who should be so classified even though their body weight exceeds 80 % of the standard weight for age.
(2) Marasmus. These are children without oedema who weigh less than 60 % of their expected weight for age.
(3) Marasmic kwashiorkor. These are children with oedema who weigh less than 60 % of their expected weight for age.

Statistical methods

Results are reported as means with their standard errors (SEM). ANOVA was used first to compare the malnourished children with the reference group. Second, ANOVA was used for each variable to compare the malnourished groups with each other. The error mean squares from these ANOVA were used in the calculation of pooled SEM for the malnourished groups. Correlation coefficients were evaluated according to Pearson. Results were considered significant when $P \leq 0.05$.

RESULTS

Protein status

The reference children had significantly higher concentrations of plasma albumin and transferrin than the malnourished children (Table 1).

Iron status by traditional criteria

Reference subjects. The traditional plasma variables for the assessment of Fe status indicated that in the reference population, Fe nutrition was sufficient. The mean values of blood Hb, plasma Fe, transferrin, ferritin and the TIS were consistent with the concentrations accepted for Fe adequacy.
   Malnourished patients. Except low Hb levels, the traditional variables also showed adequate Fe nutrition in the malnourished subjects, although most of the mean values were lower than those of the reference subjects (Table 1).

Table 1. *Plasma concentrations of several biochemical variables in malnourished children and reference subjects*

(Mean values with their standard errors for twenty children per group)

Group...	Reference		Kwashiorkor		Marasmic kwashiorkor		Marasmus	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Albumin (g/l)	37·0	1·0	24·4***	0·9	26·5***	0·9	28·2*	0·9
Iron (μmol/l)	15·6	1·18	11·7**	1·16	11·1**	1·16	13·2**	1·16
Haemoglobin (g/l)	133	3·7	82***	3·2	82***	3·2	87***	3·2
Transferrin (g/l)	1·88	0·063	1·24***	0·060	1·32***	0·060	1·53***	0·060
TIS (%)	42	5·68	47*	5·67	42	5·67	43	5·67
Ferritin (μg/l)	87	8·18	51***	6·98	62***	6·98	55***	6·98
TfR (mg/l)	6·1	0·44	4·2***	0·43	3·4***	0·43	5·2**	0·43
TfR:ferritin (μg/μg)	79	8·4	114*	22	75	22	141*	22

TIS, transferrin iron saturation; TfR, transferrin receptor.
Mean values were significantly different from those for the reference group: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.
    Comparison of means and significance tests were done using ANOVA. Pooled SEM for malnourished children were based on the error mean square from the ANOVA of the results for malnourished children.

Transferrin receptor and transferrin receptor:ferritin ratio

The mean concentration of plasma TfR in the reference children was higher than those of the malnourished children, but the reverse was the case with TfR : ferritin ratio.

In the malnourished children with plasma albumin ≥ 30 g/l and ferritin < 100 μg/l (n 12), the mean albumin concentration was 32 (SE 7) g/l, ferritin was 52 (SE 7·2) μg/l and TfR was 4·0 (SE 0·28) mg/l. However, in children with plasma albumin < 30 g/l and ferritin < 100 μg/l (n 43), the mean albumin value was 25 (SE 0·5) g/l, ferritin was 51 (SE 3·7) μg/l and TfR was 4·0 (SE 0·35) mg/l.

No significant differences were observed between malnourished groups with two exceptions. The mean plasma value of transferrin in marasmic patients was higher than those found in kwashiorkor ($P = 0.002$) and marasmic kwashiorkor patients ($P = 0.03$). Moreover, the mean plasma albumin value of marasmic patients was higher than that of kwashiorkor patients ($P = 0.003$).

Relationship of plasma transferrin receptor to plasma albumin

When plasma TfR or the TfR : ferritin ratio was plotted as a regression *v.* plasma albumin, no correlations were found in either the reference or the malnourished subjects.

DISCUSSION

We report here our findings in a study which involved sixty patients with PEM and twenty apparently healthy reference children in Nigeria. Although the plasma concentration of albumin, which is an indicator of protein status, was significantly higher in the reference population than those found in the patients, the mean concentrations fell within the low range for healthy children in developed countries. Differences in plasma concentrations of biochemical variables between tropical and temperate regions of the world abound, due to variations in diet and environment. However, our plasma albumin results for the patients

indicate severe protein malnutrition, most severe in those with kwashiorkor and the least severe in those with marasmus (Hay *et al.* 1975).

To evaluate the Fe status of our subjects we employed the traditional criteria (blood Hb, plasma Fe, transferrin, TIS and ferritin) and also measured plasma TfR, which is a new indicator of Fe status (Kohgo *et al.* 1986, 1987; Flowers *et al.* 1989; Huebers *et al.* 1990; Skikne *et al.* 1990; Beguin *et al.* 1993). The traditional variables for the assessment of Fe status were within reference ranges in both the 'healthy' and malnourished children (Siimes *et al.* 1974; Saarinen and Siimes, 1979). The only exception was the low mean Hb values which indicated Fe deficiency in the malnourished population. The plasma TfR values for the reference children were higher than those reported for Finnish children (Kivivuori *et al.* 1993). There is no published information on the concentrations of serum TfR in apparently healthy African children. However, plasma TfR levels were significantly lower than in the reference children. Our data indicate adequate Fe and protein status in the reference children. These observations suggest that protein deficiency might affect the overall expression of TfR, and consequently depress circulating levels. An explanation might be that the hypoproteinaemia observed in the patients impairs both incorporation of Fe into Hb and the rate of erythropoiesis, which also determines the individual levels of serum TfR (Beguin *et al.* 1993; Kivivuori *et al.* 1994*a,b*). If this is the case, we would have expected to find some correlation between the levels of TfR and albumin. Moreover, a simple analysis using similar ferritin conditions and differing protein status did not reveal any difference in mean TfR values. This suggests that the protein status *per se* might not be the critical factor which determines the TfR values in malnutrition.

The lower levels of plasma TfR may be partly attributed to the dilutional effect of malnutrition. However, while it is true that the plasma volume is diluted in kwashiorkor patients (due to the markedly reduced value of plasma albumin which maintains the plasma oncotic pressure), this effect is minimal in marasmic kwashiorkor patients and absent in marasmic patients (Editorial, 1970).

While plasma ferritin values are not diagnostic in the presence of infections, it has been suggested that anaemic patients with acute or chronic inflammatory diseases can be distinguished from those with Fe-deficiency anaemia by their low or normal serum TfR concentrations (Ferguson *et al.* 1992). In the malnourished subjects, while the ferritin levels were higher than expected, the transferrin levels were lower than expected. This indicates inflammation and is capable of affecting the plasma TfR values. The relatively low plasma transferrin concentration encountered in the reference population, coupled with the elevated ferritin values, suggests that the reference population was not free of mild inflammation. Since we cannot rule out infection in the malnourished children, the presence of both a compromised Fe status and infection will tend to keep the serum TfR normal.

Anaemia of chronic inflammation (Ferguson *et al.* 1992), aplastic anaemia, chronic renal failure and marrow transplantation (Huebers *et al.* 1990) have been associated with decreased levels of plasma TfR; we should perhaps add severe tropical infantile protein malnutrition.

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