Etiology of anemia in pregnancy in south Malawi[1–4]

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ABSTRACT

Background: Anemia in pregnancy is a major public health problem in developing countries. In sub-Saharan Africa, such anemia is generally accepted as resulting from nutritional deficiencies, particularly iron deficiency.

Objective: We comprehensively assessed the full spectrum of nutritional and nonnutritional factors associated with pregnancy anemia.

Design: Iron, folate, vitamin B-12, and vitamin A were measured in serum in a cross-sectional study of 150 pregnant women in Blantyre, Malawi. Bone marrow aspirates were evaluated, peripheral blood films were examined for malaria parasites, stool and urine samples were examined for helminthic infection, and tests were done for genetic disorders and for HIV infection. C-reactive protein (CRP) concentrations and erythrocyte sedimentation rates were measured as markers of inflammation.

Results: Of the 150 anemic women, 23% were iron deficient with no evidence of folate, vitamin B-12, or vitamin A deficiencies; 32% were deficient in iron and one or more of the other micronutrients; 26% were not iron deficient but had evidence of one of the other micronutrient deficiencies, most often vitamin A; and 19% were not deficient in any of the micronutrients studied. CRP concentrations were notably high in 54% of the anemic women with no nutritional deficiencies and in 73.5% of the anemic women who were iron replete by bone marrow assessment.

Conclusion: The role of chronic inflammation as a possible contributing factor to anemia in pregnancy has important implications for the clinical evaluation and treatment of women. *Am J Clin Nutr* 2000;72(suppl):247S–56S.

KEY WORDS Anemia, pregnancy, nutritional deficiency, infection, Malawi, iron, vitamin B-12, folate, vitamin A, women

INTRODUCTION

Anemia in pregnancy continues to be a common clinical problem in many developing countries, and prevalence rates of 35–75% are reported (1). In cases in which the anemia is severe and not corrected, blood transfusion may become necessary (2). Anemia has been reported to contribute significantly to maternal mortality (1, 3–5) and to both maternal and fetal morbidity (6–8). Anemia in pregnancy is a risk factor for infant iron deficiency anemia (9) that, if left uncorrected, can be associated with adverse behavioral and cognitive development (10).

Few studies have comprehensively assessed the etiologic factors responsible for anemia in pregnancy. This lack of research is probably due to 3 main factors, as follows. *1*) Adequate diagnostic facilities are lacking in many health institutions in developing countries. *2*) The etiologic pattern is often complex such that, for example, infection and nutritional deficiencies coexist. Biochemical measurement of iron status is influenced by inflammation, and clearly defined and validated cutoffs for diagnosing iron deficiency in pregnancy in the presence of coexisting infection have been lacking (11–13). The evaluation of suitably stained bone marrow aspirates, therefore, is necessary to identify nutritional deficiencies with certainty (14, 15). *3*) The contribution of each etiologic factor is difficult to assess in pregnancy because maternal physiologic changes alter the indexes used to diagnose anemia and nutritional deficiencies. For example, serum vitamin B-12 is markedly reduced toward the end of pregnancy (16–18).

Despite the lack of stringent criteria and the problems with definitions, in sub-Saharan Africa anemia during pregnancy is most often believed to result from nutritional deficiencies, especially iron deficiency (19, 20). Folate deficiency has been described in West Africa (21) and recent studies suggest that deficiencies of other vitamins can also contribute to anemia. Studies in Indonesia showed that vitamin A deficiency may contribute to anemia in pregnancy (22, 23). Until 1994 vitamin B-12 deficiency was an unrecognized—but important—cause of anemia in Zimbabwe (24). The role of chronic infection has been discussed in this connection (25), but has not been studied in pregnant women. In the past decade, HIV infection has become more prevalent and must now be considered as a possible etiologic factor (26, 27). Parasitic infections known to cause anemia include malaria, hookworm, and schistosomiasis (28). Thus, the etiologic factors responsible for anemia are multiple and their relative contributions can be expected to vary by geographic area and by season. Knowledge of the relative importance of the different causes should form the basis for intervention strategies to control anemia (29). This paper describes a cross-sectional study that identified etiologic factors associated with anemia in pregnancy in south Malawi.

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SUBJECTS AND METHODS

Subjects

Women who came to the Queen Elizabeth Central Hospital in Blantyre for antenatal care were screened for anemia, which was defined as a hemoglobin concentration < 105 g/L (2), with use of a battery-operated HemoCue photometer (HemoCue AB, Ängelholm, Sweden). Of the 265 women screened, 150 were anemic and were enrolled consecutively in the study. After a woman consented to participate in the study, she completed a structured questionnaire and underwent a general physical and obstetric examination. Gestational age was assessed by history of the last menstrual period and measurement of fundal height. None of the 150 women enrolled in this study had clinical signs or symptoms suggestive of AIDS or other serious medical or surgical conditions other than anemia.

The study was approved by the Malawi Health Sciences Research Committee before commencement. Women were informed individually about the purpose of the study, in their own language, and asked to sign a consent form if they agreed to participate. The background prevalence of HIV infection in this population of women (anemic and nonanemic) was obtained via anonymous screening and updated yearly. In accordance with guidelines provided by the Health Sciences Research Committee of Malawi, consent was obtained for anonymous screening for HIV infection. Thus, at the time of enrollment into the study, the HIV status of the patient was not known. Each woman was given the opportunity to receive counseling from an independent health care worker if she so wished and to have her serum test results made available to her then or at any time in the future. A separate consent form was obtained for the bone marrow aspiration.

Methods

Venous blood, urine, and stool samples were obtained for examination. Blood samples were stored in the dark immediately after being drawn and were processed within 24 h, after which the serum was stored at −70°C. A bone marrow aspirate was obtained from anemic women only if they had specifically given consent for this and only if they could be observed for 24 h after aspiration. Bone marrow aspirates were obtained from the anterior iliac crest under local anesthesia by using a sterile procedure.

Full blood counts were obtained by using an automated cell counter (model Onyx/CP; Coulter Electronics, Johannesburg, South Africa) calibrated regularly according to the manufacturer's specifications with appropriate controls. Reticulocyte counts were determined after staining with brilliant cresyl blue with the use of Merret tubes (Mercia Diagnostics, Guildford, United Kingdom). Transferrin concentration was measured by Tina-quant immunoturbidimetric assay (Hitachi 911 analyzer, detection limit of 0.2 g/L; Boehringer-Mannheim, Mannheim, Germany). Serum ferritin and serum transferrin receptor (TfR) concentrations were determined by immunoenzymometric assay (detection limit of 0.6 μg/L and 0.7 μg/L, respectively; Ramco, Houston). Vitamin B-12, serum folate, and erythrocyte folate concentrations were measured with the SimulTRAC Radioassay Kit (Becton Dickinson, Nairobi, Kenya). Serum retinol concentrations were measured by HPLC (Spectrasystem P1000, Resolve 5C18 column; Spectraphysics Analytic, San Jose, CA). C-reactive protein (CRP) was measured by Tina-quant immunoturbidimetric assay (detection limit of 3 mg/L; Boehringer-Mannheim). The erythrocyte sedimentation rate was measured by the Westergren method (30). HIV status was determined in duplicate by using an immunoenzymometric assay (WellcoZyme; Wellcome Diagnostics, Johannesburg, South Africa).

A thick blood film was stained and examined for malaria parasites (Field stains A and B). Stool and urine samples were examined microscopically for the presence of parasites. Hookworm loads were classified semiquantitatively as 0, +, ++, or +++ for no, mild, moderate, or severe infection, respectively.

Bone marrow films were stained for iron by using Prussian blue (Perls's method) (31). All bone marrow aspirates included in the analysis were considered representative if they contained fragments. The stained aspirates were interpreted by a hematologist blinded to the biochemical and hematologic results and were classified as 0, no iron present; 1, traces of iron only; 2, moderate amounts of iron present; and 3, abundant iron present. In addition, bone marrow aspirates were examined for megaloblastic change. The classification used was 0, no megaloblastic changes; 1, masked megaloblastic changes; and 2, megaloblastic changes in both red and white blood cell series.

DNA was obtained from bone marrow aspirate material by standard phenol chloroform extraction. The Southern blot procedure was carried out to determine thalassemia status, and polymerase chain reaction was used to determine sickle cell and glucose-6-phosphate 1-dehydrogenase (G6PD) status by using the methods described by the Molecular Haematology Unit, Institute of Molecular Medicine, Oxford, United Kingdom. At the time of recruitment, blood samples were also tested for G6PD deficiency by a simple screening method [methemoglobin reduction test (32)].

Statistical analysis

Data were analyzed by using SPSS for WINDOWS (version 6.0; SPSS Inc, Chicago) and EXCEL for WINDOWS (version 6.1; Microsoft Corp, Redmond, WA). The Cochran-Mantel-Haenszel test was used to examine the association between both serum folate and vitamin B-12 concentrations and megaloblastic change in the bone marrow. The Mann-Whitney *U* test was used to compare hemoglobin concentration, CRP, serum retinol, serum vitamin B-12, serum folate, and erythrocyte folate measurements for HIV seropositive and seronegative women. An analysis of variance was used to examine the differences in hemoglobin concentration and mean corpuscular volume in α+ and α+ homozygous patients.

RESULTS

Clinical and serologic data were obtained from 150 anemic women. Of these women, 69% were in the third, 28% in the second, and 3% in the first trimester of pregnancy. A representative bone marrow aspirate was available for 88 of the 150 women. Sixty-two women either did not give consent for bone marrow aspiration or could not stay to be observed for 24 h after the procedure.

The results of the hematologic and biochemical analyses of the entire sample are presented in **Table 1** and the results of the bone marrow aspirates are shown in **Table 2**. The distributions of hemoglobin concentration for the entire sample and for the group with bone marrow aspirates are presented in **Table 3**. Women with severe anemia (hemoglobin <70 g/L) comprised 16% of the total group and 18% of the subsample for whom bone marrow samples were available.

TABLE 1
Results of hematologic and biochemical analyses in 150 anemic pregnant women

	Mean	Median	SD	Minimum	Maximum	Skewness	*n*
Hemoglobin (g/L)							
Coulter[1]	85	86	15	28	106	−13	150
HemoCue[2]	82	87	15	28	102	−14	150
Ferritin (μg/L)	120.5	25.5	289	3.6	2657	5.6	148
Serum transferrin receptor (mg/L)	12.4	10.5	7.6	3.0	50	2.1	146
Serum folate (nmol/L)	17.7	11.8	16.1	1.1	79.3	2.3	117
Red blood cell folate (nmol/L)	1135	972	902	91	5833	3.0	149
Reticulocyte count	0.02	0.019	0.007	0.005	0.036	−0.0004	142
Vitamin B-12 (nmol/L)	412	280	502	7.4	3839	3.3	149
Retinol (μmol/L)	0.87	0.84	0.39	0.18	1.9	0.6	150
C-reactive protein (mg/L)	30.1	9.0	44.8	1.0	174.0	1.8	147
Erythrocyte sedimentation rate (mm/h)	107	109	24.8	36	218	0.2	149

[1] Coulter Electronics, Johannesburg, South Africa.
[2] HemoCue AB, Ängelholm Sweden.

Iron deficiency

Cutoffs for diagnosing iron deficiency in this population were determined previously, as was a mathematical model for predicting bone marrow iron depletion (33). After all the commonly available variables for measuring iron status were evaluated, 2 options emerged. When a single serum variable was used, ferritin was the best predictor of iron deficiency. Through use of receiver operating characteristic curves, the most appropriate cutoff for diagnosing iron deficiency in this population was found to be 30 μg/L for serum ferritin. This cutoff had a sensitivity of 90% and a specificity of 85%. For the 150 women for whom serum ferritin measurements were available, 55% were classified as iron deficient because their serum ferritin concentrations were <30 μg/L. With use of the conventional cutoff of 12 μg/L (34), only 21% of the study participants would have been classified as iron deficient (**Table 4**). Alternatively, a combination of iron variables including serum ferritin and TfR, which reflects iron status at both storage and tissue levels, can be used with CRP to determine iron status (33, 35). Values below zero from the following formula indicate the absence of stainable iron in the bone marrow with a sensitivity of 77% and specificity of 80%:

$$0.34 + 0.0043\ \text{Ferritin}\ (2.7\ \text{TfR})/\text{ferritin} + 0.007\ \text{CRP} + 0.05\ \text{TfR} \quad (1)$$

With this formula, 52% of the 150 women in this study were predicted to have no stainable bone marrow iron and were classified as iron deficient.

Examination of stained bone marrow aspirates remains the most definitive method for determining iron status. Of the subsample of 88 women for whom bone marrow aspirates were available, 44% had no demonstrable stained iron whereas 17% had a trace only (Table 2), suggesting a total of 61% with insufficient iron.

Folate deficiency

With use of a cutoff of 9.1 nmol/L (34), the prevalence of folate deficiency in these anemic women was 34%. To allow for the maximum hemodilution at term in pregnancy, a cutoff of 7.7 nmol/L was also considered (18). Alternatively, if serum folate measurements were not available, folate deficiency was defined as erythrocyte folate <317 nmol/L (34). For the latter more stringent criteria, folate deficiency was present in 21% of women (Table 4). Of the 25 folate-deficient women, 6 (24%) were not deficient in any of the other micronutrients studied, 10 (40%) were iron deficient, 4 (16%) were vitamin B-12 deficient, 4 (16%) had low serum retinol concentrations, and 1 was deficient in all 3 micronutrients (**Table 5**). A significant association was found between megaloblastic change in the bone marrow and serum folate concentrations ≤9.1 nmol/L ($P = 0.022$). The association between megaloblastic change and serum folate concentrations ≤7.7 nmol/L, however, was not statistically significant ($P = 0.08$).

Vitamin B-12 deficiency

The reported range for serum vitamin B-12 concentrations in pregnancy is 52–369 pmol/L (34). One-third of the anemic women in this study had serum vitamin B-12 concentrations <148 pmol/L, which is the accepted lower limit outside pregnancy. When deficiency was defined as serum vitamin B-12 <52 pmol/L, reflecting the observed decrease toward the end of pregnancy resulting from the active transplacental transfer from mother to fetus and the added effect of hemodilution (18, 34), 16% of women were affected (Table 4). Five (21%) of these 24 vitamin B-12–deficient women were also folate deficient (Table 5). The association between serum vitamin B-12 concentrations and the megaloblastic changes observed in the bone marrow was linear and highly significant ($P < 0.001$).

TABLE 2
Results of bone marrow aspirates examined for iron content and megaloblastic change

	n (%)
Bone marrow iron	
0 = No stainable iron	39 (44.3)
1 = Trace of iron	15 (17.0)
2 = Moderate amount of iron	16 (18.2)
3 = Abundant iron	18 (20.5)
Total	88 (100.0)
Megaloblastic change	
No change	29 (33.0)
Masked change	44 (50.0)
Change in both red and white cells	15 (17.0)
Total	88 (100.0)

TABLE 3
Distribution of hemoglobin concentration in all anemic pregnant women and in a subsample from whom bone marrow aspirates were taken

	Entire sample	Bone marrow subsample
	n (%)	
Hemoglobin (g/L)		
20–29.9	1 (0.7)	1 (1.1)
30–39.9	1 (0.7)	0 (0.0)
40–49.9	4 (2.7)	4 (4.5)
50–59.9	1 (0.7)	1 (1.1)
60–69.9	17 (11.3)	10 (11.5)
70–79.9	17 (11.3)	11 (12.5)
80–89.9	45 (30.0)	30 (34.1)
90–99.9	48 (32.0)	23 (26.1)
100–109.9	16 (10.6)	8 (9.1)
Total	150 (100.0)	88 (100.0)

Vitamin A deficiency

During pregnancy, serum retinol concentrations have been shown to drop below nonpregnancy concentrations (36). Serum retinol concentrations of 1.05, 0.7, and 0.35 μmol/L indicate inadequate, moderately inadequate, and very inadequate liver stores, respectively (37). In this study, a serum retinol concentration <0.7 μmol/L was used to define vitamin A deficiency and 39% of these anemic women fell in this category (Table 4). Thirteen percent of the women were very deficient, with a serum retinol concentration <0.35 μmol/L. Of the women with serum retinol concentrations <0.7 μmol/L, 52% (30 of 58) were also iron deficient (Table 5). Vitamin A deficiency was the only micronutrient deficiency in 15% (23 of 150) of all women, making this the second most frequent single micronutrient deficiency after iron deficiency in this group of anemic women.

Combined micronutrient deficiencies

Of the 150 anemic women, 23% were iron deficient only, 33% were deficient in iron and one or more of the micronutrients studied, and 26% were not iron deficient but had evidence of another micronutrient deficiency, most often vitamin A (Table 5). For 19% of the anemic women there was no evidence to suggest that any of the micronutrients studied contributed to their anemia.

Of the 83 anemic women with iron deficiency (serum ferritin <30 μg/L), 41% were iron deficient only; thus, iron deficiency was associated with other micronutrient deficiencies for 59% of the women. Iron deficiency and one other deficiency (folate, vitamin B-12, or vitamin A) were present in 46% of women. Iron deficiency was combined with 2 other deficiencies in 12% of women and with the 3 other deficiencies in 1 woman (Table 5).

Markers of inflammation

Markers of inflammation were high in most of the women studied. The erythrocyte sedimentation rate increases with gestational age and is known to increase in anemia (34). Only 1% of these anemic women had an erythrocyte sedimentation rate <50 mm/h; 32% had rates >50 and <100 mm/h, 67% had rates >100 and <150 mm/h, and 4% had rates >150 mm/h (Table 4).

Cutoffs for CRP are well established for pregnancy and values >190 nmol/L are considered elevated (38). The CRP concentration was >190 nmol/L in 35%, >238 nmol/L in 30%, and >476 nmol/L in 20% of the study population (Table 4). Of the anemic but iron-replete women (bone marrow iron 2 or 3, n = 34), 73.5% had a CRP concentration >190 nmol/L, suggesting chronic inflammation associated with (and possibly responsible for) their anemia.

Of the entire sample of anemic women, 19% had neither biochemical deficiencies of iron, vitamin B-12, folate, or vitamin A nor an identifiable parasitic infection at the time of study. More than one-half (54%, 15 of 28) of these women had CRP concentrations >190 nmol/L. Of these 15 women, 9 were HIV positive.

HIV status

Forty-eight percent of the anemic women tested positive for HIV (Table 4). The mean hemoglobin concentration for HIV seropositive participants was 81 g/L, which was significantly lower than the 88 g/L for seronegative participants ($P < 0.001$). The median CRP concentration was significantly higher in the HIV seropositive group than in the HIV seronegative group (167 compared with 29 nmol/L; $P < 0001$). The differences in iron, vitamin B-12, folate, and serum retinol concentrations between HIV seropositive and seronegative women were not statistically significant. Similarly, no significant differences were observed in either bone marrow megaloblastic change or iron content between seropositive and seronegative women for whom bone marrow aspirates were available.

Parasitic infestation

Falciparum malaria was identified in 8% of women. In all cases the malaria was caused by *Plasmodium falciparum*. The mean parasite load was 780 parasites/200 white blood cells (range: 1–4800). Hookworm was present in 6% of the women. A maximum worm load of ++ was found in 2 women only. *Schistosoma haematobium* was identified in the urine of 1 woman.

TABLE 4
Biochemical indexes for all women and for a subsample from whom bone marrow aspirates were taken

Biochemical indexes	Entire sample	Bone marrow subsample
	n (%)	
Serum ferritin	150	88
<30 μg/L	83 (55.3)	43 (48.9)
<12 μg/L	31 (20.7)	16 (18.2)
Serum folate	117	63
<9.1 nmol/L	40 (34.2)	23 (36.5)
≤7.7 nmol/L[1]	25 (21.4)	15 (23.8)
Serum vitamin B-12	149	87
<148 pmol/L	49 (32.9)	35 (40.2)
<52 pmol/L	24 (16.1)	20 (23.0)
Serum retinol	150	88
<0.7 μmol/L	58 (38.7)	43 (48.9)
<0.35 μmol/L	20 (13.3)	6 (6.8)
Erythrocyte sedimentation rate	149	87
<50 mm/h	2 (1.3)	2 (2.3)
50 to <100 mm/h	47 (31.6)	24 (27.6)
100 to <150 mm/h	94 (63.1)	61 (70.1)
>150 mm/h	6 (4.0)	0 (0)
C-reactive protein	147	85
>190 nmol/L	51 (34.7)	38 (44.7)
>238 nmol/L	44 (29.9)	32 (37.6)
>476 nmol/L	29 (19.7)	25 (29.4)
HIV status	150	88
Positive	72 (48.0)	43 (48.9)

[1] Or red cell folate <317 nmol/L.

TABLE 5
Distribution of micronutrient deficiencies by iron and folate status

Other deficiency	Iron deficient	Not iron deficient
	n (%)	*n (%)*
None	34 (40.9)	— (—)
+ Vitamin A	21 (25.3)	23 (58.9)
+ Vitamin B-12	7 (8.4)	3 (7.7)
+ Folate	10 (12.1)	6 (15.4)
+ Vitamin A + vitamin B-12	5 (6.0)	4 (10.3)
+ Vitamin A + folate	3 (3.6)	1 (2.6)
+ Vitamin B-12 + folate	2 (2.4)	2 (5.1)
+ Vitamin B-12 + folate + vitamin A	1 (1.2)	0 (0.0)
Total	83 (100.0)	39 (100.0)

Hemoglobinopathies

None of the women had sickle cell disease (HbSS, HbSC, or HbS[hyphen]β-thalassemia). However sickle cell trait (HbAS) was found in 15% of the participants. In addition, none of the women had α-thalassemia or β-thalassemia, although homozygous α+ (α-3.7/ α-3.7) was present in 14% and α+ (αα/α-3.7) in 25% of the women. The mean hemoglobin concentrations for the α+ and α+ homozygous women were 8.6 and 82 g/L, respectively, whereas that for the entire sample was 83 g/L; however, these differences were not statistically significant (P = 0.71). Similarly, mean corpuscular volumes for α+ and α+ homozygous women were 81.6 and 80.7 fL, respectively, and that for the entire sample was 84.3 fL. These differences, too, were not statistically significant (P = 0.54).

G6PD deficiency

On the simple methemoglobin reduction screening test for G6PD deficiency, 17% of women tested positive at entry into the study. Mutations were confirmed in 5 of the 88 women for whom DNA was available. These mutations were identified as follows: 202/202; 376/376, 202/+; 376; 202/+; and 376/376, 202/202. For all of these mutations, the women had screened positively with the first test.

DISCUSSION

Nutritional deficiencies

Anemia in pregnant women in developing countries is generally presumed to be primarily the result of iron deficiency. The definition and identification of iron deficiency has been problematic, however, especially in situations in which chronic inflammation is present (13, 25, 39). The gold standard for identifying iron deficiency anemia has been the examination of suitably stained bone marrow aspirates for storage iron as hemosiderin (33, 39). This method is invasive, though, and therefore not suitable for population screening. Serum ferritin has been shown to be a good measurement of storage iron when the cutoff is raised to 30 μg/L (33). Alternatively, a more comprehensive assessment can be achieved by using a mathematical model that includes a combination of biochemical variables (33). In the present study, iron deficiency was diagnosed both by measuring serum ferritin concentrations and serum transferrin receptor concentrations and by examining bone marrow aspirates from a subsample of women.

Of the pregnant women (n = 4646) attending the antenatal clinic at Queen Elizabeth Central Hospital, 57% were anemic (40). The prevalence of iron deficiency in this study was between 44% and 61%, confirming that this was the most common micronutrient deficiency associated with anemia during pregnancy in this population. In ≈60% of the iron-deficient women, however, other micronutrient deficiencies were also identified. Of the micronutrients studied, vitamin A deficiency (serum retinol <0.7 μmol/L) was the second most frequent micronutrient deficiency and was identified in just under 40% of all women studied. However, serum retinol is kept under homeostatic control and is generally not a good indicator of liver stores (41, 42). Dose-response tests would establish the true prevalence of vitamin A deficiency in pregnant women in south Malawi. It is becoming increasingly clear that vitamin A plays an important role in iron metabolism and that vitamin A supplementation, particularly in women with low or borderline serum retinol concentrations, may improve mobilization of iron stores (43, 44). Almost 20% of anemic women in this study were not biochemically deficient in folate, vitamin B-12, or vitamin A. For those in whom bone marrow iron was more than adequate but who had evidence of inflammation, anemia could have resulted from a blockage in the incorporation of iron into heme, which is described in association with inflammation. Vitamin A, which has been described as an antiinflammatory vitamin, may work by counteracting this block. Further work in this area is needed.

Folate deficiency was identified in 21–34% of the women, depending on the criteria used, and was frequently associated with other micronutrient deficiencies. Whether folate deficiency in this population was primarily the result of dietary insufficiency, problems with absorption, or the result of malaria is difficult to establish. Folate deficiency as a consequence of malaria can show seasonal variation (45, 46). This study was carried out during the dry season, when malaria transmission is at its lowest, and the presence of folate deficiency was not related to peripheral parasitemia.

Unlike folate deficiency, vitamin B-12 deficiency is not normally thought to be associated with anemia in pregnancy (18). Low serum vitamin B-12 concentrations, however, were present in 16–33% of the women in this population, depending on the cutoff used. A diagnosis of vitamin B-12 deficiency in this population was supported by the fact that serum concentrations of vitamin B-12 were found to correlate with bone marrow megaloblastic change. Low serum vitamin B-12 concentrations can also occur in folate deficiency, but 80% of the women with vitamin B-12 deficiency did not have deficient folate concentrations.

Serum vitamin B-12 concentrations in pregnancy are reduced, especially toward term, making the diagnosis of true deficiency more difficult when bone marrow aspirates are not available. Measurements of total homocysteine and methyl malonic acid have been proposed as more accurate biochemical measurements for assessing vitamin B-12 deficiency (47), but further work is needed to establish the validity of these measures in pregnancy.

Studies in nonpregnant women in Zimbabwe and pregnant women in India also showed that vitamin B-12 deficiency may be an unrecognized problem (24, 48). The diet of many women in developing countries contains little or no animal protein and is, therefore, effectively vegetarian. In addition, absorption could be inhibited because of undiagnosed bowel pathology.

Parasitic infestation

Established infestation with hookworm (*Ankylostoma duodenale* or *Necator americanus*), *Schistoma mansoni*, and *Trichuris*

trichuria in the intestinal tract and *Schistosoma haematobium* in the bladder can lead to chronic hemorrhage and iron deficiency (28, 49). Few of the women in this study had parasites present, however, and none had a high density of infestation; thus, parasites are unlikely to have contributed significantly to the presence of anemia in this population. In individuals with precarious iron balance, however, relatively small hookworm loads may result in anemia (50, 51).

The role of malaria in pregnancy in Malawi has been examined extensively (52) and it is universal policy to give all pregnant women 2 doses of sulfadoxine-pyrimethamine. This drug is almost always available and most women in this study received this form of prophylaxis, although data were not collected on the number of doses received or on compliance. All women were asymptomatic for malaria at the time of recruitment (ie, they had no reported clinical symptoms and were afebrile). Evidence of malaria infection was tested only by examination of one thick blood film taken at the time of recruitment. In addition, the study was conducted in the season of low malaria transmission. Further studies that encompass all seasons are necessary to assess more carefully the contribution of malaria to anemia in this population.

Hemoglobinopathies

Despite a 15% prevalence of sickle cell trait, none of the women had sickle cell disease. Access to health care is poor in Malawi. This, compounded by the inability to detect cases of sickle cell disease at an early age and to offer adequate treatment and follow-up, means that girls with this condition probably do not reach reproductive age.

Milder forms of α-thalassemia, which can now be detected more easily by using DNA methods, are associated with hypochromic and microcytic anemia and can mimic iron deficiency anemia. (53, 54). In this study, no significant difference in mean corpuscular volume between carriers for α-thalassemia and those with a normal genotype were found. Caution is needed, however, because the number of cases was small and mean corpuscular volume is not a sensitive indicator of iron deficiency in pregnancy (33). In nonpregnant population groups in whom α-thalassemia is common, particularly in childhood, states of α-thalassemia may be confused with iron deficiency anemia.

Chronic inflammation

The possibility that chronic inflammatory disease plays an important etiologic role in the anemia of pregnancy in developing countries has not been examined previously. In the group of women studied here, almost 20% of those with anemia had no demonstrable deficiencies of iron, vitamin B-12, folate, or vitamin A. In more than half of these cases, CRP was significantly raised; thus, the anemia in these women could well have been associated with chronic inflammatory disease. Similarly, in 73% of the iron-replete but anemic women, CRP was raised above normal and failure to mobilize iron stores, as discussed above, could be an important factor associated with anemia in this group (55). Again, without a control group, causality cannot be inferred. Further work in this area is needed. In particular, it would be important to establish the possible underlying infective pathology in women with raised markers of infection. Suggested possibilities are urinary tract infections, sexually transmitted diseases, and tuberculosis. High markers of infection were not limited to HIV-positive women. In this group, 36% (54 of 150) of women had elevated CRP (>190 nmol/L). Of these, 68.5% were HIV positive and 31.5% were HIV negative.

In common with other antenatal populations in this part of Africa, HIV prevalence was high. Among pregnant women coming for an antenatal examination at Queen Elizabeth Central Hospital, the yearly average range is 27.2–32.8% positive sera for HIV (n = 10 540; antenatal screening program, unpublished data, 1998). In this study, positive HIV status was significantly associated with evidence of inflammation. Opportunistic infections and dietary deficiencies in patients with AIDS are associated with anemia (56). An independent effect of HIV infection on hemoglobin concentration not associated with concurrent infection or dietary deficiency was shown previously in this population (27). Thus, it is possible that HIV status may be a major determinant of anemia in pregnancy in Malawi and other sub-Saharan countries even before a clinical diagnosis of AIDS is made.

A recognized limitation of this study is the lack of a nonanemic control group. At the time the study was designed, it was felt that the inclusion of nonanemic women in a study protocol that included bone marrow sampling was unethical. Because of the complexity—and to some extent unexpectedness—of the current findings, this will have to be reconsidered. However, cutoffs for the serum variables used to identify micronutrient deficiency were chosen strictly. The evaluation of the bone marrow aspirates is unique and helped to substantiate many of the serologic findings. Now that cutoffs for nutritional deficiency (eg, iron deficiency for this population) have been better established (33), the necessity of bone marrow aspirates for certain diagnosis can be reexamined and the possibility of enrolling a control group of nonanemic women for serum testing can be considered. An intervention study examining the effect of supplementation with a combination of vitamin A and iron on hemoglobin concentrations, iron status, infection, and vitamin A status in antenatal women is currently ongoing. This may help establish causality, because only an intervention study can provide irrefutable attribution as to the causes of anemia.

Conclusions

Anemia in pregnant women in developing countries is often primarily attributed to iron deficiency. The prevalence of iron deficiency can be easily misinterpreted if inflammatory disease is not taken into account. We found that although iron deficiency can be shown to be associated significantly with anemia, it is not usually an isolated associated deficiency. The roles of deficiencies of vitamins A and B-12 require further clarification. Inflammation is clearly likely to contribute to anemia in pregnancy in developing countries but the exact causes and mechanisms by which this could occur require further elucidation. The role of acute or chronic inflammation as a possible contributing factor to anemia in pregnancy has important implications for the clinical evaluation and treatment of women.

The often-cited reluctance of pregnant women to take their antenatal iron supplements is unlikely to be the only explanation for the disappointing results of many supplementation trials. All too often anemia in pregnancy in developing countries is thought to be a relatively simple problem for which a simple solution must soon become available. Results of this study suggest that iron supplementation alone will not provide the optimal solution.

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DISCUSSION

Dr Fleming: Why do you think these patients have vitamin B-12 deficiency?

Dr van den Broek: It is difficult to say. A paper from Zimbabwe by Savage [Savage D, Gangaidzo I, Lindenbaum J, et al. Vitamin B12 deficiency is the primary cause of megaloblastic anaemia in Zimbabwe. Br J Haematol 1994;86:844–50] starts by saying we are always "taught vitamin B-12 deficiency is not a problem." We have now looked at it. It could well be a problem.

With all these nutritional deficiencies, we are ever more looking toward the problem of what is in the food and what is being absorbed. Although you only need a little vitamin B-12 in the diet, it may not get absorbed. We would like to be able to look at whether it is not getting absorbed. We are currently doing studies to work out the methodology for looking at iron absorption in pregnancy. The diet of most women in our situation is effectively vegan. Only women with very low serum B-12 concentrations (ie, <52 pmol/L) and megaloblastic change in the bone marrow were identified as deficient.

Dr Caulfield: When you say, "hemoglobin at booking," what does booking mean in terms of the time of pregnancy?

Dr van den Broek: Most of our patients book late, around 20–24 wk. In this group of anemic women, 28% were in their second trimester and 69% were already in their third trimester. The anemic women who participated in the etiology study represent everyone who came to the clinic, whether for a first (booking) or a subsequent visit. The prevalence data I presented were screening hemoglobin values obtained at the first visit. Getting patients to come for antenatal care early in pregnancy is culturally very difficult.

Dr Rush: What are the rates of severe anemia in these populations?

Dr van den Broek: There are hardly any data published on the prevalence of severe anemia. Recent data obtained in southern Malawi [van den Broek NR, Rogerson SJ, Mhango CG, et al. Anaemia in pregnancy in southern Malawi: prevalence and risk factors. Br J Obstet Gynaecol 2000;107:445–51] show the prevalence rates of severe anemia (hemoglobin <70 g/L) to be 3.6% in the urban Blantyre antenatal clinic population (n = 4646, year-round survey) and 4.0% in a rural population (n = 2293, year-round survey). If severe anemia is taken as hemoglobin <80g/L, these percentages are 8.5% and 10.5%, respectively. Data on etiologic factors responsible for or associated with anemia are very hard to find in the literature.

Dr Yip: I would like to congratulate you for this study. The reason you have been searching the literature extensively and not finding anything is because nobody has been able even to attempt to try to look at the multiple factors for etiology that contribute to the anemia observed. This is due partly to a conceptual block among people in the field, who get confused when they see a case of severe anemia. We want to attach a single diagnosis to anemia, whether this is HIV or whether it is iron deficiency. We need to get beyond this point and say that a case with a hemoglobin of 40 g/L, or severe anemia, could be due to a combination of several things. When somebody has multiple causal factors, the hemoglobin values show a net result because hemoglobin concentration is a net or single result, which often makes it difficult to detect some biochemical indicators or the clinical indexes being used. For example, some people whom you classified as iron replete by virtue of their bone marrow still have quite a bit of iron. Let us assume that this is due to accumulated iron immobilized by an infection that is curable. Assuming that there are non-HIV infections that are severe enough to drive the hemoglobin down far enough and assuming that the infection is curable with antibiotics or treatment, you might find the bone marrow stain would disappear because the infection was treated and the iron mobilized. You might then find the ferritin concentration will be low enough to fit your definition of iron deficiency. Right now, categorizing that particular situation is very hard for you, but I am surprised that you found ≈25% of patients that you can classify as having pure iron deficiency anemia in your setting. If I had to venture to guess, I would guess probably <10%, but we are obsessed with trying to say that you have only iron deficiency.

It would be a great contribution for us to go one step further and say that much of the anemia we are seeing has more than one factor and it is not always easy to study. Perhaps our intervention program strategies have to go beyond a single approach, which currently has iron only as the cornerstone. Perhaps we need to have a multiple micronutrient push.

Dr van den Broek: That is very true—I take the approach that it is a multifactorial problem, and these data illustrate just how multifactorial (and complicated) it probably is. We used very strict criteria for defining deficiencies based on critically examined cutoff points. The combination of vitamin A and iron may help mobilize the iron as was shown to a certain extent in Indonesia, where a first trial in pregnancy was done [Suharno D, West CE, Muhilal, Karyadi D, Hautvast JG. Supplementation with vitamin A and iron for nutritional anaemia in pregnant women in West Java, Indonesia. Lancet 1993;342:1325–8]. We are currently doing a second trial, which will be concluded in 1999. I am intrigued by the possibility of unidentified inflammation. Why are all these markers of

infection so high? These are healthy women attending routine antenatal care who have no fever, no malaria, and are not all HIV positive. Yet they have findings suggestive of iron blockage, if I may call it that, presumably because of unidentified chronic or acute infection. Maybe we should treat them with antibiotics.

Dr West: This is just a note to Dr Rush's question. We are currently analyzing data from a consecutive series of ≈1200 pregnancies obtained in a population-based way in Nepal where we measured hemoglobin, erythrocyte protoporphyrin, and serum ferritin; did malaria parasite smears; and examined stools for hookworm and other helminths, all to try to partition these causes of anemia. In a large randomized trial with vitamin A or β-carotene, we found that about three-quarters of all severe anemia was due to hookworm. We also found that vitamin A and β-carotene move the hemoglobin curve to the right, but only in the mild stages of anemia (ie, between 80 and 100 g/L) and only in women who did not have hookworm. In other words, the hookworm drowns out the beneficial effect of vitamin A on hemoglobin concentration.

Dr Fleming: Hookworm infection results in micronutrient deficiencies.

Participant: Because you saw the women for the first time relatively late in pregnancy, do you know whether they received their iron and folate supplements and malaria prophylaxis?

Dr van den Broek: In 1995 the only iron tablets that could be obtained in Malawi were very expensive tablets, and these were not available in the antenatal clinic of the government hospital in which we worked. These are the typical problems encountered in the field. I do not think women included in this study received much iron before enrollment, but antimalarial prophylaxis (intermittent presumptive treatment with sulfadoxine-pyrimethamine) is almost always available and they did get that.

Participant: Your move toward trying iron and vitamin A follows very logically. However, you are only looking at one effect by adding iron and vitamin A. Because we are talking about syndromes, which have multiple effects and multiple outcomes, might your next step not be to try some multivitamin and look at more than one outcome?

Dr van den Broek: Yes, our vitamin A and iron trial was designed before I had all the results of this etiology study. Our results do not contradict a multinutrient supplement trial. We hope to set up a new project to try something else once we finish the current vitamin A and iron trial, because I do not think this trial will reduce all the anemia. I have not decided whether a next trial should be a multivitamin intervention and whether it should be targeted at both the mildly anemic and the severely anemic or whether these groups should be targeted separately. Another consideration is whether to try an antibiotic regimen in pregnant women, which may have a good effect on things such as premature labor. The microbiologists worry about resistance. Before designing any new intervention trial, however, it would be good to have more substantive data. Analysis of the currently on-going vitamin A and iron trial should help.

Participant: The interactions between vitamins increase as you have more, and I think this has been one of the single biggest problems we have had. It is hard enough to study a single micronutrient intervention and yet when we have these multiple outcomes and multiple inputs; perhaps with your kind of thinking you will be the one to make the breakthrough.

Dr Rush: I hope we can reach a consensus about the cutoff points for mild, moderate, and severe anemia. The causes of anemia will vary from region to region, and conditions change with time. Further studies like yours are, therefore, more than just interesting—they are necessary. In tropical Africa or areas where malaria is endemic, anemia is the rule. Iron and folic acid deficiencies are major problems. I was surprised you did not refer to folic acid. I assume the severity of malaria increases during pregnancy because of diminished immunity and that megaloblastic anemias are more often due to folic acid than to vitamin B-12 deficiency.

Dr van den Broek: More studies should be done; although I suspect the data presented are representative for the region we work in, they may not be. For example, I have not attempted to look at the effect of malaria in detail. Measuring the hemoglobin reduction caused by peripheral parasitemia is very difficult. Sufficient evidence now exists from some good trials showing that if you treat malaria, or if you give prophylaxis, hemoglobin concentrations are also improved [Menendez C, Todd J, Alonso PL, et al. The effects of iron supplementation during pregnancy, given by traditional birth attendants, on the prevalence of anaemia and malaria. Trans R Soc Trop Med Hyg 1994;88:590–3; Menendez C, Kahigwa E, Hirt R, et al. Randomised placebo-controlled trial of iron supplementation and malaria chemoprophylaxis for prevention of severe anaemia and malaria in Tanzanian infants. Lancet 1997;350: 844–50]. Also, our etiologic study was conducted in the season with low malaria transmission. We hope to be able to repeat the etiology study in all seasons.

I have not said too much about folate and vitamin B-12 because it is controversial. I, too, have been taught that vitamin B-12 is not a problem, but we happened to measure it because the kit (Simultrac Radioassay kit, Becton Dickinson, Nairobi, Kenya) that we used measured both. Low serum values of vitamin B-12 correlated much more strongly with megaloblastic changes in the bone marrow than did folic acid deficiency. Therefore, vitamin B-12 deficiency could be a problem in this population. There are now better ways to distinguish between vitamin B-12 and folate deficiency, for example, by measuring precursors, and we are looking into this currently.

I would also like to comment on your cutoff point for anemia. I am a bit worried about people who ask why we use a certain cutoff point. Given the extent of the problem, it could be argued that it is a matter of shifting the whole hemoglobin curve to the right. If the curve is shifted, you will get fewer people who are severely and moderately anemic. If the focus is only on the severely anemic, what cutoff point are you going to use? Whichever you use, 70 or 80 or 90 g/L, each can probably be shown to have detrimental effects for pregnancy outcomes although more work is needed in this area.

Dr Kahn: The US Agency for International Development supported a study in Kenya ≈10–12 y ago and the vitamin B-12 content of women's milk was extremely low, much lower than anybody expected, and would be considered deficient [Neumann CG, Harrison GG. Onset and evolution of stunting in infants and children. Examples from the Human Nutrition Collaborative Research Support Program. Kenya and Egypt studies. Eur J Clin Nutr 1994;48(suppl):S90–102]. This was also found in other countries [Casterline JE, Allen LH, Ruel MT. Vitamin B-12 deficiency is very prevalent in lactating Guatemalan women and their infants at three months postpartum. J Nutr 1997;127:1966–72; Allen LH, Rosado JL, Casterline JE, et al. Vitamin B-12 deficiency and malabsorption are highly prevalent in rural Mexican communities. Am J Clin Nutr 1995;62:1013–9]. Human milk is a good indicator for other micronutrients for which you require certain dietary intake. That is something you may want to

pursue—lactating women after pregnancy—which may help to get a handle on vitamin B-12 .

Dr Bothwell: On the interrelationship with vitamin A and iron, why are you not giving them together? A paper from Layrisse's group in Venezuela [Garcia-Casal MN, Layrisse M, Solano L, et al. Vitamin A and beta-carotene can improve nonheme iron absorption from rice, wheat and corn by humans. J Nutr 1998;128:646–50] showed that vitamin A affects the absorption of iron, increases the absorption presumably by binding phosphates, polyphenols, and phytates, and I wonder what other associations there are.

Dr van den Broek: The interrelationship between vitamin A and iron seems a complex one and we continue to study it as part of the ongoing vitamin A and iron intervention trial. Work in Latin America, particularly in children, has shown that vitamin A mobilizes the possibly blocked iron in bone marrow; they observed first a decrease and then an increase of ferritin concentrations. We are also studying the methodology needed to measure iron absorption in pregnancy by using stable isotopes. This may enable us then to measure absorption of iron in women with and without vitamin A supplements in the future.