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ICCNA PROGRESS REPORT AND WORK PLAN

IRON DEFICIENCY SUPPORT PROGRAM

AID COOPERATIVE AGREEMENT DAN-5115-A-00-~~7908~~-00
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1990 - 1991

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ICCNA PROGRESS REPORT

IRON DEFICIENCY SUPPORT PROGRAM

AID COOPERATIVE AGREEMENT DAN-5115-A-00-7908-00

September 1990 - August 1991

The following report summarizes activities performed under the AID Cooperative Agreement from September 1, 1990 to August 31, 1991. The overall objective of this program is to assist developing countries in their efforts to identify and implement effective strategies to combat iron deficiency anemia. The work is supported by a variety of intramural activities at the International Center for the Control of Nutritional Anemia (ICCNA) designed to develop newer techniques for assessing iron status and novel methods for delivering iron to populations at risk of nutritional anemia.

I. PUBLICATIONS

1. **Cook JD, Dassenko SA, Whittaker P. Calcium supplementation: Effect on iron absorption. Am J Clin Nutr 1991;53:106-111.**

Several studies have suggested that calcium has an inhibiting effect on the absorption of iron either as a supplement or contained in the diet. Because calcium rich foods and calcium supplements in women of the child-bearing age are promoted to slow the development of osteoporosis, the present study was undertaken to examine the effect of calcium supplementation on

iron absorption. This topic may be important in certain developing countries where combined supplementation of iron and calcium during pregnancy is under consideration. In the present study, radioisotopic measurements of iron absorption were performed in 61 normal volunteers to assess the potential inhibiting effect of calcium on iron absorption. When taken without food, calcium carbonate did not influence the absorption of ferrous sulfate with doses of either 300 mg calcium and 37 mg iron or 600 mg calcium and 18 mg of iron. However, calcium citrate and calcium phosphate both reduced absorption by approximately 50% at the higher molar ratio of calcium:iron. More importantly, all three calcium supplements inhibited the absorption of an iron supplement when taken with food. The absorption of dietary nonheme iron was also inhibited by all three supplements, the inhibition being more pronounced with a meal of high iron availability and low calcium content than with a meal of low iron availability and high calcium content. This study demonstrates that the regular use of calcium supplements significantly inhibits the absorption of iron whether given as a supplement or contained in the diet and may make it more difficult for women in the child-bearing age to absorb the required amounts of iron from their diet.

2. **Hurrell RF, Cook JD. Strategies for iron fortification of foods. Trends Food Sci Tech 1991;1:56-61.**

One of the most important obstacles in developing more effective means of supplying additional iron by food fortification relates to the selection of a suitable food vehicle and a satisfactory fortification iron compound. The objective of this review was to summarize existing information on the relative bioavailability and cost of existing compounds suitable for food fortification. The main factors governing the choice of iron compounds were identified as organoleptic problems, bioavailability, cost, and safety. The organoleptic problems arising with specific food vehicles were discussed with emphasis on the fortification of infant cereals. The major iron compounds were also ranked in relation to their relative bioavailability based on studies both in rat and man and the relative cost of these compounds were ranked on a scale of 1-5. The characteristics of individual iron compounds were reviewed with emphasis on NaFeEDTA, a promising new approach to delivering bioavailable iron. The potential risks of iron fortification were discussed including the possible risk of infection, chronic iron overload, impaired trace metal absorption, and the risk of cancer.

3. **Cook JD, Skikne BS. New approaches to the assessment of iron nutriture. In: Hercberg S, Galan P, Dupin H, eds. Recent Knowledge on Iron and Folate Deficiencies in the World. Paris:INSERM, 1990;129-136.**

This report summarizes the existing technology for assessing iron status in population studies. Each of the large variety of available laboratory iron parameters were reviewed in regard to their advantages and disadvantages. The value of serum ferritin measurements was emphasized because of its ability to identify iron deficiency unequivocally in an anemic individual. The potential impact of serum transferrin receptor measurements to detect iron deficient erythropoiesis in population studies was emphasized. Finally, analytical methods for evaluating epidemiologic surveys were discussed with emphasis on using a combination of laboratory values to estimate quantitatively the level of body iron in each surveyed individual.

4. **Shih YJ, Baynes RD, Hudson BG, Flowers CH, Skikne BS, Cook JD. Serum transferrin receptor is a truncated form of tissue receptor. J Biol Chem 1990;265:19077-19081.**

One of the major advances in recent years in detecting early tissue iron deficiency is the measurement of serum transferrin receptor. Although immunoassays have been developed to measure this key iron-related protein, there is little information about the biochemical nature of the circulating material. It was of interest to determine whether the circulating

receptor is biochemically similar in patients with iron deficiency and those with enhanced erythropoiesis. If the biochemical form of circulating receptor is different in these two conditions, it may be possible to develop immunoassays that are specific for one or either condition and therefore distinguish in developing countries between elevations due to iron deficiency and those due to the hemolysis associated with malaria or certain hemoglobinopathies such as sickle cell anemia. In this study, transferrin receptor was purified from human serum using recently developed monoclonal antibodies and then characterized biochemically. It was shown that serum receptor is distinctly smaller than intact placental receptor and actually consists of a truncated monomeric fragment lacking amino acid residues 1-19 of intact placental receptor. The circulating material therefore consists of the large extracellular domain but lacks the cytoplasmic and transmembrane portions. Clinical studies demonstrated that the same truncated form circulates both in patients with iron deficiency and those with enhanced erythropoiesis and, as a result, it is not possible to develop assays that are selective for these two causes of an elevation in circulating receptor levels.

5. **Hurrell RF, Berrocal R, Lynch SR, Dassenko SA, Cook JD. The influence of bovine milk proteins on iron absorption in man. In: Hercberg S, Galan P, Dupin H, eds. Recent Knowledge on Iron and Folate Deficiencies in the World. Paris:INSERM 1990;265-273.**

This study was undertaken to examine the reasons why iron absorption from infant formulas is much lower than from human milk. The inhibitory nature of bovine milk proteins was evaluated using radioisotopic measurements in human subjects. The inhibition by whey and casein was significantly greater than animal protein sources such as bovine serum albumin and beef muscle but somewhat less than soy protein isolate. It was shown that prior enzymatic hydrolysis of casein and whey increased iron absorption by about 2-fold. Iron absorption from formula containing whey protein that had been treated to remove inorganic calcium and phosphorous was increased nearly 3-fold.

6. **Hurrell RF, Reddy MB, Dassenko SA, Shepherd D, Cook JD. Ferrous fumarate fortification of a chocolate drink powder. Br J Nutr (In press).**

There is a mounting interest in methods for delivering fortification iron to school-aged children because of the lasting deficits in intellectual performance and learning capacity that are associated with iron deficiency in this age group. In this study, the usefulness of ferrous fumarate as an iron fortificant for an experimental chocolate drink powder used primarily by

children and adolescents was evaluated. Fortification of this product has been difficult because of organoleptic problems when ferrous sulfate or other highly soluble iron compounds are used. Ferrous fumarate was found to be organoleptically acceptable when the chocolate drink powder was reconstituted with milk or water that was heated to <80°C although higher temperatures were associated with unacceptable color changes. When added during the manufacture of chocolate drink powder, absorption of ferrous fumarate was significantly higher than ferrous sulfate and dramatically higher than ferric pyrophosphate. Ferrous fumarate was identified as a useful source for fortifying a chocolate drink powder if constituted with milk or water below the boiling point.

7. **Carriaga MT, Skikne BS, Finley B, Cutler B, Cook JD. Serum transferrin receptor for the detection of iron deficiency in pregnancy. Am J Clin Nutr (In press).**

This study was undertaken to examine the value of serum transferrin receptor measurements as an index of iron deficiency during pregnancy. Iron nutriture during pregnancy has been of particular concern in recent years because of convincing evidence that iron deficiency anemia is associated with an increased frequency of low birth weight, premature delivery, and fetal mortality. The detection of iron deficiency during pregnancy has been a vexing problem because the majority of existing laboratory iron parameters are influenced by gestation *per se*. For example,

physiological alterations in plasma volume and red cell mass during pregnancy diminish the reliability of hemoglobin and hematocrit determinations and other parameters such as the serum ferritin appear to be altered in pregnancy independently of the influence of iron status. To assess the reliability of serum receptor measurements for identifying iron deficiency in pregnancy, assays were performed together with a battery of iron related measurements in 176 women in third trimester pregnancy who were attending a university prenatal clinic. Contrary to prior reports, the mean receptor level in pregnant women did not differ significantly from non-pregnant controls. It was also shown that elevations in serum receptor above the upper limit of normal occurred only in women with depleted iron stores. Although the prevalence of iron deficiency was low in this study, 11 out of 13 women with unequivocal iron deficiency anemia had increased serum receptor levels. These findings indicate that serum receptor is not influenced by pregnancy *per se* and is a highly sensitive index of iron deficiency during gestation.

8. **Ferguson BJ, Skikne BS, Simpson KM, Baynes RD, Cook JD. Serum transferrin receptor distinguishes the anemia of chronic disease from iron deficiency anemia. J Lab Clin Med (In press).**

One of the most important obstacles in assessing iron nutriture in developing countries is the inability to distinguish anemia due to iron deficiency and that caused by chronic infection or inflammation. The present

study was undertaken to evaluate the ability of serum transferrin receptor measurements to make this distinction. As in prior studies, the mean transferrin receptor level was shown to be significantly elevated in patients with iron deficiency anemia (13.9 mg/L) as compared to normal controls (5.4 mg/L). In 20 patients with acute infection including 5 with hepatitis, the mean receptor level was within the normal range in all instances. The receptor level was also normal in 9 of 11 anemic patients with chronic liver disease. The most important finding was that in 41 patients with the anemia of chronic disease, receptor levels remained within the normal range in over 90% and only minimal elevations were observed in the remainder. It was therefore concluded that unlike serum ferritin levels which are disproportionately increased relative to iron stores in patients with chronic infection or liver disease, the serum transferrin receptor level is not influenced by these disorders. This key observation greatly enhances the utility of serum receptor measurements for the assessment of iron status in populations where chronic infection and inflammation is common.

II. PRESENTATIONS

The following activities were performed to increase awareness that iron deficiency is an important nutritional problem and to present recent advances at ICCNA such as newer intervention strategies and newer methods for evaluating iron status in prevalence surveys.

1. Dr. Cook served as an *ad hoc* expert for the Life Sciences Research Office, FASEB in October 1990 in Washington DC to develop guidelines for the assessment and management of iron deficiency in women of childbearing age. This session concluded studies that were initiated in September 1988. A monograph of the findings, published in February 1991, covered several important aspects relating to iron deficiency in women of the childbearing age with emphasis on optimal methods for screening for anemia and identifying its cause and guidelines for the treatment and follow-up of uncomplicated iron deficiency in pregnancy. Measurement of the hemoglobin concentration was identified as the optimal screening method and serum ferritin as the best overall test for etiological diagnosis in anemic women. An important conclusion in this study was the recommendation that because of the increased likelihood of iron deficiency in pregnancy, oral iron supplements containing 30 mg of ferrous iron should be given to all pregnant women during the second and third trimesters.

2. Drs. Cook, Skikne, Shih and Baynes attended the joint meeting of the International Society for Hematology and American Society for Hematology in Boston, Massachusetts, November 28 - December 4, 1990. The following eight presentations relating to work at ICCNA were presented.

   A. Cook JD, Dassenko SA, Lynch SR. Role of nonheme iron availability in iron balance.

This paper describes a method for evaluating food iron availability which incorporates the influence of both dietary enhancers and inhibitors of iron absorption.

B. Cook JD, Dassenko SA, Whittaker P. Effect of calcium supplementation on absorption of iron supplements and dietary nonheme iron.

This report describes the inhibiting effect of calcium supplements on the absorption of ferrous sulfate and dietary nonheme iron.

C. Skikne BS, Carriaga MT, Finley B, Cutler B, Cook JD. Serum transferrin receptor and the assessment of iron status during pregnancy.

This report describes the use of serum receptor levels to detect iron deficiency anemia in pregnant women.

D. Skikne BS, Ferguson BJ, Simpson K, Baynes RD, Cook JD. Serum transferrin receptor distinguishes anemia of chronic disease from iron deficiency.

This report describes the use of serum transferrin receptor levels in distinguishing iron deficiency anemia from the anemia of chronic disease.

E. Shih YJ, Baynes RD, Hudson BG, Flowers CH, Skikne BS, Cook JD. Serum transferrin receptor is a truncated form of tissue receptor.

This study demonstrates that serum receptor is a polypeptide chain resulting from truncation in the extracellular domain of tissue receptor and therefore lacks the cytoplasmic and transmembrane portions of intact receptor.

F. Baynes RD, Shih YJ, Cook JD. Site of production of soluble transferrin receptor by K562 cells.

This report describes the use of a cell culture to define the source and nature of transferrin receptor released from the cells.

G. Baynes RD, Zhuang W, Cook JD. The effect of iron storage status on the kinetics of reticuloendothelial (RE) iron release.

This study demonstrates that neither the iron status of the reticuloendothelial system nor body iron status has any influence on fractional iron release from stores.

H. Baynes RD, Shih YJ, Hudson BG, Cook JD. Characterization of immunoreactive transferrin receptor externalized by K562 cells.

This paper indicates that K562 cells provide a useful *in vitro* model for examining the production of the soluble and particulate forms of externalized transferrin receptor.

3. Dr. Cook attended a workshop at Nestlé in Vevey, Switzerland, January 7-9, 1991 for the purpose of reviewing ongoing collaborative studies of food iron availability and to develop work plans for further studies. The current emphasis of this work is in defining more effective fortification iron compounds for infant cereals.

4. Dr. Cook attended a symposium entitled "Micronutrients and Health II - Trace Metals" held in Norwich, U.K. at the AFRC Institute of Food Research on March 25-26, 1991. Dr. Cook presented a paper on the physiological role of iron and used this format to update the nutrition community in the U.K. on the value of serum transferrin receptor measurements for the assessment of iron status.

5. Dr. Cook attended the FASEB Meetings in Atlanta, Georgia, April 21-24, 1991 and presented a state-of-the-art lecture entitled "Newer Approaches to the Detection of Iron Deficiency" at the iron minisymposium. This presentation emphasized the value of serum transferrin receptor

measurements and the assessment of nutritional iron status and fostered collaborative studies with Dr. Noel Solomons in Guatemala.

6. Dr. Cook attended the 30th Nestlé Workshop on Nutritional Anemia held on June 2-5, 1991 in Toronto, Canada. He presented a paper on optimal screening strategies for detecting nutritional iron deficiency at this important international workshop.

7. Dr. Cook served as a consultant to the First National Nutritional Anemia Workshop sponsored by the Tanzania Food and Nutrition Centre (TFNC) in Dar es Salaam, Tanzania, July 1-5, 1991. The purpose of this conference was to establish a national nutritional anemia consultative group within Tanzania to combat nutritional anemia. This conference is of historic importance in the field of nutritional anemia because it represents the only country, at least in Africa, to have established a national anemia control program. Dr. Cook presented a paper on "Iron Supplementation" and assisted in developing a national strategy to combat nutritional iron deficiency in Tanzania.

8. Drs. Cook and Baynes attended the 10th International Conference on Iron and Iron Proteins held in Oxford, England, July 27-31, 1991. This meeting provided an opportunity to hold a meeting of the Expert Iron Panel of the International Committee for Standardization in Hematology which is assigned the responsibility of standardizing iron related laboratory

measurements. This panel is chaired by Dr. Cook. Discussions on standardization focussed on the preparation of a new ferritin standard and initiating efforts at interlaboratory standardization of serum transferrin receptor measurements.

III. FIELD PROGRAMS

1. GDS Evaluation, Kingston, Jamaica

This activity involved a trial of the gastric delivery system (GDS) for iron supplementation in pregnant women. The objective was to determine the hematological efficacy of GDS as compared to conventional iron when administered for three months to pregnant women. Subjects were randomized into three groups: a control group receiving folic acid only, a conventional treatment group given 65 mg of elemental iron twice daily, and a GDS group given a single capsule containing 50 mg of iron once daily. Of the 376 women admitted to this study, 325 completed 6 weeks of supplementation and 275 completed the entire 12 weeks.

The major activity on this project during the past year involved completion of all laboratory measurements and detailed analysis of the hematologic data. The major obstacle in preparing this work for publication is that at the time of entry into the study, the mean hemoglobin concentration of the control group was significantly higher than in women given either

conventional ferrous sulfate or GDS suggesting a problem with the randomization procedure. An extensive effort was made in Kingston, Jamaica to identify the reasons for the inadequate randomization but no explanation was found. After consultation with various statistical consultants, it was decided to proceed with the analysis of the trial with conventional statistical methods. It was the feeling that if the hemoglobin level was higher in the control group rather than lower, inadequate randomization would not compromise the findings of the study.

A first draft of this report has now been made (Appendix 1). It was initially planned to prepare separate manuscripts, one for the overall design of the study (appendix) and a second for description of the biochemical parameters. However, we are currently planning to develop a single manuscript. The salient finding in the study is that the hematological response obtained with a single GDS capsule is comparable to that observed with two ferrous sulfate tablets daily. Compliance, measured by tablet count, and side-effects, assessed by weekly questionnaires, did not differ in the three study groups but it is highly likely that both these aspects of this study were influenced by the repeated questioning during the 12 week period of supplementation. There was a high dropout rate in the study, but the rate was similar in all three groups and will not compromise the interpretation of the data. It should be noted that the hematologic response to ferrous sulfate was slightly greater at 6 weeks as measured by hemoglobin and slightly better at 12 weeks as measured by serum ferritin

(data not included) but these differences were not statistically significant. Serum transferrin receptor measurements were performed on the total sample and appeared to be the most sensitive index of hematologic response. This report, when completed, will represent the first study in which serum transferrin receptor measurements have been used to assess the results of a field trial in a developing country. It is planned to finalize the manuscript in January 1992 at the time of a planned visit by Dr. Cook to Kingston, Jamaica.

2. Wheat Fortification Project - Grenada

This project is an outgrowth of an anemia survey in this country conducted in 1985-86 which showed that iron deficiency anemia was a serious problem throughout the population and that as many as two-thirds of certain segments of the population had absent iron stores based on serum ferritin measurements. Wheat flour and wheat products are the staple foods in this country. Roughly one-half of the wheat is imported and milled locally. Baking flour, which is used commercially, is fortified with iron to a level of 37 mg/kg flour. Counter flour, on the other hand, is sold for home consumption and is not fortified with iron. Consequently, approximately 45% of the flour consumed is not fortified with iron.

During the past year there has been further refinement of a project proposal that was first drafted following a visit to Grenada by Drs. Cook and

Simmons in the spring of 1990. Several revisions of the proposal were made during the past year and a copy of the proposal, which is now funded, is included in the appendix (Appendix 2). The study will begin in the fall of 1991 when a systems analyst (Keith Bingham) will visit Grenada to assist the local statistician in choosing a sample of 300 families for a household survey defining wheat flour consumption, work output, and hematologic status. Following this baseline survey all counter flour will be fortified with iron and follow-up surveys at 18 and 36 months will be performed to assess the impact on wheat flour consumption, work output, and hematologic status. It is planned to perform the hematologic measurements (hemoglobin, serum ferritin, serum transferrin receptor) at CFNI, Jamaica. ICCNA will serve as a backup for CFNI providing immunologic reagents, standardization, and depending on funding for the project beyond the first year, performing some of the immunoassays.

3. Anemia Control Program - Tanzania

One of the major activities during the past year was to develop an effective interface with efforts in Tanzania to develop a national program for the control of nutritional anemia. The interaction with ICCNA and Tanzania Food and Nutrition Centre (TFNC) was begun in 1987 when Dr. Cook served as a UNICEF consultant in Tanzania. The focus of that evaluation was a possible trial of GDS in rural Tanzania. A study protocol was drafted and submitted at the conclusion of that consultation.

Since that time, anemia has continued to be identified as one of the leading nutritional problems in the country. The leader in efforts to develop an anemia program is Dr. Kavishe, who took over as head of TFNC in 1988. Following the submission of a proposal to develop a national strategy to combat nutritional anemia, World Bank awarded a modest amount of money ($400,000 US) to establish a 5-year program to control nutritional anemia in the country. One of the first projects in this program was to conduct a workshop on nutritional anemia which was held in Dar es Salaam, July 1-5, 1991 (Appendix 3). This workshop was organized by Dr. Kavishe's staff at TFNC and attended by approximately 60 physicians from all major regions within the country. Local representatives from UNICEF, WHO, World Bank, and government ministries attended this conference. External consultants included Dr. Mehari Gebre-Medhin, Department of Pediatrics, University Hospital, Upsala, Sweden, and Dr. Cook, ICCNA. The first three days of this workshop were devoted to a detailed review of knowledge about anemia within the country. The salient features are summarized as follows.

There was complete agreement from all regions represented at this workshop that anemia was a leading cause of mortality in all age groups but particularly in infants, children, and pregnant women. The mean hemoglobin in certain segments was <6 g/dl and in one series of 59 children between 2 and 6 months of age, the mean hemoglobin was 4.7 g/dl. In many hospital surveys, anemia was listed as the third or fourth most common cause of death. Overall, it was generally estimated that anemia as defined

by a hemoglobin <10 g/dl (note the lower criteria as compared to WHO criteria) involved 80% of pregnant women and 45% of children under the age of 5. Those attending the conference were in total agreement that anemia was an urgent and serious problem in the country and required aggressive intervention strategies.

An important finding in this workshop was that the causes of anemia in the country are exceedingly complex. It is believed that a large portion of the population suffers from iron deficiency anemia but there are virtually no laboratory measurements such as serum ferritin or serum iron to confirm this impression. The only exception is a series of 61 infants <6 months of age who had a mean serum ferritin of 29 ng/ml with a mean hemoglobin of 10 g/L suggesting that iron deficiency is a major factor. Obstetricians attending the conference, however, believed that most of the anemia occurring during pregnancy responds to iron therapy. Within hospitals the treatment usually consists of total dose infusion (TDI) of iron using intravenous iron dextran (Imferon) but no reliable studies were presented on the proportion of women given Imferon that responded. A large study in which 1500 pregnant women were given TDI was just concluded using Imferon supplied by the pharmaceutical manufacturer (Fison).

In addition to iron deficiency, there are numerous other causes of anemia. The next most important is malaria. There is little evidence that this parasitic infection has been controlled in recent years despite a national

malarial control program. It was estimated that 60-80% of pregnant women develop active malaria during their first pregnancy and that roughly 90% of infants have had at least one attack of malaria by three months of age. There was no agreement at the conference about the most effective means of controlling malaria but routine prophylaxis is generally not advised. There is also doubt about the optimal antimalarial compound because of increasing resistance to chloroquin. UNICEF representatives believe that impregnated bed nets (IBN) distributed widely in the population is the most effective control measure, especially if coupled with efforts at environmental (mosquito) control. Hookworm is highly prevalent in many sections of the country ranging as high as 40-60% of the population by some estimates and is certainly a major contributor to iron deficiency. Likewise, schistosomiasis occurs widely throughout the population although reliable estimates of prevalence were not available. In certain regions, PEM (protein energy malnutrition) is a major problem and undoubtedly contributes to the anemia. Finally, HIV infection involves 20-25% of the population in certain regions where testing has been performed. Apparently many of the HIV+ individuals are not clinically ill and the extent to which HIV infection contributes to the anemia problem is not clear at this time. Folate deficiency is known to be a significant problem and one investigator suggested that nutritional vitamin B_{12} is a major problem (low serum B_{12} levels were reported in a small group of pregnant women). Hemoglobinopathies such as sickle cell anemia are not believed to be a major factor, sickle cell anemia occurring in approximately 1% of the population. However, it would

not be surprising if asymptomatic hemoglobinopathies including certain thalassemic syndromes are common.

A good deal of discussion at the conference was held on whether the control program should be focussed only on nutritional anemia or on all forms of anemia. The preference of many local physicians to include all forms of anemia probably reflects their inability to distinguish various causes because of limited laboratory facilities. The only laboratory measurement available is the hemoglobin, which is probably very unreliable, in most hospital laboratories and based on inadequate visual techniques (e.g. Tahlquist, Sahli hemoglobinometers) in community areas. Diagnosis of malaria is based on the association of anemia with fever and occasionally on microscopic examination of the peripheral blood. Whether the program to control anemia will focus on all causes or will be directed to the two major causes, iron deficiency and malaria, was not fully resolved during this workshop.

The latter two days of the workshop were devoted to developing a national program to control anemia. A working draft of this proposal is included in the appendix (Appendix 4). In drafting this proposal, the major elements of discussion related to the criteria of anemia. Most physicians felt that the WHO criteria are unreasonably high and that lower levels should be used in Tanzania. Many physicians felt that the hemoglobin measurements in their hospital were highly unreliable. It was recommended that a central

reference lab for hemoglobin measurements be established at TFNC using cyanomethemoglobin standards and that blood samples be distributed as unknowns to regional hospitals at regular intervals in an attempt to develop more reliable estimates of anemia prevalence.

There appears to be few options for iron fortification in the country, mainly because of limited economic resources. However, an interesting pilot project was completed recently suggesting that extended germination and fermentation of certain cereals (maize, sorghum) may improve food iron availability, presumably by reducing the phytate content of the food. Another aspect of discussion was the problem of standardization of hemoglobin measurements. Detailed discussions were held on the optimal approach to controlling parasitic disease with emphasis on hookworm, schistosomiasis, and malaria and specific recommendations were drafted in regard to the optimal drug for each disorder.

A review of possible reasons for reduced efficacy of iron supplementation in Tanzania identified several possible factors. Late booking for prenatal evaluation was considered a major problem. It was also shown that in several regions there is a very limited supply of iron tablets for supplementation. A limited supply of ferrous sulfate is distributed through the essential drug program supported by UNICEF but many of the iron tablets never reach the community level. No reliable data on compliance with iron therapy was available at this conference. A final recommendation

was made that all women receive two iron tablets daily from the time of their first prenatal visit until delivery. This represents a significant change from the existing policy of screening for anemia and providing iron only to those women with severe anemia.

At the conclusion of this conference, a group was appointed to draft the final recommendations which should be available in the early fall of 1991. It was agreed that an annual workshop be held for the next five years to review progress on the various recommended intervention measures. The key elements of the intervention are an effective distribution of ferrous sulfate tablets for pregnant women and the supply of IBN to as many individuals in the population as resources permit.

IV. INTRAMURAL PROGRAM

1. Assessment of Iron Status

Laboratory evidence continues to accumulate that serum transferrin receptor measurements will play a major role in the assessment of iron status in field studies. Work has continued during the past year to define the precision, sensitivity, and specificity of this new laboratory parameter of iron status. A large study of receptor measurements in pregnant women was concluded and is now in press for the American Journal of Clinical Nutrition (see publications). One of the limitations of this study was the fact

that only 13 of 176 women were shown to have iron deficiency anemia. There is, consequently, a need for further observations in a population where iron deficiency anemia is more common. We have therefore analyzed, in the past year, transferrin receptor data performed in connection with the Jamaican trial of GDS. After 12 weeks of therapy, the mean transferrin receptor level in the placebo group was significantly elevated to 10.4 mg/L and was significantly higher than in groups given GDS (8.3 mg/L) or ferrous sulfate (7.3 mg/L). This highly significant difference indicated that the transferrin receptor is a sensitive index of iron deficiency in pregnant women with a high prevalence of iron deficiency anemia.

One potential complication in using the transferrin receptor to assess iron status in developing countries is if iron deficiency coexists with folate deficiency. Because folate deficiency is associated with ineffective erythropoiesis and will therefore elevate receptor levels because of the expansion in the erythroid marrow, the pattern of laboratory abnormalities when iron deficiency and folate deficiency coexist is an important issue. We have approached this problem during the past year in two ways. First, we have undertaken a series of measurements of serum receptor in patients with pernicious anemia, a deficiency in vitamin B_{12} due to malabsorption which results in megaloblastic anemia almost indistinguishable from folic acid deficiency. Although this work is still in progress, preliminary results suggest that the elevation in transferrin receptor is modest even in severe untreated pernicious anemia. Thus, folate deficiency may not produce a

significant number of false positive receptor values when it occurs in association with iron deficiency anemia. The second approach is to measure transferrin receptor levels in patient populations where iron deficiency and folic acid deficiency are common. One such group is patients with sickle cell anemia who often develop folate deficiency due to chronic hemolysis. We have therefore initiated collaborative studies with Dr. Graham Serjeant at the MRC Sickle Cell Anemia Unit in Kingston, Jamaica. Dr. Serjeant has collected several hundred samples from children with sickle cell anemia over the past several years and has forwarded these specimens to our laboratory for transferrin receptor measurements. Once the baseline observations are obtained, we plan to provide folic acid to patients with folate deficiency to observe the influence on serum receptor levels. With these two approaches we hope to better define the diagnostic value of serum receptor measurements when iron and folic acid deficiency coexist.

Another important area where little is known about the value of serum receptor levels is in assessing iron status in infancy. Last year we undertook a collaboration with Dr. Susan Fairweather-Tait at the Agriculture and Food Research Council (AFRC) Institute of Food Research in Norwich, U.K. In the first phase of this study which began in February 1990, children aged 4, 8, 12, and 18 months are sampled for measurements of hemoglobin, MCV, RDW, erythrocyte protoporphyrin, serum ferritin, and serum transferrin receptor. Serum receptor measurements will be performed at

ICCNA. In the second phase of this project, the impact of different iron fortification levels of weaning food on iron status will be examined using serum ferritin and serum transferrin values performed at ICCNA. The children will be randomized at 4 months of age to receive weaning foods that contain either 7.5 mg of iron/100 g or three times this level of fortification. This study should clearly define the role of transferrin receptor measurements in the assessment of iron status in infancy.

2. Iron Fortification Strategies

More effective means of delivering iron to infants and children continues to be the focus of collaborative studies with Nestlé, Vevey, Switzerland. During the past year, radioiron absorption studies in human volunteer subjects proceeded in two general areas. The first related to the effect of **cereal composition and processing** on the iron availability of infant cereals. To examine the possible effect of processing, wheat cereals were prepared by four separate processing methods: roller dried wheat, extruded wheat, wheat baked into a bread roll, and wheat served as a gruel. As in several recent studies in this area, absorption was uniformly <1% making it impossible to identify any significant differences in the effect of processing. Low absorption has also been a problem when comparing the iron availability from different cereals, a project that has been ongoing for the past two to three years. In an attempt to more clearly define possible differences in the inhibiting effect of various cereals, absorption studies

were performed in which four different cereals (maize, wheat, rice, oats) were fed with and without ascorbic acid. Although without ascorbic acid absorption is uniformly low from these cereals, it was reasoned that differences in their inhibiting effect would become more apparent when vitamin C was added to the formula. With all four cereals, the relative enhancing effect of vitamin C was similar and as in studies without added ascorbic acid, we have failed to find major differences in the inhibiting effect of different cereals with the exception of quinoa which has a strong inhibitory influence.

The second general area of iron fortification is to examine **absorption from different forms of fortification iron**. To develop studies in this area, a variety of potential fortification iron compounds were labelled by Lohmann Chemical Co. under contract with Nestlé. In this manner, large supplies of radiolabelled forms of iron were prepared including NaFeEDTA, ferrous fumarate, ferrous sulfate, ferrous succinate, and ferrous saccharate. In one series of absorption measurements, we compared iron absorption from cerosoy, a Nestlé soy based cereal, when fortified with either NaFeEDTA, ferrous fumarate and ferrous sulfate. Absorption was again uniformly <1% except when NaFeEDTA was used, percentage absorption increasing to >2%. Other studies with this strongly inhibitory cereal (cerosoy) failed to give absorptions >1% and thereby impaired our ability to define significant differences in absorption from different forms of fortification iron.

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AID COOPERATIVE AGREEMENT DAN-5115-A-00-7908-00

SEPTEMBER 1991 - AUGUST 1992

The following is a 12-month work plan proposed for the Iron Deficiency Support Project beginning in September 1991. The rationale for the following studies is outlined in detail in the preceding progress report. The work plan is divided into two major sections: Extramural Programs or field studies, and Intramural Programs to develop and refine methods for assessing iron status and providing fortification iron.

I. EXTRAMURAL PROGRAMS (Field Studies)

1. Compliance with Iron Supplementation - Tanzania

Efforts have developed over the last four years to perform a study of compliance with iron supplementation in pregnancy in Tanzania and to include the possible benefit of using a gastric delivery system (GDS) rather than conventional ferrous sulfate. Funds for such a study have now been identified by Eva-Charlotte Ekstrom, a post-graduate student from Cornell University who has recently been assigned as WHO Nutrition Officer at TFNC, Dar es Salaam, Tanzania. Ms. Ekstrom has obtained $15,000 US to support a field study of compliance in a rural area in Tanzania. The

proposed protocol was evaluated in detail during the National Nutritional Anemia Workshop in Tanzania in July, 1991 and a current draft of the project is included in the appendix (Appendix 5). The study will be performed in the Ilula village approximately 45 km from Iringa town. Both villages are located about 6 hours by car from Dar es Salaam. This area was used in the JSNP project conducted by WHO and UNICEF that finished in 1988. Health care is provided in the Ilula village by a Roman Catholic dispensary and a Lutheran dispensary. This area is characterized by high antenatal coverage and is comparable to other rural villages in that there is neither electricity nor telephones. The proposed study is divided into three phases the first of which will include an assessment of the coverage and attendance of pregnant mothers at the Ilula MCH clinic. All women who have given birth during the past three months will be visited and interviewed. In the second phase of the study, a pre-test will be made of a questionnaire developed to assess side-effects with iron supplementation during pregnancy. In this pilot phase, data will be collected for a two month period to define the household characteristics, sociologic aspects of compliance, and morbidity.

In the third and final phase of the study, all women registering at the Ilula MCH clinic with a gestational age of <27 weeks will be recruited for study. Women with a hemoglobin <7 g/L will be excluded. Those with lesser anemia who agree to participate will be randomized to receive either a single tablet of GDS daily or two tablets of ferrous sulfate daily. It is

calculated that between 12 and 14 months are needed before a sufficient number of pregnant women are recruited for the evaluation. Women will be treated for hookworm infestation at the outset of the study by receiving a single dose of albendazol. For each of the recruited subjects, data will be collected at registration and at 2, 4, 8, and 12 weeks following supplementation. This data will include information on morbidity, sociological aspects of compliance, and hematology measurements including a hemoglobin, sickle cell prep, reticulocyte count, and erythrocyte protoporphyrin. A unique feature of this study is that compliance will be monitored by a microprocessor which is placed in the cap of the bottle used to distribute the supplementation tablets. This specialized microprocessor is referred to as a "Medication Event Monitor System" or MEMO. The microprocessor registers each time and date the pill bottle is opened and the data is recovered by computer software developed for use with the microprocessor. The assumption with this monitoring approach is that every time the pill bottle is opened, a tablet is actually taken. To assess whether the women open the pill bottle regularly but throw away the tablet, the iron content of stool samples will be examined periodically and a riboflavin marker will be given in the iron supplement that can be detected in urine samples.

One question with this proposed study is whether Roche is willing to provide the GDS tablets required. It is estimated that 25,000 tablets are required for this proposal. Ms. Ekstrom will travel to Roche in August, 1991 to obtain

their commitment to supply the tablets required for the investigation. In recent telephone conversions, Dr. Ackermann, with the Swiss Pharmaceutical Division, is optimistic that Roche will provide the GDS preparation required for this important study.

2. Wheat Fortification - Grenada, WI

This project has now been initiated and is outlined in detail in the appendix (Appendix 2). Keith Bingham will travel to Grenada in October, 1991 to establish a statistical framework for the initial phase of the study. In January, 1992 Drs. Simmons and Cook will review the proposed sampling design in Kingston, Jamaica and implement plans for the hematologic monitoring of this study. Our laboratory program will maintain a close interface with the wheat fortification project in Grenada as it develops. This will be the first opportunity to evaluate the efficacy of wheat fortification in a field setting.

3. Effect of Coffee Consumption on Iron Status - Guatemala City

ICCNA has agreed to provide the laboratory backup for a proposed collaborative study between the University of California, Davis (Kathryn Dewey) and CeSSIAM, Guatemala (Noel Solomons). The rationale for the proposed study is outlined in the appendix (Appendix 6). The object of this study is to examine the possible adverse effect of coffee consumption on

iron status in children living in Guatemala City. In Guatemala, coffee is given as early as two months of age either with other foods or by bottle or cup. In a recent study to determine the extent of coffee intake and the doses consumed by infants in Guatemala, an informal infant feeding survey of 100 mother/infant pairs recruited through outpatient clinics at three hospitals serving low income families was conducted in Guatemala City. Of 100 Guatemalan infants surveyed, 28 were not given coffee, 41 were given <0.18 g/kg (approximately 3 cups/day) and 31 were given equal or greater quantities. The age at which coffee was introduced was between 2 and 24 months.

To determine the possible association between coffee consumption and iron status in Guatemalan infants, it is planned to survey 200 mothers of infants between the ages of 6 and 24 months. Blood samples will be collected by venipuncture for the measurement of hematocrit, erythrocyte protoporphyrin and plasma ferritin. In addition, serum transferrin receptor measurements will be performed at ICCNA and possibly plasma ferritin measurements as well. Preliminary samples have already been collected from 43 subjects and will be forwarded to ICCNA within the next two months.

Following this baseline study, a randomized intervention trial will be initiated in infants 12 to 18 months of age who have been given coffee for at least two months. Subjects will be recruited from pediatric hospital clinics serving low income families. In the control group, mothers will continue to feed their

infants the usual foods including coffee while in the intervention group, mothers will be asked to discontinue giving coffee for four months. A substitute consisting of an instant coffee colored, fruit flavored powder will be provided as a replacement.

The full details of this study are outlined in the appendix. An important component will be the assessment of iron status in the two groups employing measurements of iron status and including transferrin receptor levels. This study provides important baseline data for serum receptor measurements in a developing country and will further define the sensitivity and specificity of serum receptor measurements in the assessment of intervention field trials. It is planned to recruit two to three subjects weekly between March 1992 and February 1993. The laboratory analysis will run concurrently.

II. INTRAMURAL PROGRAMS

1. Assessment of Iron Status

One of the exciting new possibilities for assessing iron status is the use of **filter paper technology**. It is planned to undertake an intensive laboratory program to define the potential utility of this technology for iron measurements. This area is an outgrowth of programs that have been in operation for several years involving screening newborn infants for

phenylketonuria and other metabolic birth defects. Blood obtained by capillary sampling is allowed to drop onto a specially designed filter paper. After the sample has dried, a circle is punched from the central portion of the blood spot and placed in a measured volume of diluent. The blood is eluted from the filter paper during the next one to two hours. Samples are then obtained from the diluent for measurement of various laboratory measurements including enzyme-linked immunoassays. This technology has been used successfully not only for the newborn screening of inherited metabolic defects but also in screening for subclinical iodine deficiency by measurements of thyroid stimulating hormone (TSH).

There are several phases in the development of filter paper technology for iron related measurements. We plan to use blood eluted from the filter paper for simultaneous measurements of hemoglobin concentration, whole blood ferritin and whole blood transferrin receptor levels. It will first be necessary to establish, both in normal and various disease states, the correspondence between whole blood ferritin and serum ferritin levels. Preliminary studies in our laboratory in normal individuals have shown that whole blood ferritin levels are virtually identical to serum ferritin levels when corrected on the basis of the hematocrit. It is, however, uncertain whether serum transferrin receptor measurements will correlate directly with whole blood transferrin receptor measurements, particularly in hematologic disorders associated with hemolysis and an elevated reticulocyte count.

Therefore more extensive studies will be needed to define the correspondence between serum and whole blood transferrin receptor levels.

In regard to the filter paper technology, labelled samples of blood will be distributed on the filter paper to monitor the recovery with various elution conditions. Several dilutions will be used in an attempt to employ a single eluate for measurements of hemoglobin, whole blood ferritin, and whole blood transferrin receptor.

The potential of this filter paper technology for assessing iron status in developing countries is enormous. Blood could be obtained by capillary sampling, placed on a filter paper, and transported to a central laboratory through the mail. If our laboratory studies with this approach are successful, we will undertake collaborations with Dr. Van Meus in the Kilimanjaro region of Tanzania who has agreed to send us whole blood and filter paper samples obtained from infants in this region. If a close correspondence between conventional whole blood and filter paper measurements of ferritin receptor can be established, the approach can be widely implemented in developing countries for the assessment of iron nutriture.

2. Development of Fortification Programs

Efforts to develop more effective delivery methods for iron fortification will continue in collaboration with Nestlé in Vevey, Switzerland. The first area of proposed evaluation will be to define a form of iron to fortify a chocolate milk drink which is not adversely affected when boiling water or milk is used to prepare the product. Ferrous fumarate proved to be a satisfactory fortificant for a chocolate milk powder except when boiling water or milk was used. Preliminary organoleptic studies have indicated that ferrous saccharate and ferrous succinate are both suitable compounds for the fortification of a chocolate powder. We have prepared radiolabelled forms of these iron fortificants and will undertake radioisotopic studies in human subjects to compare absorption of these forms of iron with ferrous sulfate. As in prior studies, the relative availability of these iron compounds will be compared when added before and following the commercial preparation of this chocolate powder.

A secondary activity will relate to the **rice fortification project** now underway at Rutgers University. In developing a synthetic rice granule containing the iron fortification compound, it has been shown that EDTA and iron pyrophosphate can be mixed with little or no adverse color changes. It is assumed with this approach that in the presence of EDTA a greater proportion of iron pyrophosphate will be released and that the absorption will be facilitated with EDTA. Before undertaking a trial of radiolabelled rice,

we will examine this assumption by determining the effect of adding EDTA to radiolabelled iron pyrophosphate mixed with either a rice or wheat based cereal. If we can demonstrate a facilitating effect of EDTA on the absorption of this highly unavailable form of iron, we will proceed directly to studies of iron absorption from the synthetic rice granule.

We will also continue our efforts to define the optimal use of NaFeEDTA for the fortification of infant cereals. Large quantities of labelled FeEDTA have now been obtained for these investigations. In addition to comparing the relative availability of FeEDTA with other forms of iron, we will perform studies in which the molar ratio of EDTA:iron is progressively reduced. This is based on the assumption that there will be less resistance to the use of EDTA in infant cereals if the quantity of the chelate can be reduced. Although a 1:1 molar ratio is commonly assumed as the optimal amount of EDTA to promote iron absorption, it is possible that smaller quantities of EDTA will be sufficient. Another area of investigation stems from prior observations that EDTA promotes the absorption of iron from inhibitory foods when the inhibiting compound is phytate but not tannin. We will therefore undertake studies to determine whether EDTA confers a higher degree of bioavailability of iron contained in tea as compared with high phytate cereals.

APPENDIX A

Calcium supplementation: effect on iron absorption[1-3]

James D Cook, Sandra A Dassenko, and Paul Whittaker

ABSTRACT The influence of calcium supplements on the absorption of dietary nonheme iron and of iron supplements was evaluated in 61 normal volunteer subjects by use of a double-radioisotope technique. When taken without food, calcium carbonate did not inhibit the absorption of ferrous sulphate with doses of either 300 mg Ca and 37 mg Fe or 600 mg Ca and 18 mg Fe. However, at the latter levels, calcium citrate and calcium phosphate reduced iron absorption significantly by 49% and 62%, respectively. All calcium supplements inhibited absorption of the iron supplement when taken with food. The absorption of dietary nonheme iron was also inhibited by all three supplements. This inhibition was less pronounced from a meal of high iron availability and low calcium content (28%) than from a breakfast meal of low iron availability and high calcium content (55%). These results suggest that taking regular calcium supplements with meals makes it more difficult for women to meet their daily iron requirement. *Am J Clin Nutr* 1991;53:106–11.

KEY WORDS Iron absorption, calcium, supplementation

Introduction

The most common nutritional deficiencies now affecting adult women involve iron and calcium. The use of a daily calcium supplement by women in their childbearing years and beyond is believed to reduce the risk of postmenopausal osteoporosis. Iron supplementation is also widely recommended in adult women, particularly when iron requirements are increased because of pregnancy, lactation, or endurance training. When both iron and calcium supplements are indicated, compliance is improved when these supplements are taken together. However, recent clinical observations suggest that iron absorption from prenatal supplements is adversely affected by other minerals such as calcium (1) and that calcium supplementation reduces the absorption of dietary iron (2).

The present investigation was undertaken to assess more fully the effect of calcium supplements on iron absorption. We studied the effects of calcium carbonate, calcium citrate, and calcium phosphate, all of which are now widely used for calcium supplementation. We examined their effect on the absorption of nonheme food iron and the absorption of ferrous sulphate taken with or without food. Other variables included in our evaluation were the relative amounts of added calcium and iron, the iron status of the subjects, and the bioavailability of iron from the meal.

Subjects and methods

Subjects

The volunteer subjects included 28 women and 33 men between the ages of 20 and 39 y. All were in good health and denied a history of disorders known to influence iron absorption. None of the subjects were anemic, but eight had depleted iron stores as defined by a serum ferritin concentration ≤ 12 μg/L. Written informed consent was obtained from each volunteer before the investigation and all experimental procedures were approved by the Human Subjects Committee at the University of Kansas Medical Center.

Iron-absorption measurements

Iron absorption was measured from four separate test meals in each subject by use of sequential ^{55}Fe and ^{59}Fe labels. All test meals were given between 0700 and 0900 after an overnight fast. Only water was permitted for 3 h after each test meal. The test meals were labeled with either 111 MBq ^{55}Fe or 37 MBq ^{59}Fe (New England Nuclear, North Billerica, MA). On the day preceding the first test, blood was drawn for measurement of packed cell volume, serum ferritin concentration (3), and background radioactivity. The first pair of test meals, tagged separately with either ^{55}Fe or ^{59}Fe, were then fed on the following two mornings. Fourteen days after the second test dose, blood was drawn for measurement of incorporated red cell radioactivity. The second pair of test meals, again labeled with either ^{55}Fe or ^{59}Fe, were fed on the next two mornings, and a final blood sample was obtained 2 wk later to measure the increase in red cell radioactivity. Measurements of blood radioactivity were performed on duplicate 10-mL specimens of whole blood by use of a modification of the method of Eakins and Brown (4). Percentage absorption was calculated on the basis of blood volume estimated from height and weight (5, 6). Red cell incorporation of absorbed radioactivity was assumed to be 80% in all subjects (7).

Preparation of test meals

Labeled iron supplements were prepared by crushing Feosol tablets (Smith, Kline, and French Laboratories, Philadelphia)

[1] From the Division of Hematology, Department of Medicine, Kansas University Medical Center, Kansas City, KS.

[2] Supported by NIH grant DK39246 and AID Cooperative Agreement DAN-5115-A-00-7908-00.

[3] Address reprint requests to JD Cook, Division of Hematology, Kansas University Medical Center, 39th and Rainbow, Kansas City, KS 66103.

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TABLE 1
Effect of calcium carbonate (300 mg Ca) on absorption of ferrous sulphate (37 mg Fe) given with and without a hamburger meal

Subject (sex)	Age	Packed cell volume	Serum ferritin	Iron absorption (%) With food −Ca	Iron absorption (%) With food +Ca	Iron absorption (%) Without food −Ca	Iron absorption (%) Without food +Ca	Absorption ratio (+Ca/−Ca) With food	Absorption ratio (+Ca/−Ca) Without food
	y	%	*μg/L*	*% of dose*					
1 (F)	21	40	11	5.93	6.00	7.77	10.27	1.01	1.32
2 (F)	22	42	12	2.40	1.32	6.55	5.70	0.54	0.87
3 (F)	20	39	13	4.52	2.82	13.32	8.40	0.62	0.63
4 (F)	20	40	16	1.56	1.67	6.00	4.41	1.07	0.73
5 (F)	21	41	23	1.93	1.35	5.06	5.52	0.69	1.09
6 (F)	20	40	28	0.60	0.53	3.92	3.67	0.88	0.93
7 (F)	22	43	33	1.25	0.98	6.58	4.02	1.27	0.61
8 (F)	20	41	35	2.98	1.68	11.72	11.71	0.56	0.99
9 (F)	20	44	37	2.58	1.32	15.02	9.98	0.51	0.66
$\bar{x}$*	21	41	21	2.12	1.61†	7.68	6.50‡	0.76	0.85
−1 SEM			18	1.67	1.30	6.60	5.61	0.68	0.77
+1 SEM			25	2.69	2.01	8.95	7.53	0.85	0.92

* Geometric means except for age and packed cell volume.
† Significantly different from with-food value, without calcium, $P = 0.043$.
‡ Not significantly different from without-food value, without calcium, $P > 0.10$.

to a fine powder and mixing thoroughly with the radioactive label. The powder was then pressed through a 100-mesh screen three times and placed in a #4 gelatin capsule. The variation in radioactivity between different batches of labeled capsules was < ±2%. Commercially available calcium supplements were given separately. Calcium carbonate was given as Caltrate (Lederle Laboratories, America Cyanamid, Pearl River, NY), calcium citrate was given as Citracal (Mission Pharmaceutical Co, San Antonio, TX), and calcium phosphate was given as Posture (Ayerst Laboratories, New York).

When assessing the effect of calcium supplements taken with food, two meals were selected to represent the extremes in iron bioavailability. The enhancing meal consisted of a hamburger patty (94 g) and bun (70 g). The meal contained 1708 kJ, 26 g protein, and 5.1 mg Fe, of which 1.4 mg was heme and 3.7 mg nonheme. When the absorption of the native nonheme iron was measured, this meal was tagged extrinsically by pipetting 0.5 mL 0.1 mol HCl/L containing 0.1 mg Fe as [^{55}Fe]chloride onto the bun (8). The inhibiting meal was a typical breakfast meal containing an egg, muffin, 40% bran flakes, sugar, 2% lowfat milk, and coffee (9). The meal weighed 562 g and contained 2467 kJ, 31 g protein, and 4.7 mg Fe. The meal was labeled extrinsically in the same manner as the enhancing meal.

Statistical analysis

Percentage absorption values and absorption ratios were converted to logarithms for statistical analysis and the results were reconverted as antilogarithms to recover the original units (10). Paired *t* tests were used to compare absorption from meals given with and without a calcium supplement by determining whether the mean log absorption ratio differed significantly from zero. Differences in mean absorption ratios between studies were evaluated by analysis of variance and by Sheffé's test. The *ABSTAT* program was used for the statistical analyses (AndersonBell Corp, Parker, CO).

Results

In our initial study we examined the effect of adding a modest supplement of calcium carbonate (300 mg Ca) to a therapeutic dose of ferrous sulphate (37 mg Fe). When taken with the enhancing meal, iron absorption fell from 2.12% to 1.61%, a modest but significant 24% decrease ($t = 2.36$, $P = 0.043$) (**Table 1**). When the calcium and iron supplements were taken with water, mean absorption fell by only 15%, from 7.7% to 6.5% ($P > 0.10$). Because of the marginal inhibitory influence of calcium with these relative amounts of added calcium and iron, we used a higher level of Ca (600 mg) and a lower level of Fe (18 mg) in subsequent studies.

The next series of studies was performed with the same experimental design. The first of two studies with calcium carbonate was performed in a group of volunteers with low iron reserves as reflected by a mean serum ferritin of 24 μg/L (**Table 2**). The addition of calcium carbonate to the hamburger meal reduced absorption from 13.0% to 7.3%, a significant 44% decrease ($P < 0.001$). As in the first study, no significant effect was seen when iron and calcium supplements were taken with water; a slight increase in iron absorption from 18.0% to 21.5% occurred. This same study was repeated in volunteers with normal iron reserves as indicated by a serum ferritin of 74 μg/L. Calcium carbonate produced a slight 16% decrease in iron absorption from 3.9% to 3.3% when taken with food and even less effect when taken with water. In neither case was the inhibitory effect statistically significant ($P > 0.10$). If the iron absorption ratios with and without calcium carbonate taken with food in subjects with normal and reduced iron stores are pooled, a mean ratio

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TABLE 2
Effect of different calcium supplements (600 mg Ca) on absorption of ferrous sulphate (18 mg Fe) given with or without a hamburger meal

Subject (sex)	Age	Packed cell volume	Serum ferritin	Iron absorption (%) With food −Ca	Iron absorption (%) With food +Ca	Iron absorption (%) Without food −Ca	Iron absorption (%) Without food +Ca	Absorption ratio (+Ca/−Ca) With food	Absorption ratio (+Ca/−Ca) Without food
	y	%	*μg/L*	*% of dose*					
Calcium carbonate (reduced iron stores)									
1 (F)	20	38	6	39.7	17.5	58.5	61.4	0.44	1.04
2 (F)	25	40	12	26.7	14.4	20.4	49.6	0.53	1.68
3 (F)	28	41	13	34.7	20.9	37.1	29.2	0.60	0.78
4 (F)	28	44	23	3.7	3.4	11.8	7.7	0.90	0.65
5 (F)	21	37	26	15.6	11.3	14.6	37.0	0.72	2.53
6 (F)	21	39	26	18.1	6.4	27.7	25.0	0.35	0.90
7 (F)	20	39	29	5.2	4.3	9.9	14.8	0.82	1.48
8 (M)	22	43	73	8.2	3.4	9.9	10.6	0.41	1.07
9 (F)	21	40	75	6.2	3.1	6.6	9.9	0.49	1.49
$\bar{x}$*	23	40	24	13.0	7.3†	18.0	21.5‡	0.56	1.20
−1 SEM				9.7	5.6	14.1	16.7	0.50	1.04
+1 SEM				17.3	9.4	23.0	27.6	0.63	1.38
Calcium carbonate (normal iron stores)									
1 (M)	23	44	19	34.0	25.6	41.1	28.3	0.75	0.68
2 (M)	22	45	25	7.5	8.0	8.9	10.1	1.06	1.13
3 (M)	23	49	75	11.4	6.6	12.5	11.0	0.57	0.88
4 (M)	25	46	80	1.6	3.1	12.7	8.9	1.93	0.69
5 (M)	24	46	95	2.5	1.1	6.4	7.7	0.44	1.20
6 (M)	22	44	96	2.2	0.8	6.2	5.2	0.36	0.83
7 (M)	23	46	98	3.6	4.3	9.9	11.9	1.19	1.21
8 (M)	23	47	104	1.8	1.6	5.0	5.8	0.92	1.16
9 (M)	23	45	115	2.3	2.2	9.2	6.9	0.95	0.74
10 (M)	21	45	171	2.2	2.5	12.4	9.7	1.16	0.78
$\bar{x}$*	23	46	74	3.9	3.3§	10.3	9.4‡	0.84	0.91
−1 SEM				2.8	2.4	8.5	8.1	0.72	0.85
+1 SEM				5.3	4.5	12.3	10.9	0.99	0.98
Calcium citrate									
1 (M)	23	41	28	6.9	3.8	15.7	6.7	0.67	0.42
2 (F)	22	44	33	12.2	10.1	19.5	5.3	0.82	0.27
3 (F)	26	40	46	4.5	5.0	12.9	6.5	1.11	0.50
4 (M)	22	46	54	13.6	2.6	11.4	3.6	0.19	0.31
5 (M)	23	41	56	30.1	20.4	21.9	23.7	0.67	1.08
6 (M)	22	44	64	18.5	11.0	10.0	8.4	0.59	0.83
7 (M)	22	44	164	3.9	2.8	5.9	3.3	0.71	0.55
$\bar{x}$*	23	43	54	10.1	6.1‖	12.9	6.6¶	0.60	0.51
−1 SEM				7.6	4.5	10.9	5.2	0.49	0.43
+1 SEM				13.5	8.2	15.2	8.5	0.74	0.62
Calcium phosphate									
1 (M)	28	43	47	14.9	2.9	19.4	4.9	0.19	0.25
2 (F)	24	39	50	2.8	1.2	15.6	5.3	0.42	0.33
3 (M)	23	46	66	10.1	2.1	24.4	5.4	0.20	0.22
4 (M)	23	46	79	10.1	5.7	29.1	13.1	0.56	0.44
5 (M)	26	47	96	8.1	8.4	23.4	15.3	1.03	0.65
6 (M)	23	43	105	8.7	7.1	12.9	3.9	0.81	0.30
7 (M)	24	43	108	3.8	1.3	4.1	2.6	0.33	0.61
$\bar{x}$*	24	44	75	7.3	3.2**	16.0	6.0††	0.43	0.38
−1 SEM				5.8	2.3	12.5	4.7	0.34	0.32
+1 SEM				9.2	4.3	20.5	7.6	0.55	0.44

* Geometric means except for age and packed cell volume.
†** Significantly different from with-food value, without calcium: † $P < 0.001$, ** $P = 0.015$.
‡ Not significantly different from without-food value, without calcium, $P > 0.10$.
§‖ Not significantly different from with-food value, without calcium: § $P > 0.10$, ‖ $P = 0.058$.
¶†† Significantly different from without-food value, without calcium: ¶ $P = 0.013$, †† $P < 0.001$.

TABLE 3
Comparison of the effect of different calcium supplements' (600 mg Ca) absorption of nonheme iron from enhancing and inhibitory meals

Subject (sex)	Age	Packed cell volume	Serum ferritin	Iron absorption (%): No added calcium	With: Carbonate	With: Citrate	With: Phosphate	Absorption ratio (+Ca/−Ca): Carbonate	Absorption ratio (+Ca/−Ca): Citrate	Absorption ratio (+Ca/−Ca): Phosphate
	y	%	*μg/L*		*% of dose*					
Enhancing meal (with meat)										
1 (F)	22	37	5	33.1	21.0	31.3	21.5	0.63	0.94	0.64
2 (F)	22	37	11	25.8	21.0	45.6	43.8	0.81	1.76	1.69
3 (F)	23	39	14	30.5	31.0	26.1	9.9	1.01	0.86	0.32
4 (F)	22	39	24	36.2	29.0	15.0	21.3	0.80	0.41	0.58
5 (M)	24	44	43	8.6	5.8	6.2	4.9	0.66	0.72	0.56
6 (F)	22	41	54	13.5	7.9	11.0	7.7	0.58	0.81	0.57
7 (M)	24	44	60	12.4	5.1	5.1	3.0	0.40	0.40	0.24
8 (M)	23	46	80	5.3	3.0	6.7	4.4	0.57	1.27	0.83
9 (M)	27	46	184	1.8	1.2	3.7	2.3	0.66	2.00	1.23
10 (M)	26	49	409	14.3	12.1	12.3	6.3	0.84	0.85	0.44
$\bar{x}$*	23	42	42	13.4	9.1†	11.9‡	8.2§	0.68	0.89	0.61
−1 SEM				10.0	6.5	9.2	6.1	0.63	0.75	0.51
+1 SEM				18.1	12.8	15.5	11.1	0.74	1.05	0.74
Inhibiting meal (no meat)										
1 (F)	24	40	12	4.7	4.8	1.9	1.7	1.03	0.40	0.35
2 (F)	22	38	12	1.6	1.1	1.1	0.6	0.70	0.59	0.37
3 (F)	30	39	32	5.0	4.8	1.4	0.6	0.95	0.28	0.12
4 (M)	23	43	56	0.5	0.4	0.2	0.3	0.71	0.32	0.47
5 (M)	25	44	79	0.8	0.6	0.5	0.4	0.75	0.65	0.49
6 (M)	22	48	126	0.7	0.5	0.4	0.5	0.63	0.50	0.72
7 (M)	20	46	127	0.4	0.3	0.6	0.5	0.77	1.71	1.28
8 (M)	39	47	200	0.4	0.1	0.2	0.1	0.13	0.40	0.21
9 (M)	22	43	359	2.9	1.0	0.3	0.6	0.32	0.09	0.19
$\bar{x}$*	25	43	66	1.2	0.7‖	0.5¶	0.4¶	0.58	0.43	0.37
−1 SEM				0.8	0.4	0.4	0.3	0.47	0.33	0.29
+1 SEM				1.7	1.1	0.7	0.6	0.72	0.56	0.47

* Geometric means except for age and packed cell volume.
†§‖¶ Significantly different from no-added-calcium value: † $P = 0.001$; § $P = 0.03$, ‖ $P < 0.05$, ¶ $P < 0.01$.
‡ Not significantly different from no-added-calcium value, $P > 0.10$.

of 0.69 is obtained, which indicates significant inhibition ($P < 0.01$).

The same protocol was used to evaluate the effect of calcium citrate and calcium carbonate on the absorption of ferrous sulphate. Calcium citrate reduced iron absorption from 10.1% to 6.1% when taken with food, but this 40% decrease was not significant ($P = 0.058$) (Table 2). On the other hand, when taken without food, calcium citrate reduced absorption by 49%, from 12.9% to 6.6% ($P < 0.013$). The greatest inhibition occurred with calcium phosphate, which reduced absorption of ferrous sulphate when taken with food by 57%, from 7.3% to 3.2% ($P = 0.015$), and when taken with water by 62%, from 16.0% to 6.0% ($P < 0.001$). Thus, the reduction in iron absorption by calcium phosphate exceeded 50% in both cases.

When the results of these studies are taken together, it is apparent that the effect of calcium supplements on the absorption of an iron supplement depends on whether they are taken with food. Without food, calcium carbonate has no inhibitory effect on the absorption of ferrous sulphate, whereas calcium citrate and calcium phosphate are both inhibitory. All three forms inhibit the absorption of an iron supplement if taken with food, although the degree of inhibition was not statistically significant with calcium citrate. When taken with food, the pooled mean absorption ratio with and without added calcium was 0.53 (±1 SEM, 0.47–0.59), reflecting a highly significant inhibitory effect ($t = 5.26$, $P < 0.0001$).

In the final series of studies, we examined the effect of calcium supplementation on dietary nonheme iron absorption. Mean absorption from the enhancing meal taken without added calcium was 13.4% (Table 3). Iron absorption was reduced significantly by 32% with calcium carbonate ($P < 0.001$) and 39% with calcium phosphate ($P < 0.03$) but by only 11% with calcium citrate ($P > 0.10$). However, when the mean absorption ratios of the three calcium forms were compared by analysis of variance, the differences were not significant ($F = 1.63$, $P = 0.21$). The pooled mean absorption ratio of 0.71 (±1 SEM, 0.65–0.78) indicated a highly significant inhibiting effect of calcium ($t = 3.78$, $P < 0.0001$).

The inhibiting effect of calcium supplements on food iron absorption was more pronounced with a breakfast meal containing several known inhibitors of iron absorption. When fed without calcium, mean absorption from this meal was only 1.2% as compared with 13.4% from the hamburger meal (Table 3). The degree of inhibition was similar for all three calcium supplements: 32% with calcium carbonate ($P < 0.05$), 57% with calcium citrate ($P < 0.01$), and 63% with calcium phosphate ($P < 0.01$). When these results were compared by analysis of variance, however, differences among the three calcium supplements were not significant ($F = 0.9$, $P = 0.41$). The pooled mean absorption ratio was 0.45 (±1 SEM, 0.39–0.52), indicating a highly significant inhibiting effect ($t = 5.79$, $P < 0.0001$). The degree of inhibition with the breakfast meal was also significantly greater than with the hamburger meal ($t = 2.871$, $P = 0.003$). Thus, the inhibiting effect of calcium on nonheme iron absorption is similar with all three supplements and is significantly greater when the meal itself contains poorly available iron (**Fig 1**).

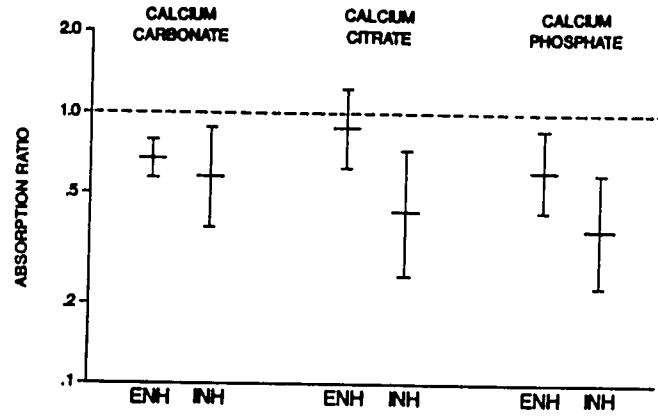

FIG 1. Effect of calcium supplements (600 mg Ca) on nonheme iron absorption from an enhancing (ENH) and inhibiting meal (INH). Mean absorption ratios ± 2 SEM are shown.

Discussion

Despite investigation for more than a half century, the effect of calcium on iron absorption is still unclear. The majority of investigations have been performed on laboratory animals and these findings were recently reviewed (11). There is surprisingly little clinical information, particularly about the effect of the larger amounts of calcium used for supplementation. At the level of calcium commonly used for supplementation, an inhibitory effect was demonstrated by Seligman et al (1), who added different forms of calcium to various prenatal multivitamin and mineral supplements. Iron absorption was determined indirectly in healthy, fasting women from the postabsorptive rise in serum iron, a method that cannot be used to measure food iron absorption. A significant decrease in iron absorption from 9.0% to 6.8% was observed when 300 mg Ca as calcium carbonate was added to 65 mg Fe as ferrous fumarate.

These results differed from our present study, where an insignificant 15% decline in iron absorption occurred when 300 mg Ca as calcium carbonate was given with 37 mg Fe as ferrous sulphate. Moreover, no effect was seen when the amount of calcium was doubled and the iron halved. On the other hand, both calcium citrate and calcium phosphate decreased absorption of ferrous sulphate when given with water. There are several possible explanations for the difference in findings in these two studies. Our iron and calcium supplements were given in separate capsules, whereas in the previous report these minerals were formulated in the same preparation. Their commercial prenatal preparation contained several other minerals and vitamins, some of which may have augmented any inhibitory effect of calcium carbonate. It is also possible that ferrous fumarate, which was used in the prenatal supplement, is more influenced by calcium carbonate than is the ferrous sulphate evaluated in our studies. At neutral pH, ferrous fumarate is far less soluble in water than is ferrous sulphate, although it dissolves rapidly at the more acid pH of gastric juice.

Whereas calcium carbonate failed to inhibit iron absorption when taken with water, we detected no obvious difference in the inhibiting effects of calcium carbonate, calcium citrate, and calcium phosphate when taken with food. A similar inhibiting effect of these supplements was observed with meals of both high and low iron availability and with or without an added ferrous sulphate supplement. The degree of inhibition was similar to that observed when a breakfast meal was fed to postmenopausal women (2). In this study, basal absorption of 6.3% was reduced by 57% when 500 mg Ca as calcium carbonate was added and by 54% when the same amount of calcium was added as hydroxyapatite. This degree of inhibition was almost identical to that observed with our inhibitory meal in the present study; inhibition ranged between 42% and 63%, with a pooled mean of 55%.

An unexpected finding in the present study was that the degree of inhibition in nonheme iron absorption varied with the nature of the meal. The relative inhibition was considerably less with a hamburger meal that promoted nonheme iron absorption, compared with a breakfast meal that contained several inhibitors of iron absorption. There are several possible explanations for the differences in inhibition with these two meals. It is possible that the inhibitory effect of calcium may have been partially offset by the meat in the hamburger meal, which facilitated the absorption of nonheme iron (12). The estimated calcium content of the two meals also differed markedly, 597 mg for the breakfast meal as compared with 141 mg for the hamburger meal. If the inhibitory effect of calcium is proportional to the amount contained in the meal and if dietary and nondietary calcium are comparable in their effect, a greater inhibition would be expected with the breakfast meal. Finally, the ability to quantify reliably an inhibitory effect with the breakfast meal is less because of the low basal absorption of 1.2% as compared with 13.4% for the hamburger meal. The difference in the inhibiting effect of calcium with different meals is important in predicting the long-term effects of regular calcium supplementation on iron balance. Although the inhibiting effect of calcium is greater with a meal that inhibits absorption, iron absorption from our breakfast meal is too low to account for significant quantities of absorbed iron.

Predictions about the potential influence of regular calcium supplementation on iron balance must take into account the specific fraction of dietary iron and the number of meals with which the calcium supplements are consumed. Heme iron is believed to account for approximately one-third of the absorbed dietary iron, and because dietary ligands do not influence heme absorption, any inhibiting effect of calcium supplements is limited to nonheme dietary iron. Recent studies showed that 0.73 mg of nonheme iron is absorbed daily by women with depleted

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iron stores (13). Thus, only 0.1–0.2 mg would be furnished by a breakfast meal containing numerous inhibitors of iron assimilation. If calcium supplements are taken only with a breakfast meal, reduction in absorbed nonheme dietary iron would be only 10–15%/d. A decrease of 20–30% could occur if calcium supplements are ingested with each meal of the day. When calcium carbonate was taken with food in the present study, the inhibitory effect was greater in women with reduced iron stores (Table 2).

Our present observations permit some tentative conclusions about the effect of calcium supplementation on iron balance. If combined iron and calcium supplementation is required, no adverse effects on iron absorption will occur if calcium carbonate is used and the supplements are taken between meals. If both are taken with food, the calcium supplement reduces the absorption of ferrous sulphate by one-third. Calcium supplements also inhibit the absorption of dietary nonheme iron. All forms of calcium share this property, but the degree of inhibition varies with the nature of the meal. The relative inhibition is greater with a breakfast meal of low iron availability (55% inhibition) than with a hamburger meal of high iron availability (28% inhibition). If taken with meals, calcium supplements used regularly may make it more difficult to meet daily iron requirements.

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Strategies for iron fortification of foods

R.F. Hurrell and J.D. Cook

Iron deficiency is a major nutritional problem in the world today. It affects hundreds of millions of people and is especially prevalent in infants, young children and women of child-bearing age[1]. Although lack of iron remains one of the few nutritional deficiencies in affluent societies, iron deficiency anaemia is most common in poor populations where it has serious public health implications. In children, it can retard psychomotor development and mental performance. In pregnant women, it can result in premature delivery as well as foetal and maternal mortality. In addition, iron deficiency can reduce work capacity, impede body temperature regulation, impair immune response and perhaps lead to a higher incidence of infection[2-4].

Iron deficiency can be overcome by increasing iron intake, either by supplementing individuals with medicinal iron, or by fortification of the food supply. Iron supplementation has the advantages of producing rapid changes in iron status and of directing the iron to those segments of the population in greatest need. However, its effectiveness is reduced by the gastrointestinal side effects of oral iron, and the difficulty in maintaining compliance. Consequently, food fortification is generally regarded as the best long-term approach to reducing the incidence of iron deficiency[5]. Once the prevalence of iron deficiency in a population is known, the dietary iron intake measured and the foodstuffs likely to influence iron bioavailability determined, the desired level of iron fortification can be calculated. However, a successful fortification programme still depends on the correct choice of food vehicle and the selection of suitable fortification compounds. The choice of food vehicle was reviewed extensively by Cook and Reusser[5]. Therefore, this review will deal mainly with the criteria involved in the selection of suitable iron fortification compounds.

Food vehicles for iron fortification

The selected food vehicle must be consumed by a high proportion of the population at risk, with little potential for excessive intake. Foodstuffs that contain few inhibitors of iron absorption are preferred and, in developing countries, monitoring is easier if the foodstuff is produced in a limited number of factories.

The most common iron-fortified staple foodstuff is wheat flour, which is subjected to mandatory fortification in the USA, UK and Sweden. The added iron reaches most segments of the population. Rice would be an ideal vehicle in many developing countries. However, until now, iron fortification of rice has not been successful, as the consumer can usually detect and remove the fortified grains which are mixed with normal grains. Coated grains have been commonly used, although the use of fortified grains made from extruded rice flour is also a possibility. Other food vehicles evaluated in developing countries include salt in India, sugar in Guatemala, and various condiments.

Some iron-fortified foods are directed towards the needs of specific population groups. These foods include formulae and cereal weaning foods for infants and young children, breakfast cereals, chocolate drink powders and beverages for older children and adolescents, and special dietetic products. The efficiency of infant formula fortification is well documented[6] and, in the USA, the consumption of iron-fortified infant formulae instead of cow's milk has resulted in a substantial reduction in the prevalence of iron deficiency in infancy[7].

Iron compounds

The major iron compounds used in food fortification can be divided into four groups (see Table 1): those that are water-soluble, those with poor water solubility but which are readily soluble in dilute acids, those that are insoluble in water and poorly soluble in dilute acids, and experimental compounds. The major factors governing the choice of iron compound are organoleptic problems, bioavailability, cost and safety. Ideally, one would like to have a safe, cheap, highly bioavailable iron compound that causes no organoleptic problems. Unfortunately, this is often not possible since the water-soluble compounds that are the most bioavailable cause unacceptable colour and flavour changes in many foods. The more insoluble compounds are organoleptically inert, but are poorly absorbed. The cost of iron compounds may vary about tenfold, with elemental iron and ferrous sulphate being the least expensive; safety is really only of concern for the experimental compounds sodium iron EDTA and bovine blood.

Organoleptic considerations

When selecting an iron compound for food fortification, the first consideration is its influence on the organoleptic properties of the food vehicle. If colour and/or flavour are substantially altered, the food is not likely to be eaten and the iron will not be consumed. Many iron compounds are coloured and cannot be used to fortify lightly coloured foods (e.g. rice grains). In addition, the more soluble iron compounds often react with substances in the food to give coloured products. Infant cereals have been found to turn grey or green on addition of ferrous sulphate[8], and dark blue if bananas

R.F. Hurrell is with Nestec Ltd, Nestlé Research Centre, Vers-chez-les-Blanc, PO Box 44, CH-1000 Lausanne 26, Switzerland. **J.D. Cook** is at the Kansas University Medical Center, Kansas City, KS 66103, USA.

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are present; polyphenolic compounds are thought to be responsible. Similarly, salt fortified with ferrous sulphate or other soluble iron compounds becomes yellow or brown[9].

Off-flavour can result from the metallic taste of soluble iron, particularly in beverages. However, the catalytic effect of iron on fat oxidation in cereals during storage is the major problem. As in the case of product discolouration, it is the water-soluble compounds such as ferrous sulphate that promote fat oxidation and reduce product shelf life. A convenient method to measure the potential of iron fortification compounds to promote fat oxidation in cereals is to measure pentane formation in the headspace of sealed cans containing the iron-fortified product[8]. Pentane is the major hydrocarbon formed on the oxidative degradation of linoleic acid, and its formation correlates with off-flavour formation. Figure 1 shows the rate of pentane formation during storage at 37°C of a precooked whole wheat flour containing various iron salts (at a concentration of 15 mg Fe per 100 g flour). Ferrous sulphate and ferrous gluconate rapidly generated pentane, and were judged unacceptable by a taste panel after four to six weeks of storage. Ferric pyrophosphate and reduced elemental iron generated far less pentane and were still organoleptically acceptable after seven weeks of storage.

Fat oxidation during storage can be effectively prevented by encapsulation of the soluble iron compounds with coatings such as hydrogenated oils or ethyl cellulose[10]. Unfortunately, hot water or milk can remove the coatings, and colour may still develop in susceptible products such as infant cereals. The bioavailability of ferrous sulphate has been little affected by most coatings tested, although any new material should be carefully checked.

Table 1. Major iron compounds used in food fortification[a]

Iron compound	Relative bioavailability[b] Rat	Man	Relative cost[c]
Water-soluble			
Ferrous sulphate	100	100	1
Ferrous gluconate	97	89	5
Ferric ammonium citrate	107	NA	5
Ferrous ammonium sulphate	99	NA	2
Poorly water-soluble			
Ferrous succinate	119	92	4
Ferrous fumarate	95	100	1
Ferric saccharate	92	75	4
Water-insoluble			
Ferric orthophosphate	6–46	31	4
Ferric pyrophosphate	45	39	4
Elemental iron	8–76	13–90	1
Experimental			
Sodium iron EDTA	Depends on the food vehicle		10
Bovine haemoglobin	NA	NA	NA

[a] Adapted from Refs 10 and 12
[b] Bioavailability is expressed as a percentage relative to that of $FeSO_4 \cdot 7H_2O$
[c] Relative to the cost of $FeSO_4 \cdot 7H_2O$
NA, Data not available

Bioavailability

Once it has been ascertained which iron compounds are acceptable organoleptically, the next step is to check their bioavailability. The absorption of fortification iron depends primarily on its solubility in the gastric juice. Water-soluble compounds such as ferrous sulphate dissolve instantaneously in the gastric juice, whereas the more insoluble compounds such as elemental iron rarely dissolve completely. Once dissolved, fortification iron enters the common pool, where its absorption (like that of all pool iron) depends on the content of enhancing or inhibitory ligands in the meal and on the iron status of the subject. Phytate and polyphenols, for example, and a satisfactory iron status will diminish absorption; ascorbate or a low iron status will enhance absorption.

Because iron status and various food components may markedly affect iron absorption, the absorption of a single iron compound can vary from less than 1% to almost 100%. Therefore, when comparing different iron compounds, bioavailability must be measured relative to a standard compound. The standard is usually ferrous sulphate, to which has been designated a relative bioavailability (RBV) of 100. It has recently been demonstrated that the haemoglobin repletion test in rats and the measurement of dialysable iron *in vitro* are good predictors of iron bioavailability in man[11]. The RBV of many commercial iron compounds is already well known (Table 1). New compounds can be evaluated by

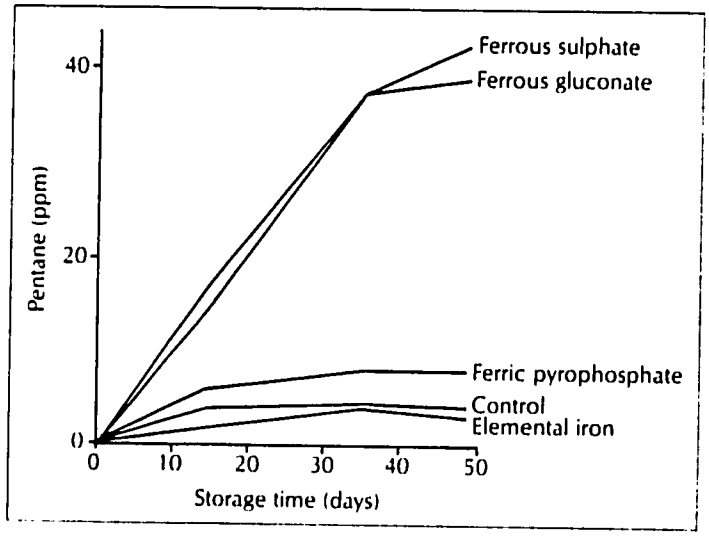

Fig. 1
Pentane formation in stored wheat flour fortified with different iron compounds (adapted from Ref. 8).

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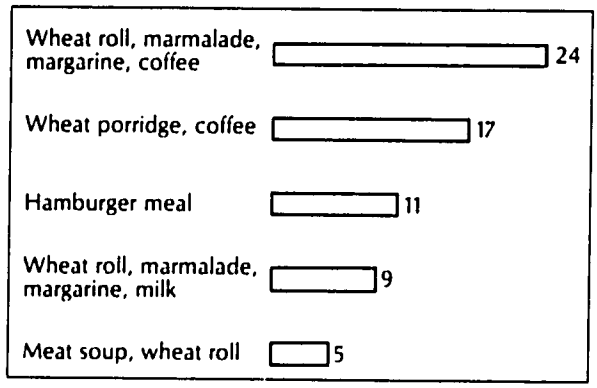

Fig. 2

Bioavailability in human subjects of carbonyl iron added to wheat flour and consumed with different meals. Bioavailability is expressed as a percentage relative to that of $FeSO_4 \cdot 7H_2O$ (adapted from Ref. 13).

animal or *in vitro* assays, but absorption studies on humans, using radioisotopes, are ultimately necessary if the impact of iron fortification has to be determined.

Characteristics of individual iron compounds

Compounds that are freely soluble in water, such as ferrous sulphate and ferrous gluconate, have a high RBV in both rats and humans, but often cause colour and flavour problems in foodstuffs. Ferrous sulphate is the cheapest and, for most applications, there seems little reason to use the other more expensive iron compounds in preference to ferrous sulphate. It is widely used to fortify infant formulae.

Ferrous succinate and ferrous fumarate are compounds with poor water solubility that are soluble in dilute acids. They do not promote fat oxidation during the storage of infant cereals, yet they are readily soluble in the gastric juice and have an RBV in rat and man similar to that of ferrous sulphate[12]. Although they are useful for infant cereal fortification, we have found them to promote fat oxidation in stored wheat flour. This is presumably because of the higher moisture content of wheat flour.

Compounds such as ferric orthophosphate, ferric pyrophosphate and various forms of elemental iron are water insoluble and are widely used to fortify food products, especially cereals. They cause few organoleptic problems, but bioavailability is mediocre to low and often variable (Table 1). The absorption of ferric pyrophosphate has been reported to be 40–50% of that of ferrous sulphate in both rat and man, whereas that of ferric orthophosphate is usually lower and more variable due to the effects of particle size differences on its solubility in the gastric juice. The RBV of elemental iron also varies considerably, both with the type of elemental iron used and its particle size. Carbonyl iron and electrolytic iron are the commercial compounds of smallest particle size and highest solubility in dilute acids, and they have been given RBV values in rats from 30 to 50 (Refs 8 and 12). Reduced iron is generally less well absorbed.

Very few absorption studies on humans have used elemental iron, due to the difficulty in obtaining radioactive compounds identical to the commercial ones. However, a recent study in humans, using radioactively labelled carbonyl iron[13], has raised some doubts on the usefulness of elemental iron as a food additive. Wheat flour fortified with carbonyl iron was fed with different meals, either as a bread roll or porridge. The measured RBV of the carbonyl iron was universally low and, unexpectedly, varied from 5 to 20, depending on the meal composition (Fig. 2). This presumably reflects differing dissolution rates of carbonyl iron in the gastric juice.

At the present time, there appears to be no alternative to the use of an insoluble iron compound for the

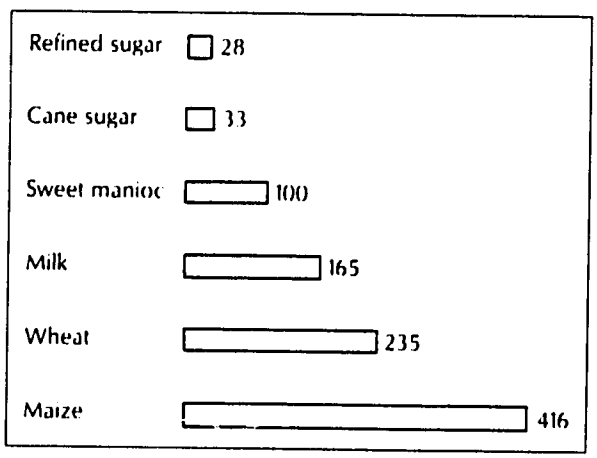

Fig. 3

Bioavailability in human subjects of sodium iron EDTA added to different foods. Bioavailability is expressed as a percentage relative to that of $FeSO_4 \cdot 7H_2O$ (adapted from Ref. 15).

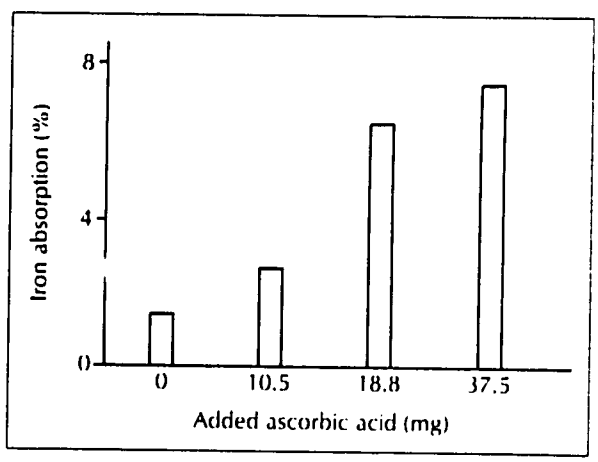

Fig. 4

Influence of ascorbic acid on iron absorption by adult women from ferric pyrophosphate-fortified infant cereals (adapted from Ref. 16).

fortification of salt or wheat flour and other cereals, which are stored for long periods. We would recommend that the chosen compound have an RBV of at least 50 in the haemoglobin repletion assay in rats, that individual batches be rigorously controlled, that an absorption enhancer such as ascorbic acid be added when possible, and that human studies be conducted to determine the amount of iron absorbed. It may be that raising the level of fortification is all that is needed to ensure that enough iron is absorbed. Ultimately, the efficacy of fortification should be validated by monitoring iron status in a field trial.

It has long been known that haem iron is not absorbed by the same mechanism as inorganic iron, and that the intact haem molecule enters the cell before releasing its iron. Iron absorption from haemoglobin is relatively constant (10–25%) and is little influenced by meal composition or by iron status. Recently, in Chile, bovine haemoglobin concentrate has been added to cookies, with 20% iron absorption, and to infant cereals, with 14% absorption[14] (almost three times that of ferrous fumarate[12]). Unfortunately, however, there are many disadvantages that rule out the widespread use of bovine haemoglobin as an iron fortificant. The most important disadvantage is its intense colour and low iron concentration (5% haemoglobin must be added to infant cereal to give 14 mg of iron per serving)[14]. In addition, there are the technical difficulties involved in the collection, drying and storage of animal blood, the possibility of bacterial and viral contamination, and difficulties in obtaining a supply of animal blood in countries where meat is not widely consumed or where religious beliefs forbid the consumption of blood.

The use of sodium iron EDTA is somewhat more promising. Iron from this compound is less well absorbed by humans than ferrous sulphate from foods containing few inhibitors of absorption, but better absorbed from foods containing many inhibitors of iron absorption[15] (Fig. 3). As with haemoglobin, iron absorption from sodium iron EDTA varies little with diet composition, and several pilot fortification trials of this compound in fish sauce, sugar and curry powder have reported an improvement in the iron status of individuals consuming the fortified food. Sodium iron EDTA causes fewer organoleptic problems than do soluble iron salts, but is about ten times more expensive than ferrous sulphate, and there are some concerns over its long-term safety, due to its possible influence on the metabolism of other trace elements. A small fraction (5%) of the EDTA is absorbed and excreted in the urine, while the majority passes through to the stools[16]. No evidence of an influence on zinc status has been found in pilot studies, but no systematic study of the urinary and faecal excretion of other trace elements in subjects fed sodium iron EDTA has been reported. Sodium EDTA is an accepted additive in many countries, but the use of sodium iron EDTA has been discouraged in industrialized countries as the consumption of EDTA is already close to the recommended acceptable daily intake. Sodium iron EDTA could still prove very useful in developing countries.

Influence of food processing

Processing can influence the absorption of the more insoluble iron compounds, but appears to have little effect on the absorption of ferrous sulphate. Therefore, RBV can be modified. Heat sterilization of ferric pyrophosphate in a liquid infant formula has been shown to increase its RBV in rats from 39 to 93 (Ref. 17), whereas we have found that vacuum drying of a chocolate drink powder reduces the RBV of ferric pyrophosphate in humans from 60 to 21. The influence of the processing technology on the solubility of the iron fortification compound in the gastric juice is presumably the critical factor.

Enhancers of absorption

Ascorbic acid is the only absorption enhancer commonly added to foods. Once the fortification iron is dissolved in the gastric juice, it can be reduced by ascorbic acid to its more soluble ferrous state, and then be combined in soluble chelates which are available for absorption. Ascorbic acid addition has been reported to increase iron absorption from many different foods fortified with most types of iron compound, including ferrous sulphate, ferric pyrophosphate[18], elemental iron and ferric orthophosphate[11]. In infant cereals, for example, adding different amounts of ascorbic acid to a meal containing 3.75 mg of ferric pyrophosphate progressively increased iron absorption by adult women from 1.6% to 7.5% (see Fig. 4, Ref. 16). The main problem with ascorbic acid is that it is sensitive to processing and storage losses, although overaddition to the product and careful storage can ensure that sufficient ascorbic acid remains until the end of the product's shelf life. Encapsulation is an alternative in developing countries where adequate storage facilities do not exist. Other absorption promoters have been investigated, but have met with only limited success[9].

Risks of iron fortification

When supplying additional iron to large segments of a population there are four areas of potential concern: increase in infection rate, chronic iron overload, cancer development and impaired assimilation of other essential trace elements. Some of these concerns relate more to the larger amounts of iron provided by supplementation or iron therapy than to the limited quantities supplied by fortification but, when considering strategies for iron fortification, the potential risks must be considered.

Risk of infection

Because all microorganisms require iron for their growth, it has been postulated that additional iron could increase the frequency or severity of certain infections. The major support for this was a reported increase in malarial infection observed when nomads with undefined immunity for malaria were given iron therapy[19]. This observation was not confirmed in a more recent study in Papua New Guinea, where malaria is endemic[20]. In prepubescent anaemic school children given

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130 mg elemental iron daily, no influence on parasite infection rate, parasite density or levels of antimalarial IgG was observed. It is far more likely that correction of the defect in immunity in iron-deficient subjects by iron fortification will decrease, rather than increase, the frequency or severity of infectious illnesses.

Chronic iron overload

A more significant safety issue is the progressive accumulation in body iron stores that might occur when iron-replete individuals are exposed to raised levels of dietary iron over several years. This issue was highly debated in the early 1970s when enhanced fortification of wheat flour in the USA was being considered[21]. Because of the rigid control over iron absorption exerted by the gastrointestinal tract, healthy individuals have never been considered at risk. The major concern is for patients with disorders in iron balance. Idiopathic haemochromatosis is an inherited defect in the regulation of iron absorption that, eventually, leads to widespread tissue iron excess and death from liver cirrhosis or heart failure. Once considered rare, this autosomal recessive disease has recently been shown to be present in 3–5 individuals per 1000 in some Caucasian populations[22]. Iron fortification does not cause this disease, but it could induce clinical manifestations at an earlier age[23]. If patients are diagnosed before irreversible organ damage has occurred, the excess iron can be removed by a vigorous phlebotomy programme. Hopefully, efficient laboratory screening methods will become available to permit early detection and treatment. Thalassemia major, an inherited defect in haemoglobin synthesis, is also associated with severe gastrointestinal iron-loading and premature death from iron overload. Because patients with thalassemia major are severely anaemic, the diagnosis is usually made during infancy. Iron fortification will accelerate the rate of iron accumulation by these patients, but the frequent blood transfusions required to treat their anaemia is a more important source of their iron excess in countries where regular transfusions are feasible. Excessive iron absorption also occurs in certain acquired disorders such as sideroblastic anaemia, but iron overload is not the major clinical problem in these patients.

Impaired trace metal absorption

Assimilation of certain trace metals that share a common absorptive pathway may be competitively inhibited by iron. There is evidence that ferrous iron given as an aqueous solution impairs the absorption of zinc when the ratio of iron to zinc is 2:1 or greater and when the total amount of iron plus zinc consumed exceeds 25 mg (Ref. 24), although this observation is more relevant to iron supplementation than to iron fortification. It has been more difficult to demonstrate a competitive interaction in the presence of food[25], and there is no clear-cut evidence for an adverse effect of iron fortification on zinc status. When infants were given an iron-fortified formula, there was a significantly greater growth rate and weight gain at six months in those who were also given added zinc[26], suggesting that iron fortification impairs zinc nutrition. In another study, the plasma zinc concentration was lower in infants fed an iron-fortified rather than an unfortified cow's milk formula[27]. On the other hand, Mahloudji *et al.*[28] observed that schoolboys grew better when supplemented with 20 mg iron daily than when supplemented with both iron and 20 mg of zinc daily, suggesting that zinc impaired iron status.

This issue will not be resolved until more precise measurements of iron status become available, but it would be prudent in the interim to provide additional zinc in infant foods that are heavily fortified with iron.

Risk of cancer

The fundamental importance of iron for cellular growth and multiplication has prompted theoretical concerns that an excess in body iron will enhance the survival and proliferation of malignant cells[29] or promote carcinogenesis by the formation of toxic free radicals. There is a striking increase in primary liver cancer in patients with idiopathic haemochromatosis, due to their underlying liver cirrhosis, but there is no apparent increase in other malignancies[30]. Some evidence that iron may act as a carcinogen was provided by a recent epidemiologic study in which 14 000 adults were re-evaluated ten years after a national nutritional survey[31]. Baseline laboratory measurements in the survey included percentage iron saturation of transferrin, the specific protein carrier for iron in the circulation. The mean iron saturation was significantly higher and the transferrin and serum albumin concentrations were lower in 242 men who developed cancer in the next decade than in the more than 3000 men who remained free of malignant disease. These relationships were not significant in adult women. The greatest association between transferrin saturation and the incidence of cancer was observed with cancers of the colon, bladder and oesophagus. While this retrospective observation raises the possibility of an association between iron status and cancer risk, the evidence is equivocal. The relationship with a low serum albumin level was not explained, and transferrin saturation is not a reliable index of iron status in healthy subjects. Prospective studies employing more specific methods of determining iron status, such as measurements of serum ferritin levels, are needed to define further the relationships between dietary iron intake, body iron reserves and the development of neoplasia.

Therefore, at the present time, the liabilities of iron deficiency, in particular the decreased psychomotor development and mental performance in iron-deficient children, and the association of increased premature deliveries and higher infant and maternal mortality rates with iron deficiency in pregnant women, appear to far outweigh any adverse effects of iron fortification. Over 50% of the women and children in developing countries, and around 10% in the more industrialized countries, are anaemic, and many would benefit from the iron fortification of foods.

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Aspects actuels des carences en fer et en folates dans le monde
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NEW APPROACHES TO THE ASSESSMENT OF IRON NUTRITURE

J.D. Cook, B.S. Skikne

Department of Medicine, Kansas University Medical Center
39th & Rainbow Blvd., Kansas City, Kansas, 66103, USA

INTRODUCTION

Our ability to assess iron nutriture in population studies has improved remarkably in recent years. Earlier surveys were based on hemoglobin or hematocrit determinations which provided only a rough guideline to the prevalence of iron deficiency in a population and no information whatsoever about the cause of the anemia or strategies to alleviate it. Several nutrient deficiencies, including iron, folate, and protein, may contribute to the prevalence of anemia in a given geographic area or segment of a population, but iron deficiency has been identified as the most important factor in the vast majority of surveys. The assessment of iron status has been refined in recent years by the development of more accurate laboratory techniques, the introduction of newer iron measurements, and an improvement in statistical methods for interpreting survey data.

Iron-Related Laboratory Measurements

Laboratory techniques for assessing iron status are best characterized by their ability to detect iron in specific body compartments (1)(Table 1). Measurements of storage iron are important because the presence of iron in this compartment excludes iron deficiency. While bone marrow examinations are still used by clinicians to evaluate storage iron, especially in hospitalized patients, the only practical survey technique for assessing storage iron is the serum ferritin. The serum ferritin is an important iron parameter in population studies because a value below 12-15 ug/L in the presence of anemia provides unequivocal evidence of iron deficiency while significantly elevated levels above 100 ug/L exclude this diagnosis (2,3). The serum ferritin is especially useful in distinguishing iron deficiency anemia from anemias due to chronic infection, thalassemia, or sickle cell disease. These latter disorders may contribute significantly to the prevalence of anemia in certain geographic regions of the world. The major drawback of the serum ferritin is that normal

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values are seen when iron deficiency occurs in the presence of infection, chronic inflammation, or liver disease. Bone marrow examination remains the only definitive method of identifying iron deficiency in these situations.

SURVEY MEASUREMENTS OF IRON STATUS

	Cut-off Value
Measurement of Storage Iron	
Serum ferritin	<12 ug/L
Serum transferrin (TIBC)	>400 ug/dl
Measurement of Functional Iron	
Transferrin saturation	<16%
Erythrocyte protoporphyrin	>70 ug/dl RBC
Mean cell volume	<80 fl
Serum transferrin receptor	>9 mg/L

Measurements of functional iron are important in assessing the severity of the deficit in iron after stores are fully exhausted and before overt anemia has developed. Plasma iron transport measurements include the serum iron, serum transferrin or total iron binding capacity (TIBC), and percent saturation of transferrin. These parameters provide indirect evidence of functional iron deficiency. The transferrin saturation is a useful screening test because normal values above 16% exclude an impairment in cellular iron supply. However, the transferrin saturation is a labile measurement and has both low specificity and sensitivity. Because the developing red cell is the body tissue which is most dependent on a continuous supply of iron, methods that identify iron deficient erythropoiesis are used extensively to assess iron nutriture in population studies. Measurements of free erythrocyte protoporphyrin in circulating red cells and mean red cell volume (MCV) using electronic counting techniques are more reliable indices of iron deficient erythropoiesis than plasma transport determinations because several weeks or even months are required before the levels become unequivocally abnormal. By the same token, the MCV and red cell protoporphyrin fail to identify iron deficiency of recent onset and do not distinguish true iron deficiency from secondary iron deficient erythropoiesis seen in patients with the anemia of

chronic disease. One advantage of the erythrocyte protoporphyrin over the MCV is that a specialized instrument for its measurement, referred to as a hematofluorometer, is particularly suited for survey work. The instrument requires little or no technical experience, provides a very rapid result, and can be used in field studies where there is no access to electricity.

Serum Transferrin Receptor

There have been major insights in recent years into the mechanisms by which iron is transported from the plasma compartment to an intracellular location. This is a highly refined process in which iron-loaded transferrin binds to a specific receptor on the cell membrane followed by endocytosis (4). As the pH in the endocytotic vesicle falls, iron is released to the cytosol and the apotransferrin is returned to the surface to acquire more iron. Cell culture studies have demonstrated that receptor synthesis increases sharply when iron supply to the cell is diminished. This provides an eloquent mechanism by which the iron needs of a cell are communicated to its environment.

In a series of recent reports, a soluble protein has been identified in sera which reacts with antibodies developed against transferrin receptor (5,6). Preliminary studies in our laboratory indicate that this circulating material is saturated with transferrin and represents only the extracytoplasmic portion of the receptor. In the original reports, distinct elevations in circulating receptor were observed primarily in patients with increased red cell production such as autoimmune hemolytic anemia, but 2 to 3-fold elevations were also seen in patients with iron deficiency anemia. In the latter patients, correlations were observed between serum receptor levels and other iron-related measurements such as TIBC, serum iron, and serum ferritin. Red cell precursors did not appear to be the only source of serum receptor because in patients with aplastic anemia, the level fell by only 50%.

We have recently developed an enzyme-linked immunoassay using specific monoclonal antibodies prepared against soluble transferrin-saturated receptor isolated from human placenta (7). One major advantage over the original assay is that the sensitivity of the assay is not influenced by the binding status of the receptor. The mean level in 82 normal male and female subjects of 5.6 mg/L increased sharply to an average of 18 mg/L in patients with iron

deficiency anemia and to an average of 33 mg/L in patients with sickle cell anemia. In patients with iron deficiency anemia we could detect no correlation with the receptor level and the circulating reticulocyte count indicating that iron deficiency produces an elevation in serum receptor independent of the total mass of red cell precursors.

Controlled phlebotomy studies to induce iron deficiency in healthy volunteer subjects in our laboratory indicate that serum receptor measurements will play a major role in assessing the iron status of a population. Receptor levels begin to increase as iron stores become fully depleted. With advancing iron deficiency, there is a good correlation with the increase in serum receptor level and the magnitude of the deficit in functional iron. This elevation can be detected long before the development of anemia and in advance of significant changes in MCV or erythrocyte protoporphyrin. If these observations are confirmed, population surveys of iron status could be greatly simplified by limiting survey measurements to the serum ferritin, serum receptor, and hemoglobin. The assays required to measure serum ferritin and receptor are identical except for the immunologic reagents. All these measurements can be performed readily on capillary blood obtained by finger stick.

Analysis of Survey Data

Improvements in techniques for interpreting survey data have also occurred, but existing methods are still relatively crude. Earlier surveys often relied on a single measurement such as the hemoglobin determination, and defined abnormality on the basis of an arbitrary cut-off level. This approach is seriously flawed by the marked overlap in the distribution of hemoglobin levels between normal and iron deficient segments of a population (8). Even relatively small changes in criteria result in marked differences in estimated prevalence and proportion of abnormal and normal subjects. The difficulties encountered in using the hemoglobin to assess anemia applies equally to other iron parameters used to detect iron deficiency; no single measurement, including the serum ferritin, completely separates an iron deficient from an iron replete population. While this is partly explained by the fact that iron stores form a continuous distribution in a population (9), there is also a marked overlap between normal and iron deficient populations.

The information gathered in population surveys can be improved by interpreting laboratory measurements in an integrated fashion rather than in isolation. In one study (10) the transferrin saturation, erythrocyte protoporphyrin, and serum ferritin were used to estimate the prevalence of iron deficiency. If only one of these parameters was abnormal, the prevalence of anemia increased only slightly from 8 to 11%. If two parameters were abnormal, the prevalence rose to 28% and if all three were abnormal, to 63%. Thus, the specificity of an isolated hemoglobin measurement can be enhanced by requiring that additional, more specific, measurements of iron deficiency are considered jointly. This approach was used in a recent analysis of a large nutritional survey in the United States conducted between 1976 and 1980 (11). One model used the transferrin saturation, erythrocyte protoporphyrin, and serum ferritin, and another model substituted the serum ferritin with the MCV. Impaired iron status was defined as occurring in individuals having at least two of these three parameters abnormal, whereas iron deficiency anemia was considered present when the hemoglobin was abnormal as well.

A further refinement in this approach is to estimate iron stores quantitatively in each surveyed individual (9). In the iron-replete segment of a population, the size of residual iron stores were estimated from the serum ferritin level using a logarithmic transformation. In anemic patients in whom iron deficiency was identified by an abnormal transferrin saturation, erythrocyte protoporphyrin, and serum ferritin, the deficit in functional iron was estimated from the reduction in circulating hemoglobin level. Between these population segments, combinations of the transferrin saturation, erythrocyte protoporphyrin, and serum ferritin were used to estimate milder deficits in the functional iron before anemia had developed. Iron stores in adult men and adult women before and following menopause appeared to be normally distributed. This approach was far more sensitive in identifying iron deficiency anemia than earlier techniques based only on a single laboratory definition. The technique lends itself to ongoing evaluation of field trials. Because iron stores are determined in each subject individually, field trials can be monitored while in progress to determine the end point of the trial. The approach also lends itself to analysis of factors which might contribute to iron deficiency in subsets of the population such as method of contraception, nature of the diet, socio-economic status, and use of iron supplements. The definition of mild iron deficiency in this model is still

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largely empirical. Our preliminary experience with measurements of the serum receptor suggest that this laboratory parameter will provide more reliable estimates of mild iron deficiency.

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SUMMARY

There have been many important refinements during the past decade in techniques to detect iron deficiency in the population and assess its magnitude. The measurement of storage iron using the serum ferritin assay is particularly important because the presence of iron in this compartment excludes iron deficiency. Serum ferritin values below 12 ug/L in anemic individuals provide unequivocal evidence of iron deficiency, but normal values do not exclude iron deficiency associated with infection or other chronic disease. Additional laboratory measurements are needed to assess the severity of deficits in functional iron. The transferrin saturation is a useful screening test to detect diminished tissue iron supply, but the sensitivity and specificity of the measurement are low. Because of the dependence of the developing red cell on a constant supply of iron, methods to identify iron deficient erythropoiesis are useful in assessing iron status. Erythrocyte protoporphyrin and mean red cell volume determined electronically are reliable measures, but neither of these distinguish true iron deficiency from the anemia of chronic disease. Our early experience with the measurement of serum transferrin receptor suggests that it will become a valuable adjuvant in the laboratory evolution of iron status. Elevations are seen in patients with either increased erythropiesis or iron deficiency, conditions which apparently exert independent effects on circulating receptor levels. Important advances have also been made in refining techniques for interpreting the results of iron surveys. Epidemiologic methods which divide the sampled population on the basis of a single laboratory criteria are inadequate because no one measurement can reliably distinguish between iron deficient and iron replete segments of a population. The optimal analytical approach is to use a combination of laboratory values to estimate body iron in each surveyed individual. Serum ferritin measurements can be used in iron replete individuals to determine the size of iron stores while the hemoglobin concentration can be used in anemic individuals to estimate the deficit in functional iron. Between these limits, serum receptor measurements may provide the most reliable estimate of mild deficits in functional iron which are difficult to quantify with existing laboratory measurements.

RESUME

De nombreux perfectionnements ont eu lieu durant la dernière décennie dans les techniques de détection de la carence en fer dans les populations, et dans l'évaluation de son amplitude. La mesure du fer de stockage à l'aide du dosage de la ferritine sérique est particulièrement importante parce que la présence de fer dans ce compartiment exclut la carence en fer. Des valeurs de ferritine sérique inférieures à 12 µg/l chez les individus anémiques sont une preuve sans équivoque de la carence en fer, mais des valeurs normales n'excluent pas l'association de la carence en fer avec une maladie infectieuse ou une autre maladie chronique. D'autres mesures de laboratoire sont nécessaires pour évaluer la sévérité des déficits en fer fonctionnel. La saturation de la transferrine est un test de dépistage utile pour détecter la diminution d'apport en fer tissulaire, mais la sensibilité et la spécificité de la mesure sont faibles. A cause de la dépendance des globules rouges en développement envers un apport constant de fer, les méthodes qui identifient une érythropoïèse déficiente en fer sont utiles pour évaluer le satut en fer. La protoporphyrine érythrocytaire et le volume globulaire moyen déterminés électroniquement, sont des mesures fiables, mais aucune des deux ne sépare la vraie carence en fer de l'anémie par maladie chronique. Notre récente expérience de la mesure des récepteurs sériques à la transferrine suggère qu'elle deviendra un accessoire utile en laboratoire pour rendre compte de l'évolution du statut en fer. Des élévations sont observées chez les patients présentant soit une augmentation de l'érythropoïèse, soit une

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carence en fer, conditions qui apparamment exercent des effets indépendants sur les niveaux des récepteurs circulants. Des progrès importants ont aussi été faits dans le perfectionnement des techniques d'interprétation des résultats d'enquêtes sur le fer. Les méthodes épidémiologiques qui divisent une population échantillon sur la base d'un unique critère de laboratoire ne sont pas adaptées parce qu'aucune mesure isolée ne permet une distinction fiable entre les segments carencés en fer et ceux au statut normal en fer dans une population. L'approche analytique optimale est d'utiliser une combinaison des valeurs de laboratoire pour estimer le fer corporel de chaque individu étudié. Les mesures de ferritine sérique peuvent être utilisées chez des individus non déplétés en fer pour déterminer la taille des stocks de fer, alors que la concentration en hémoglobine peut être utilisée chez les individus anémiques pour estimer le déficit en fer fonctionnel. Entre ces limites, la mesure des récepteurs sériques peut représenter l'estimation la plus fiable de déficits légers en fer fonctionnel, qui sont difficiles à quantifier avec les mesures de laboratoire existantes.

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Serum Transferrin Receptor Is a Truncated Form of Tissue Receptor*

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Yuan J. Shih, Roy D. Baynes, Billy G. Hudson, Carol H. Flowers, Barry S. Skikne, and James D. Cook‡

From the Departments of Medicine and Biochemistry, Kansas University Medical Center, Kansas City, Kansas 66103

Recent studies have provided immunological evidence for the existence of transferrin receptor in human serum and have revealed that its concentration is a sensitive measure of erythropoiesis and iron deficiency. The present study was undertaken to establish the molecular identity of this immunoreactive component. Purification from human serum was accomplished by immunoaffinity chromatography using, as the ligand, monoclonal antitransferrin receptor antibody. The receptor preparation contained two major components with M_r of 75,000 and 85,000, which were identifed as transferrin and transferrin receptor, respectively. The physicochemical and immunochemical properties of the 85,000 serum receptor were compared with those established for intact placental transferrin receptor. The serum receptor exhibited an apparent M_r = 85,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis under non-reducing conditions, as compared with 190,000 for placental transferrin receptor. Upon reduction, the M_r of serum receptor was unaltered, whereas, the 190,000 placental receptor dimer decreased to the expected monomer value of 95,000. Amino-terminal amino acid sequence analysis revealed that residues 1–19 of serum receptor were identical to residues 101–119 of intact receptor. These findings provide physicochemical evidence for the existence of transferrin receptor in human serum, establish its molecular identity as a truncated form lacking the cytoplasmic and transmembrane domains (residues 1–100) of intact receptor, and demonstrate that it exists as a transferrin-receptor complex in serum.

The process by which cells acquire iron for growth and synthesis of iron-containing proteins has been defined extensively in recent years (1-3). Iron uptake is mediated by a specific receptor for transferrin, the transport protein for iron. Diferric transferrin binds to its receptor at the cell surface and is then rapidly internalized in nonlysosomal vesicles. The acidic environment in these compartments causes the iron to dissociate while transferrin remains bound to its receptor. The complex of transferrin and receptor is promptly returned to the cell surface where the apotransferrin rapidly dissociates at a neutral pH. Transferrin receptor has been purified, cloned, and its primary structure determined from cDNA analysis (4, 5). The receptor is a transmembrane glycoprotein composed of two disulfide-linked monomers. Each polypeptide subunit contains 760 amino acids which are divided into the following three major portions: a large C-terminal extracellular domain of 671 amino acids, a transmembrane domain of 28 amino acids, and an N-terminal cytoplasmic domain of 61 amino acids. Transferrin receptor is expressed on all dividing cells, and its rate of synthesis is closely linked to the requirements of the cell for iron.

Recently, evidence was obtained for the existence of transferrin receptor in human and rat sera (6-9). The evidence was based on the immunological reactivity of serum with monoclonal or polyclonal antibodies that were directed against intact transferrin receptor. The concentration of this serum receptor appears to be related to tissue receptor mass and thus provides a clinical measure of erythropoiesis or iron deficiency (7-11). Potentially, this serum receptor may provide important clues about the overall metabolism of transferrin receptor, and its concentration will assume importance in the detection and management of patients with hematological disease (12).

The molecular identity of human serum transferrin receptor is unknown. On the basis of SDS-PAGE[1] analyses, Kohgo *et al.* (6) concluded that the serum receptor (non-reduced) is a polypeptide of M_r = 80,000–100,000, a value which is compatible with a monomeric form of the membrane-bound transferrin receptor. Based on a subsequent study of the electrophoretic behavior of the reduced receptor, these workers suggested that it represents nicked dimers of intact receptor (7). Huebers *et al.* (9) concluded that the serum receptor is an intact transferrin receptor based on the behavior of the receptor-transferrin complex on gel filtration chromatography. At present, no definitive information is available regarding the chemical nature of this serum transferrin receptor or its structural relationship with the membrane-bound transferrin receptor.

The present study was undertaken to establish the molecular identity of this putative transferrin receptor in serum. Our findings provide physicochemical evidence for the existence of transferrin receptor in human serum, establish its molecular identity as a truncated form lacking the cytoplasmic and transmembrane domains (residues 1–100) of intact receptor, and demonstrate that it exists as a transferrin-receptor complex in serum.

EXPERIMENTAL PROCEDURES

Materials—Serum and plasma were obtained from normal subjects and patients with iron deficiency or sickle cell anemia. Plasma samples were prepared from blood collected into tubes containing heparin. Diethanolamine, Tween 20, Tween 80, Tris, diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride, sodium azide, 4-

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‡ To whom reprint requests should be addressed: Division of Hematology, Kansas University Medical Center, 39th & Rainbow, Kansas City, KS 66103.

[1] The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay.

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chloro-1-naphthol, concanavalin A, peroxidase, cyanogen bromide-activated Sepharose 4B, and transferrin were obtained from Sigma.

Monoclonal antibodies against transferrin receptor were developed against soluble transferrin-saturated receptor purified from human placenta as described previously (11). The following two monoclonal antibodies were employed: A4A6 that is used in the enzyme-linked immunosorbent assay (ELISA) and D3A12 that is equally immunoreactive in this assay. The antibodies were conjugated to horseradish peroxidase as described previously (13). Monoclonal antibodies against human transferrin were prepared as previously described (14).

Purification of Human Transferrin Receptor—Human transferrin receptor was isolated from sera by affinity chromatography using, as the ligand, monoclonal antibodies against transferrin receptor. Human serum was prepared for chromatography by diluting it with an equal volume of equilibrium buffer containing phosphate-buffered saline, pH 7.2, 0.5% Tween 80, 0.5 mM diisopropyl fluorophosphate or 0.5 mM phenylmethylsulfonyl fluoride and 0.02% sodium azide. The diluted serum was centrifuged at 10,000 × *g* for 20 min at 4 °C and the pellet discarded.

Monoclonal antibody to transferrin receptor was coupled to cyanogen bromide-activated Sepharose 4B according to the manufacturer's instructions. Serum was mixed with the derivitized Sepharose 4B resin at an approximate ratio of 1.5 parts antibody to 1 part receptor. The mixture was placed on an end-over-end rotor overnight at 4 °C, poured onto a column, and washed with the same buffer until the A_{280} approached zero.

Quantitative recovery of the receptor from the affinity column was monitored with an ELISA (11). Because recovery varied depending on the elution buffer, preliminary studies were performed to determine the stability of serum receptor in different buffer systems. Human serum was diluted 1:5 with phosphate-buffered saline, pH 7.2, 0.5% Tween 20 and mixed in a ratio of 1:3 with the test buffer. Three acidic buffers (0.1 M glycine, pH 2.5; 0.1 M acetic acid, pH 2.8; 0.15 M acetic acid, 0.15 M NaCl) all gave recoveries of less than 3%. A polarity reducing agent, 10% dioxane, gave a somewhat higher recovery of 7.7%. Recoveries with high salt concentration buffers varied widely from 0% with 5 M KI and 27% with 3 M NaI, to 61.9% with 2.8 M $MgCl_2$. Recoveries with basic buffers were consistently higher. With 1 M NH_4OH, recovery was 63.9% whereas 0.02 M diethanolamine, 0.5% Tween 80, pH 11, resulted in no loss of receptor as measured by the ELISA. Consequently, elution of the receptor from the affinity column was accomplished by incubating the affinity resin in this buffer system for 10 min and then collecting the eluate in a test tube containing 0.5 M Tris, pH 7.5.

For control purpose, intact transferrin receptor was prepared from human placenta using the same affinity purification method. The placental tissue was homogenized and solubilized with 1% Triton X-100 (11) prior to affinity chromatography.

Electrophoretic Analysis—Various samples were analyzed on SDS-PAGE by loading onto 4–20% gradient minigels and electrophoresing by the method of Laemmli (15) at 150 V for 1.5 h. The gels were then fixed in 40% methanol, 10% acetic acid for 1 h and stained with 0.2% Coomassie Blue R-250 in 40% methanol, 10% acetic acid for 1 h. The gels were destained in 40% methanol, 10% acetic acid overnight. For Western blotting, proteins were transblotted immediately after electrophoresis to nitrocellulose as previously described (16). The nitrocellulose membrane was then blocked by incubation with 1% non-fat dry milk in phosphate-buffered saline for 1 h and incubated for 2 h with monoclonal antibody. The latter was conjugated to horseradish peroxidase and diluted in 0.1% non-fat dry milk in phosphate-buffered saline. After extensive washing of the membranes with phosphate-buffered saline, the immunoreactive bands were detected by adding 4-chloro-1-naphthol as the substrate for the peroxidase.

Detection of Glycoprotein—Glycoprotein was detected using the method of Clegg (17) after transblotting to nitrocellulose membranes as described above. The membrane was first blocked by incubating with 2.5% bovine serum albumin in phosphate-buffered saline, pH 7.2, for 1 h at room temperature. The membrane was then incubated with concanavalin A at a concentration of 10 μg/ml in phosphate-buffered saline containing 10 μM Ca^{2+}, Mg^{2+}, Mn^{2+}, and 0.5% Triton X-100 for 1 h followed by extensive washing in the above buffer. After incubating the membrane with horseradish peroxidase (50 μg/ml) for 1 h and washing with the same buffer, the glycoprotein was detected by incubating the membrane with 3.4 mM 4-chloro-1-naphthol in 50 mM Tris-saline, pH 7.4.

Amino Acid Sequence Analysis—In the purification of serum transferrin receptor, we observed that repeated elution of the receptor from the affinity column progressively decreased the amount of contaminating transferrin in the eluant. By performing repeated elutions with the same elution buffer, receptor was obtained which was essentially free of transferrin. The receptor was then electrophoresed on a 7.5–20% gradient of polyacrylamide and transblotted onto polyvinylidene difluoride membrane for sequencing according to the procedure of Matsudaira (18). The sequencing was performed at University of California, Los Angeles, Department of Biological Chemistry, UCLA School of Medicine.

RESULTS

Purification of Serum Transferrin Receptor—In preliminary studies, we attempted to purify serum receptor with techniques that are commonly used to isolate intact receptor but these methods were unsuitable. Affinity chromatography using, as the ligand, human transferrin (19) yielded a recovery of only 6.7% and less than 5% of the purified protein was immunoreactive as measured by ELISA. We also tried the combined use of DEAE-Sephacel anion-exchange and gel filtration chromatography (20) which yielded a somewhat higher recovery of 36%. However, less than 1% of the isolated protein was immunoreactive by ELISA. Affinity chromatography using, as the ligand, monoclonal antibody (A4A6 or D3A12) directed against solubilized intact placental receptor was highly satisfactory, yielding recoveries of over 80%. The high recovery indicated that the monoclonal anti-transferrin receptor antibodies extracted virtually all of the immunoreactive transferrin receptor present in the serum. This immunoreactive receptor preparation was used in subsequent studies to determine its molecular identity.

SDS-PAGE Analysis of Serum Transferrin Receptor Preparation—This receptor preparation was analyzed by SDS-PAGE to determine the number, identity, and M_r of protein components (Fig. 1). Under non-reducing conditions, the preparation was found to be composed of two major components with apparent M_r of 75,000 and 85,000, along with a faint diffuse component of M_r = 180,000. The 75,000 component was identified as transferrin, the 85,000 component as serum transferrin receptor and the 180,000 component as contaminating monoclonal antibody, as described below. Under reducing conditions, the M_r of the 85,000 transferrin receptor was unaltered. In contrast, the electrophoretic profile of intact transferrin receptor preparation from placenta, under non-reducing conditions, revealed a 75,000 transferrin component, the expected 190,000 transferrin receptor dimer and a minor 85,000 component. Upon reduction, the 190,000 dimer yielded a monomer component of M_r = 95,000. These

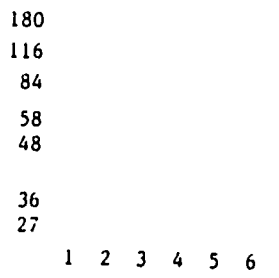

FIG. 1. **SDS-PAGE of the serum and placental transferrin receptor preparations.** The proteins were isolated by immunoaffinity column using, as the ligand, monoclonal antitransferrin receptor antibody. The proteins were eluted from the column and analyzed by 4–20% SDS-PAGE with 0.2% Coomassie Blue staining. *Lanes 2* and *5* contained serum receptor preparation, *lanes 3* and *6* contained placental receptor preparation. *Lanes 1* and *4* contained molecular weight standards. Samples in *lanes 5* and *6* were reduced prior to electrophoresis.

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results showed that the M_r of the serum receptor is about 10,000 less than that of the monomeric form of the intact receptor. Moreover, the results suggested that the serum receptor 1) is a single polypeptide chain, because its size is not influenced by reduction of disulfide bonds as is the intact placental transferrin receptor, and 2) exists in serum as a complex with transferrin because transferrin copurifies with it on the immunoaffinity column.

The electrophoretic patterns (non-reduced and reduced) of the serum receptor were unaltered by a variety of conditions. These included 1) the use of different monoclonal antibodies, directed against transferrin receptor, as the ligand for the affinity purification of the serum receptor, 2) the addition of proteolytic inhibitors to serum or whole blood prior to harvesting serum or plasma, or 3) the use of plasma rather than serum. Moreover, ELISA measurements using monoclonal antibody revealed that the recovery of receptor from serum by the immunoaffinity chromatography procedure was consistently over 80%. Thus, the major component of serum that is detected by monoclonal antibodies directed against intact transferrin receptor is the 85,000 component.

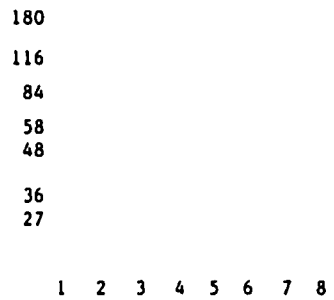

FIG. 2. **Identification of components of the serum transferrin receptor preparation by SDS-PAGE.** *Lanes 2* and *6* contained serum transferrin receptor preparation, *lanes 3* and *7*, purified transferrin, and *lanes 4* and *8*, monoclonal anti-transferrin receptor antibody. *Lanes 1* and *5* contained molecular weight standards. *Lanes 2–4* were electrophoresed under non-reducing conditions, and *lanes 6–8* under reducing conditions.

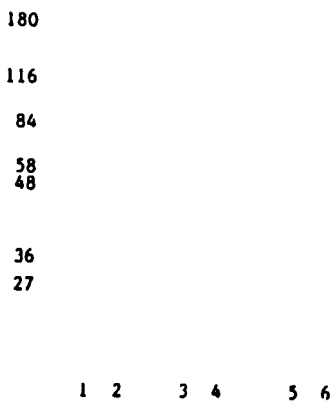

FIG. 3. **Identification of components of the serum transferrin receptor preparation by Western blotting.** Serum receptor preparation was electrophoresed on SDS-PAGE under non-reducing conditions and analyzed by both Coomassie Blue and immunostaining. *Lanes 1, 3,* and *5* contained molecular weight standards. *Lane 2,* Coomassie Blue staining; *lane 4,* immunostaining with monoclonal antitransferrin receptor antibody; *lane 6,* immunostaining with monoclonal antitransferrin antibody.

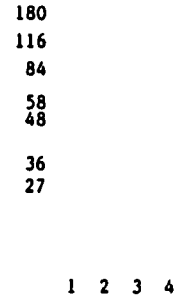

FIG. 4. **Glycoprotein detection using concanavalin A and horseradish peroxidase.** Serum transferrin receptor was transblotted onto a nitrocellulose membrane after SDS-PAGE. The glycoproteins were detected by sequential incubation with concanavalin A, peroxidase, and 4-chloro-1-naphthol. *Lanes 2* and *4* were non-reduced and reduced samples, respectively. *Lanes 1* and *3* were molecular weight standards.

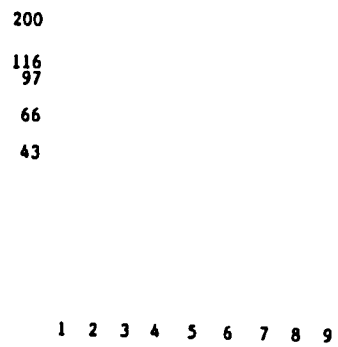

FIG. 5. **Resolution of the 85,000 serum receptor from transferrin.** The 85,000 receptor was dissocciated from the immunoaffinity column with stepwise elution. Elution *fraction numbers 2–9* were analyzed by SDS-PAGE under non-reducing conditions. *Fraction numbers 7–9* contained receptor which was essentially transferrin-free. *Lane 1* was the molecular weight standard.

Identification of the Components of the Serum Receptor Preparation—The identity of the two major (75,000 and 85,000) components and the minor (180,000) component of the immunoaffinity purified preparation of serum transferrin receptor was established by Western blotting analysis using antibodies directed against transferrin, transferrin receptor, and rabbit anti-mouse IgG. The 75,000 component comigrated with transferrin (Fig. 2, *lanes 3* and *7*) and reacted with monoclonal antitransferrin antibody (Fig. 3, *lane 6*), which established it as transferrin. The 85,000 component reacted specifically with monoclonal antitransferrin receptor antibody (Fig. 3, *lane 4*) as did the 190,000 dimer and 95,000 monomer of placental transferrin receptor (data not shown). Hence, these results established the identity of the 85,000 component as the serum transferrin receptor. The minor components of the preparation, M_r 180,000 (non-reduced) and 52,000 and 25,000 (reduced) corresponded to impurities derived from the monoclonal antitransferrin receptor antibody that was used as the ligand for affinity chromatography as shown in Fig. 2 (compare *lane 2* with *lane 4* and *lane 6* with *lane 8*). Overall, these results revealed that 1) the component of serum which reacts with monoclonal antitransferrin receptor antibody is an 85,000 protein and 2) that the affinity purified preparation of serum receptor contains about equal amounts of transferrin and transferrin receptor. In addition,

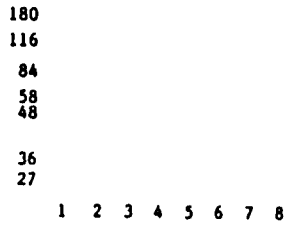

FIG. 6. **SDS-PAGE of serum receptor isolated from a normal subject and patients with elevated serum receptor levels.** *Lanes 2* and *6*, normal; *lanes 3* and *7*, sickle cell anemia; *lanes 4* and *8*, iron deficiency; *lanes 2–4*, non-reduced samples; *lanes 6–8*, reduced samples; *lanes 1* and *5*, molecular weight standards.

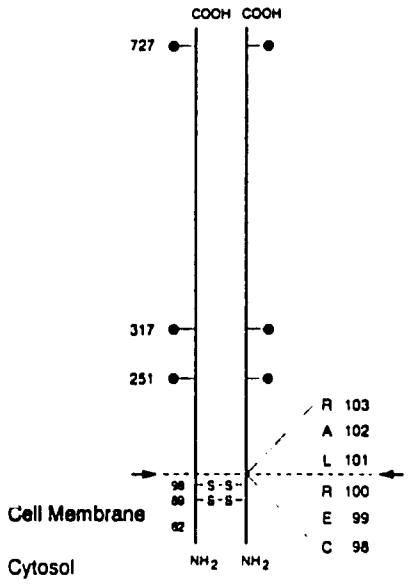

FIG. 7. **Schematic representation of serum transferrin receptor.** The intact transferrin receptor is composed of two disulfide-linked monomers, each containing 760 amino acid residues. The disulfide linkages are located at amino acid residues 89 and 98. The N-terminal intracellular domain of the polypeptide consists of 61 amino acids and the transmembrane portion has 28 amino acids, residues 62–89. The remaining 671 amino acids are extracellular. The circulating form of the receptor is truncated between amino acid residue 100 (Arg) and 101 (Leu). *Solid circles* indicate glycosylation sites.

the 85,000 component was shown to be a glycoprotein (Fig. 4).

Sequence Analysis of the 85,000 Serum Transferrin Receptor—The amino-terminal amino acid sequence of the 85,000 serum transferrin receptor was determined to chemically identify the receptor and to investigate its primary structure in relation to the intact transferrin receptor. Serum receptor was resolved from the transferrin component of the receptor preparation by successive elutions of the affinity column (Fig. 5). After seven successive elutions, the 85,000 receptor was essentially transferrin-free. The N-terminal sequence of the serum receptor was compared with that deduced from the cDNA data of the intact transferrin receptor (5). Sequence identity was observed between residues 1–19 of the 85,000 serum transferrin receptor and residues 101–119 of the intact receptor. *X*s designate the non-identified amino acid residues.

SDS-PAGE of Serum Transferrin Receptor from Patients with Hematological Disease—Clinical studies have shown that the concentration of serum receptor is increased in patients with enhanced erythropoiesis such as sickle cell anemia or with iron deficiency (7–11). It was, therefore, important to determine the biochemical nature of the immunoreactive component in these disorders. As shown in Fig. 6, the immunoreactive component of serum from patients with sickle cell anemia or iron deficiency is the 85,000 serum receptor, therefore, this component is responsible for the increased serum receptor concentration in these disorders.

DISCUSSION

The present study provides physicochemical evidence for the existence of transferrin receptor in human plasma and serum and establishes its molecular identity. The size and behavior of the serum receptor on SDS-PAGE, under reducing and non-reducing conditions, contrast sharply with that previously established for intact placental transferrin receptor. The serum receptor exhibits a M_r of 85,000 under non-reducing or reducing conditions. In contrast, placental transferrin receptor exhibits a M_r of 190,000 for the dimer under non-reducing conditions and 95,000 for the monomer upon reduction (4, 21). These results indicate that the serum receptor is a single polypeptide chain and that its M_r is about 10,000 less than that of the monomeric form of intact placental receptor.

The structural relationship between the serum and tissue forms of the receptor was established by amino-terminal amino acid sequence analysis and molecular weight values. Residues 1–19 of the N-terminal sequence of the 85,000 serum receptor were found to be identical to residues 101–119 of intact placental receptor, indicating that residues 1–100 are absent from the serum receptor. The M_r of the serum receptor of 85,000 agrees well with a value of 83,900 calculated from the M_r of the intact receptor monomer minus the first 100 amino acid residues. Glycoprotein analysis indicated that the M_r of the serum receptor included its carbohydrate residues. The lack of an effect of reduction on apparent M_r is also consistent with the absence of cysteine residues at positions 89 and 98 which are involved in intermolecular disulfide bonds (22) in the intact receptor. Thus, these findings establish that serum transferrin receptor lacks the first 100 amino acid residues of intact receptor. The truncation occurs in the extracellular domain between arginine 100 and leucine 101 (Fig. 7). A related fragment has been generated by tryptic digestion *in vitro* in which the main cleavage site is at residue 121 (19, 23).

The identification of transferrin receptor in human serum by Kohgo and co-workers (6) was prompted by an earlier observation that *in vitro* culture of sheep erythrocytes is accomplished by the release of vesicles termed exosomes containing transferrin receptor (24). These vesicles have been shown recently to contain a number of plasma membrane activities including acetylcholine esterase, cytochalasin B binding, nucleoside binding, and Na^+-independent amino acid transport as well as transferrin receptor (25). These findings differ from ours because the exosomal receptor had the same electrophoretic mobility on SDS-PAGE as intact receptor isolated from reticulocyte membranes (two polypeptide bands

85,000 serum transferrin receptor (residues 1-19) L-A-G-X-E-S-P-V-X-E-E-P-G-E-D-F-P-A-A-•••
Intact transferrin receptor (residues 95-122) •••-L-T-E-C-E-R-L-A-G-T-E-S-P-V-R-E-E-P-G-E-D-F-P-A-A-R-R-L-•••

of 186,000 and 93,000). While the soluble form of the receptor identified in the current study is clearly distinct from the intact membrane-bound form in the exosome, the latter may possibly contribute to the production of the soluble receptor.

A number of other cell surface receptors have been shown to exist in soluble or truncated form. These include the insulin receptor (26), HLA antigens (27), the epidermal growth factor receptor (28), the interleukin-2 receptor (29), and the IgM immunoglobulin molecule (30). The most extensively studied of these is the interleukin-2 receptor with findings very similar to ours with the serum transferrin receptor. The truncation of the interleukin-2 receptor occurs just distal to the transmembrane region of the molecule and involves the Cys-Leu residues at positions 192 and 193 (31). Several mechanisms have been proposed to account for these soluble forms. Some workers have suggested that they are truly secretory forms of the protein, lacking transmembrane regions and produced by either multiple genes (27) or alternate mRNA splicing pathways (30). It has also been suggested that they arise from proteolytic cleavage of the intact peptide (31, 32). This latter mechanism may be operative in the case of transferrin receptor because significant proteolytic activity exists in the low pH environment or the prelysosomal endocytotic vesicle (33, 34). However, the mechanism of production of soluble transferrin receptor in human serum remains an important unanswered question.

In serum, the truncated receptor exists as a transferrin-receptor complex. This conclusion is based on the findings that transferrin co-purified with serum receptor in roughly equal amounts on the monoclonal antibody affinity column. The affinity of the truncated form of the receptor for transferrin has not been studied to date, but it is of interest that soluble receptor produced by HL60 cells in culture has roughly half the affinity of the intact receptor (35). The normal concentration of serum transferrin receptor is 6 mg/liter (11) as compared with 3000 mg/liter for transferrin, indicating that <1% of circulating transferrin is complexed with soluble receptor. Although transferrin complexed with its receptor fragment is likely to impair delivery of iron to cells, the proportion of transferrin complexed in this manner is too small to influence iron transport significantly. Additional studies on the kinetics of serum receptor and its binding characteristics with transferrin are needed.

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Aspects actuels des carences en fer et en folates dans le monde
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THE INFLUENCE OF BOVINE MILK PROTEINS ON IRON ABSORPTION IN MAN

R.F. HURRELL[a], R. BERROCAL[a],
S.R. LYNCH[b], S.A. DASSENKO[b], J.D. COOK[b]

[a]Nestlé Research Centre, Nestec Ltd.,
Vers-chez-les-Blanc, CH-1000 Lausanne 26, Switzerland

[b]Kansas University Medical Center, Kansas City KS 66103, USA

SUMMARY

Iron is more readily absorbed from human milk than from infant formula. Many studies with human milk have failed to demonstrate clearly that lactoferrin is the promoter of iron absorption. We have now investigated the inhibitory nature of bovine milk proteins, the major proteins in infant formula, on iron absorption in adult human volunteers. Bovine casein, bovine whey and a series of other commercial protein sources were added (30 g crude protein) to a liquid formula meal containing 35 g corn oil, 67 g hydrolysed maize starch, 12 ml vanillin and 200 ml water. Each volunteer received 3 test meals and a reference meal containing egg white as the protein source. The meals were labelled sequentially with ^{55}Fe and ^{59}Fe and iron absorption was measured relative to egg white (relative absorption (RA)=100). Mean iron absorption from the egg white meal was 2.5 - 6.7%. The protein-free meal gave the highest relative absorption of 353. The addition of all protein sources inhibited iron absorption. The degree of inhibition ranged from soy protein isolate (RA 20), whey (RA 40) and casein (RA 55), to BSA (RA 190) and beef muscle (RA 300). Prior enzyme hydrolysis of the casein and whey increased absorption about 2 fold, as did 80% enzymatic dephosphorylation of the casein. Iron absorption from the formula containing whey protein, which had been treated to remove inorganic calcium and phosphorus and 50% of its organic phosphorus (mainly phospholipids), increased 3 fold.

These results indicate that the phosphopeptides from bovine casein and higher levels of calcium and phosphorus, are both partly responsible for the low iron absorption from infant formula.

Iron is more readily absorbed from human milk than from infant formula. Studies in human infants using the balance method, the extrinsic tag technique, or the increase in total body iron, have indicated that iron absorption is 45-75% from human milk, 10-30% from cow's milk or 3.9-12% from infant formula (1-5). The much higher content of iron in fortified infant formula (8-12 mg/l) than in human milk (0.2-0.7 mg/l) accounts in part for the difference but, even at a similar iron concentration (0.8 mg/l), iron

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absorption from an unfortified milk-based infant formula was 12% compared to 49% from human milk (2). Studies in adults using the extrinsic tag technique show a similar pattern of results but the differences in absorption have been less pronounced. For instance, at the same iron concentration (0.7 mg/l), McMillan et al (6) reported 15.4% absorption from human milk compared to 9.0% absorption from an adapted formula.

The question arises as to whether the greater iron absorption from human milk than from cows milk based infant formula is due to enhancing substances, such as lactoferrin, in human milk, or to inhibitory substances, such as bovine milk proteins or calcium and phosphorus in the formula. Calcium for instance is 300 mg/l in human milk compared to 500-800 mg/l in formula; and phosphorus is 140 mg/l in human milk compared to 300-600 mg/l in the formula.

Human milk protein differs from cow's milk protein in relation to total protein content, the relative amounts of total casein and total whey (35:65 in human milk versus 79:21 for cow's milk) and in the nature of the individual caseins and whey proteins (Table 1). An adapted infant formula has a much closer protein content to human milk, a similar casein to whey ratio (40:60), but still differs with respect to individual proteins. There are three major differences in the content of individual proteins. Firstly lactoferrin is virtually absent from formula but is one of the most abundant proteins in human milk; secondly, β-lacto-globulin is almost half the total whey protein in the formula but is absent from human milk; and thirdly, over half of the total casein in the formula is in the form of αS1 and αS2 caseins, which are virtually absent from human milk. Human milk contains β-casein almost exclusively. This may be important in terms of iron absorption since α-caseins are more phosphorylated than β-casein. Bovine αS1 and αS2 casein contain 8-9 and 10-13 phosphate groups per mole respectively compared to 5-6 per mole for β-casein and 1 per mole for k-casein (9). Human β-casein appears to be even less phosphorylated than bovine β-casein. It has from 0-5 phosphate groups per mole but 3 per mole is most abundant (10). Another important difference is that infant formulas have been heat-processed and any cow's milk proteins with physiological activity will be denatured and de-activated.

Table 1

Typical Protein Composition of Human Milk, Cows Milk and an Adapted Milk-Based Infant Formula.

	Human		Cow		Adapted Formula (Theoretical)	
	g/100 g	%	g/100 g	%	g/100 g	%
αS1 casein		--		35		18
αS2 casein		--		10		5
β casein		34		26		13
k casein		1		8		4
Total Caseins	0.31	35	2.8	79	0.68	40
α-Lactalbumin		17		3.5		10
Lactoferrin		17		0.3		1
β-lactoglobulin		0		9		25
Serum albumin		6		1		3
Lysosyme		6		--		--
Immunoglobulins		11		3		9
Others		8		4.2		12
Total Whey Proteins	0.57	65	0.7	21	1.02	60
Total Protein	0.88	100	3.5	100	1.7	100

Adapted from References 7, 8 and 9.

To explain the differences in iron absorption between human milk and infant formula, most workers have concentrated on the iron-binding protein lactoferrin as the possible promoter of iron absorption from breast milk see review Lonnerdal (10). The exact role of lactoferrin however will remain unclear until iron absorption studies have can be made with human lactoferrin in human infants. In adults, McMillan et al (6) have reported that subjects consuming boiled human milk or untreated human milk had the same iron absorption. This indicates that lactoferrin does not play a role in iron absorption in adults since the iron-binding capacity of lactoferrin is irreversibly lost whenever temperatures above 70°C are applied even for a short time (11). In addition, iron bound to bovine lactoferrin is absorbed no higher than ferrous sulphate or ferric chloride in rats, mice, pigs or infants (10, 12, 13). On the other hand, human lactoferrin is little digested in the alimentary tract of infants and it retains its iron at low pH; and the finding of lactoferrin receptors in the human jejunum still

points to a role for lactoferrin as the delivery system of iron in breast milk (10).

We have now investigated (14) the inhibitory nature of bovine casein and bovine whey protein on iron absorption by using the in vitro dialysable iron technique (15) and the extrinsic tag technique in adult man. Using the dialysable iron technique, we have investigated the effect of substituting different protein sources and pre-hydrolysed proteins into a liquid formula meal. The meal, consisting of the protein source, maltodextrin, corn oil and water, was digested for 2 h at pH 2 with pepsin, followed by a 2 h pancreatin digest during which dialysis took place with a molecular weight cut-off of 6-8000. Soluble iron passed into the dialysis bag from the digest and bicarbonate passed in the opposite direction raising the pH of the digest to 7.

The results are shown in table 2. With no protein in the formula, 5.3% of iron was dialysed. The addition of most protein sources reduced dialysable iron. Bovine whey, bovine casein, soy isolate and wheat gluten showed the strongest inhibition. Beef muscle had little effect on this system and only BSA was enhancing. This study shows that iron can bind to the digestion products of bovine casein and whey and that the iron-binding peptides from these proteins were too insoluble or too big to pass through a membrane with a cut-off point of between 6000-8000 molecular weight.

Table 2

Influence of Different Protein Sources on Dialysable Iron In Vitro

	% mean dialysable iron
Bovine serum albumin	13.3
Beef muscle	5.8
No protein	5.3
Egg albumen	3.2
Zein	2.8
Gelatin	2.0
Whey protein	1.0
Casein	0.6
Soy protein isolate	0.5
Wheat gluten	0.5
Pooled Standard error of the mean	0.11

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In a subsequent study we investigated the influence of prior enzyme hydrolysis on casein and whey proteins. Commercial products, pre-hydrolysed to different extents, were substituted into the formula meal. With both protein sources, increasing the extent of prior protein hydrolysis progressively increased the dialysable iron (Table 3). This study confirms that iron binds to the larger peptides produced during the digestion of casein and whey proteins and shows that the smaller peptides and amino acids formed on further digestion still chelate iron but in soluble forms small enough to pass the membrane.

Table 3

Influence of prior hydrolysis of bovine casein and whey on dialysable iron

	Apoprox. % Hydrolysis	% Mean dialysable Iron
CASEIN		
- intact	0	0.6
- enzyme-hydrolysed	8	0.4
	41	3.4
	59	7.5
	63	5.4
	84	14.0
- acid-hydrolysed	88	12.5
WHEY PROTEIN		
- intact	0	0.9
- enzyme hydrolysed	8	1.6
	11	0.6
	28	7.2
	60	11.8
	66	12.8
Pooled Standard error of the mean		0.3

For the human studies, we used the extrinsic tag technique with ^{55}Fe and ^{59}Fe and made 7 separate studies with 6-12 subjects per study. In each study, 4 separate iron absorption measures were made on each subject consuming 4 different meals. One meal in each study was a standard liquid formula meal containing egg white, maltodextrin, corn oil and water. Iron was adjusted to 4.1 mg. In the test meals, other protein sources were substituted for egg white isonitrogenously.

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Table 4 shows the relative effect of different protein sources on non-haem iron absorption by man from the liquid formula meal. The results are given as the geometric mean of iron absorption for both the substitute protein and the egg white control, and as the relative absorption of the test protein compared to egg white. This allowed us to make comparisons between studies in which the iron status of the subjects were different. With the exception of BSA, the relative effects of the different proteins on iron absorption were similar to their effect on dialysable iron. In the absence of protein, 10.6% of iron was absorbed from the formula meal. Addition of egg white reduced iron absorption to 3.0%. The relative absorption of the no protein meal compared to egg white was 350. The addition of beef muscle had little effect on relative iron absorption whereas the addition of all other proteins clearly inhibited iron absorption, with casein having a relative absorption of 55 and whey 40. Soy protein isolate was the most inhibitory with a relative absorption of 20. Prior-enzyme hydrolysis of whey (16% and 36%) increased iron absorption slightly and 84% pre-hydrolysis of casein doubled iron absorption.

Table 4

Relative effect of different protein sources on the non-haem iron absorption by man from a liquid formula meal

Substitute Protein	Mean % Fe Absorption: Substitute (A)	Egg White (B)	Relative Absorption (A/B x 100)
No Protein	10.6*	3.0	350
Beef Muscle	5.1*	1.7	300
Bovine Serum Albumin	5.7*	3.0	190
Casein	3.7	6.7	55
Whey protein	1.0*	2.5	40
Wheat gluten	2.1*	6.7	31
Soy protein isolate	0.5*	2.5	20

* Significantly different from egg white ($P<0.05$)

Hydrolysed protein

Whey (16%)	1.1	2.5	44
Whey (36%)	1.7	2.5	68
Casein (84%)	7.7**	6.7	115

** Significantly different from unhydrolysed protein ($P<0.05$)

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The inhibitory nature of bovine casein is presumably due to the binding of iron to the phosphopeptides formed in the alimentary tract on digestion. In further studies, we have shown that 80% dephosphorylation of casein prior to feeding increased the relative absorption of iron two fold. The inhibitory nature of bovine whey however does not appear to be a protein effect. It appears to result mainly from the relatively high level of inorganic calcium and phosphorus in whey and perhaps also partly from the organic phosphorus compounds present. The organic phosphorus, which represents 20-25% of the total phosphorus content in whey, is found mainly in phosphoserine esters, phosphorylated sugars and phospholipids (16). We have shown recently that removal of the inorganic calcium and phosphorus from whey by ion exchange resins increased relative iron absorption by a factor of 3. Removal of about 50% of the organic phosphorus (the phospholipids) by microfiltration further increased relative iron absorption to a value similar to that from the formula containing bovine serum albumin (Table 4).

These results in adults indicate that phosphopeptides from bovine casein, inorganic calcium and phosphorus and perhaps also phospholipids are probably responsable for the low iron absorption from infant formula. By careful technology, it should be theoretically possible to manufacture a milk-based formula from which iron can be absorbed by adults to the same extent as from human milk. In infants, however, the possibilities of modifying the present formulas are more restricted since the infant requires certain minimum quantities of calcium and phophorus for optimum bone formation. It might be possible however to reduce the organic phosphorus in the casein and whey, and the total calcium content in the present formulas, both by some 20-30%, and still produce a formula containing the recommended levels of calcium and phosphorus. Whether this would improve iron absorption in infants or have any adverse effect on bone formation or other parameters remains to be investigated.

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RESUME

Le fer est plus facilement absorbé à partir de lait humain qu'à partir d'une formule infantile. De nombreuses études utilisant le lait humain n'ont pas pu démontrer clairement que la lactoferrine etait le promoteur de l'absorption du fer. Nous avons à présent étudié la nature inhibitrice des protéines du lait de vache, les principales protéines d'une formule infantile, chez des volontaires humains adultes. La caséine bovine, le petit lait et une série d'autres sources commerciales de protéines ont été ajoutées (30 g de protéines brutes) à un repas liquide contenant 35 g d'huile de maïs, 67 g d'amidon de maïs hydrolysé, 12 ml de vanille et 200 ml d'eau. Chaque volontaire a reçu 3 repas-test et un repas de référence contenant du blanc d'oeuf comme source de protéines. Les repas ont été marqués séquentiellement avec 55Fe et 59Fe et l'absorption du fer a été mesurée relativement à celle du repas à base de blanc d'oeuf (absorption relative (AR) = 100). L'absorption moyenne du fer du repas à base de blanc d'oeuf était 2.5 - 6.7 %. Le repas ne contenant aucune protéine a donné la plus forte absorption relative : 353. L'ajout de toutes les sources de protéines a inhibé l'absorption du fer. Le degré d'inhibition diminuait graduellement de l'isolat de protéines de soja (AR 20), au petit lait (AR 40) et à la caséine (AR 55), au BSA (AR 190) et au muscle de boeuf (AR 300). L'hydrolyse préalable de la caséine et du petit lait a environ doublé l'absorption, tout comme la déphosphorylation enzymatique à 80 % de la caséine. L'absorption du fer de la formule contenant des protéines du petit lait a triplé lorsqu'elles ont été traitées pour ôter le calcium et le phosphore inorganiques et 50 % du phosphore organique (phospholipides essentiellement).
Ces résultats indiquent que les phosphopeptides issus de la caséine bovine et les niveaux élevés en calcium et phosphore, sont tous deux partiellement responsables de la faible absorption du fer contenu dans les formules infantiles.

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Ferrous fumarate fortification of a chocolate drink powder

BY RICHARD F. HURRELL

Nestec Ltd, Nestlé Research Centre, Vers-chez-les Blanc, PO Box 44, CH-1000 Lausanne 26, Switzerland

AND MANJU B. REDDY, SANDRA A. DASSENKO AND JAMES D. COOK

University of Kansas Medical Centre, 39th and Rainbow Boulevard, Kansas City, Kansas 66103, USA

AND DAVID SHEPHERD

Nestec Ltd, Linor, Centre Développement Alimentaire, 1350 Linor, Switzerland

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An evaluation was made into the usefulness of ferrous fumarate as an iron fortificant for an experimental chocolate drink powder targetted to children and adolescents. Organoleptically ferrous fumarate was acceptable when the chocolate drink powder was reconstituted in milk or water that was heated to < 80°. Unacceptable colour changes occurred, however, when boiling milk or water were used. In human Fe absorption studies when the Fe compounds were added to the chocolate drink immediately before consumption, ferrous fumarate was 3·31 % absorbed compared with 2·82 % for ferrous sulphate and 2·11 % for ferric pyrophosphate. When the Fe compounds were processed during the manufacture of the chocolate drink powder, the absorption of ferrous fumarate was 5·27 %, ferrous sulphate 2·62 % and ferric pyrophosphate 0·55 %. Ascorbic acid had little or no effect on the absorption of ferrous fumarate. It is concluded that food processing can influence the relative absorption of fortification Fe and that, if not reconstituted with boiling milk or water, ferrous fumarate could be a useful compound for the fortification of chocolate drink powders.

Iron fortification: Iron bioavailability: Food processing

Iron deficiency continues to be a common nutritional problem at the present time. There is evidence, however, that its prevalence is declining in certain segments of the population in industrialized countries due mainly to more effective intervention programmes. For example, in the US consumption of fortified infant formulas instead of cow's milk has resulted in a significant reduction in the prevalence of Fe deficiency during infancy (Yip *et al.* 1987*a,b*; American Academy of Pediatrics, Committee on Nutrition, 1989), one of the most vulnerable periods of life with respect to Fe balance. There is also some evidence that the prevalence of Fe deficiency in menstruating women is declining in industrialized countries (Pilch & Senti, 1984). The one segment of the population that remains highly vulnerable to Fe deficiency, however, is school-aged children because their Fe requirements for growth often exceed the dietary supply of Fe (Bothwell *et al.* 1979). Fe requirements for adolescents may be increased further by strenuous athletic programmes and, in turn, their athletic performance may be impaired by the development of Fe deficiency (Rowland *et al.* 1988; Rowland & Kelleher, 1989).

Several strategies have been proposed to reduce the prevalence of Fe deficiency in schoolchildren. Fe supplements are effective, but the prevalence of Fe deficiency is not usually high enough to justify the use of medicinal Fe. The Fe intake of children can be increased by fortifying a dietary staple such as wheat products or by fortifying a widely

consumed foodstuff such as sugar, but this may be too costly for Third World countries. The most cost-effective approach to increasing the Fe intake of children is targetted fortification of a speciality food item that is used selectively in this age-group. The vehicle should be appealing to children and ideally should provide other nutritional benefits. One vehicle that has several potential advantages is a chocolate-flavoured milk beverage. The consumption of this product is largely limited to school-age children and would not be consumed extensively by adult men who seldom require additional Fe. Furthermore, this product would also increase the intake of vitamins and other minerals such as zinc and calcium which is also desirable in children.

Organoleptic problems, however, are common when foods are fortified with Fe (Hurrell, 1984), and chocolate milk drinks have proved particularly difficult to fortify because of unacceptable colour problems with ferrous sulphate and other highly soluble Fe compounds (Douglas *et al.* 1981). In the present study, we have assessed the usefulness of ferrous fumarate as an Fe fortificant in a chocolate drink powder. This Fe compound was recently found to be suitable for the fortification of infant cereals, another foodstuff which is difficult to fortify with Fe because of organoleptic problems (Hurrell *et al.* 1989*a*). After first evaluating the colour and flavour of the chocolate drink with added ferrous fumarate, radio-Fe absorption measurements were made in human subjects to determine the bioavailability of ferrous fumarate relative to ferrous sulphate and ferric pyrophosphate, to evaluate the influence of heat processing on the relative absorption (RA) of the different fortification Fe compounds, to quantify the influence of added ascorbic acid, and to estimate the amount of absorbed Fe that would result from fortification.

MATERIALS AND METHODS

Test materials

Chocolate drink powder. The test meal was based on an experimental malted chocolate drink powder designed to be added to hot or cold milk or water. The experimental batches were manufactured in a pilot plant (Linor, Orbe, Switzerland) by drying under vacuum for 3 h at 95° an aqueous slurry of malt, cocoa, skim milk powder, sugar, butter oil, mineral salts and vitamins. The mineral and vitamin mixtures were calculated to provide about 20% of the recommended dietary allowances (National Academy of Sciences, 1980) for 11- to 14-year-old boys in one serving.

Chocolate drink powders for Fe absorption studies. Seven different batches of chocolate drink powder were produced. All were of identical composition except for the levels of Fe and ascorbic acid. Batch 1 contained all the normal ingredients except the fortification Fe (the test Fe sources were added to the product before consumption). In batches 2 and 3, ^{55}Fe-labelled ferrous fumarate and ^{55}Fe-labelled ferric pyrophosphate respectively were added to the slurry before processing. Batches 4 and 5 contained ^{59}Fe-labelled ferrous sulphate added to the slurry before processing. Batch 6 contained ^{55}Fe-labelled ferrous fumarate but no ascorbic acid; and batch 7 contained no added Fe and no added ascorbic acid. The Fe and ascorbic acid contents of the different products are shown in Table 1; the amount of radioactivity was such as to provide about 37 MBq ^{59}Fe or about 80 MBq ^{55}Fe in two 25 g servings.

Radioactive Fe compounds. The ^{55}Fe-labelled ferrous fumarate and ferric pyrophosphate were the same compounds as used previously by Hurrell *et al.* (1989*a*). They were prepared by the Dr Paul Lohman Co. (Emmerthal, Germany) using a scaled-down version of the normal manufacturing procedures. The radioactive Fe sources were similar in appearance, Fe content, particle size, and solubility in dilute hydrochloric acid to their commercial counterparts. The measured Fe content of ferrous fumarate was 320 g/kg and of ferric

Table 1. *Iron and ascorbic acid contents (mg/25 g serving) of experimental chocolate drink powders*

Batch no.		Fe	Ascorbic acid
1	No added Fe	0·5	26·4
2	^{55}Fe-labelled ferrous fumarate	4·7	24·8
3	^{55}Fe-labelled ferric pyrophosphate	5·1	26·6
4	^{59}Fe-labelled ferrous sulphate	5·1	28·4
5	^{59}Fe-labelled ferrous sulphate	4·8	25·4
6	^{55}Fe-labelled ferrous fumarate no added ascorbic acid	4·7	4·2
7	No added Fe, no added ascorbic acid	0·5	8·6

pyrophosphate 251 g/kg. The labelled ferrous sulphate was prepared by dissolving 1·4 g hydrated ferrous sulphate ($FeSO_4.7H_2O$) in 50 ml deionized water, adding the requisite amount of ^{59}Fe-labelled ferrous sulphate (New England Nuclear), evaporating under vacuum to approximately 10 ml and then freeze-drying overnight. The Fe content of the dried radiolabelled ferrous sulphate was 330 g/kg, indicating that it was in the anhydrous form.

Organoleptic evaluation

The organoleptic tests were made with chocolate drink powders fortified with non-radioactive ferrous fumarate and ferric pyrophosphate. Ferrous sulphate has long been known to cause unacceptable colour changes in such products and was not investigated further. Ferric pyrophosphate, on the other hand, causes no organoleptic problems and is added to similar products sold commercially.

Non-radioactive ferrous fumarate and ferric pyrophosphate were obtained from the Dr Paul Lohman Co. The fortified chocolate drink powders were reconstituted at 150 g/l with cold, hot (80°) and boiling milk or water. Colour and taste were evaluated by an experienced panel.

Fe absorption studies

Four Fe absorption studies were made and, in each study, four separate Fe absorption measurements were performed on each of eight to eleven subjects. The feeding protocol for studies 1–3 is shown in Table 2. In the first study, the subjects were given the chocolate drink powder containing no added Fe (batch 1), but supplemented at the time of feeding with ^{55}Fe-labelled ferrous fumarate, ^{55}Fe-labelled ferric pyrophosphate or ^{59}Fe-labelled ferrous sulphate. Meal 4 in the first study was ^{59}Fe-labelled ferrous sulphate added before processing (batch 4). In the second study, the radioactive Fe compounds were added to the chocolate drink powders before heat processing (batches 2, 3 and 5). Meal 4 in the second study was a reference meal. In the third study the influence of added ascorbic acid was investigated with and without ^{55}Fe-labelled ferrous fumarate added before processing (batches 1, 2, 6 and 7). An extrinsic tag containing 37 MBq $^{59}FeCl_3$ with 0·1 mg $FeCl_3.6H_2O$ in 1 ml 0·01 M-HCl was added to the chocolate drinks made from batches 1 and 7 before feeding.

The fourth study was made with a semi-synthetic liquid formula meal and not with the chocolate milk drink. It was made to investigate whether non-processed ^{55}Fe-labelled ferrous fumarate consumed with a meal enters the common Fe pool and to determine to what extent the absorption of this Fe is influenced by added ascorbic acid.

Table 2. *Studies 1–3. Feeding protocol for chocolate drink powders*

Meal	Days	Study 1	Study 2	Study 3
1	1, 2	Batch 1* plus ^{55}Fe-labelled ferrous fumarate	Batch 2	Batch 1 plus extrinsic tag of ^{59}Fe-labelled ferric chloride
2	3, 4	Batch 1 plus ^{59}Fe-labelled ferrous sulphate	Batch 5	Batch 2
3	15, 16	Batch 1 plus ^{55}Fe-labelled ferric pyrophosphate	Batch 3	Batch 7 plus extrinsic tag of ^{59}Fe-labelled ferric chloride
4	17, 18	Batch 4	Reference meal	Batch 6

* For details of different batches, see Table 1.

Test meals. The test meals in studies 1–3 consisted of 25 g chocolate drink powder which was thoroughly mixed with 200 ml cold milk (20 g fat/l) immediately before serving. The Fe-fortified meals contained about 5 mg Fe and those fortified with ascorbic acid contained about 25 mg ascorbic acid (Table 1). To permit extrapolation of the absorption data obtained in the present report to other investigations, one group of subjects in study 2 was given a standard reference dose of inorganic Fe. This test dose contained 3 mg Fe ^{50}Fe-labelled $FeSO_4.7H_2O$ and 18·9 mg ascorbic acid (molar ratio 2:1) in 50 ml water.

The semi-synthetic liquid formula meal fed in study 4 was the same meal as fed in previous studies (Hurrell *et al.* 1989*b*) and contained 35 g egg white (Monark Egg Corp., Kansas City, MO), 67 g hydrolysed maize starch (Fro-Dex 36, American Maize Products Co, Hammond, IN), 35 g maize oil (Nugget Brand, Stockton, CA), 12 ml vanillin extract (McCormick and Co., Inc., Baltimore, MD), and 200 ml deionized distilled water. This meal contained about 0·6 mg intrinsic Fe.

Subjects. Subjects ranged in age from 20 to 40 years with a mean age of 29 years. There were ten men and twenty-eight women. There was a wide range of Fe status as reflected by their serum ferritin levels. However, none was anaemic as defined by a packed cell volume below 0·36 in women and 0·39 in men. All of the subjects were in good health and denied a history of disorders that are known to influence gastrointestinal absorption of Fe.

Written, informed consent was obtained from each volunteer before beginning the investigation and all experimental procedures were approved by the human subjects committee at the University of Kansas Medical Center.

Absorption measurements. All radioactivity-labelled meals were given between 07.00 and 09.00 hours following a 10 h fast. Water, but not food, was allowed for 3 h following each meal. On the day preceding the first test meal, 15 ml blood were obtained for measurement of packed cell volume, serum ferritin, and background blood radioactivity. In studies 1–3, a test meal tagged with 41 MBq ^{55}Fe was given on the following two mornings and a second test meal tagged with 18·5 MBq ^{59}Fe was given on the third and fourth mornings. At 2 weeks following administration of the first test meal, 25 ml blood were drawn for measurements of blood radioactivity and serum ferritin. Two additional test meals labelled separately with the same amounts of ^{55}Fe and ^{59}Fe were again fed in the same way on four consecutive days and a final blood sample was drawn 2 weeks following the last test meal so as to measure the increase in blood radioactivity.

In study 4 only two meals were fed, each on one occasion only, and each was labelled simultaneously with both ^{55}Fe and ^{59}Fe. Thus in contrast to the previous three studies in which each meal contained one tag only, in this study both radio-Fe tags were present in

the same meal, one labelling the fortification Fe and the other labelling the native Fe. The semi-synthetic liquid formula meal labelled with 7·2 mg Fe as ^{55}Fe-labelled ferrous fumarate (74 MBq) and with 37 MBq $^{59}FeCl_3$ added as an extrinsic tag with 0·1 mg $FeCl_3.6H_2O$ was given on day 1. After 2 weeks 25 ml blood were withdrawn for analysis and the formula meal was again given, labelled simultaneously with 7·2 mg ^{55}Fe-labelled ferrous fumarate and 37 MBq $^{59}FeCl_3$. On this occasion 100 mg ascorbic acid were also added. The final blood sample was drawn 2 weeks later.

Measurements of erythrocyte ^{55}Fe and ^{59}Fe were performed on duplicate 10 ml samples of whole blood using a modification of the method of Eakins & Brown (Bothwell *et al.* 1979). Percentage absorption was calculated on the basis of blood volume estimated from height and weight (Wennesland *et al.* 1959; Brown *et al.* 1962). Erythrocyte incorporation of absorbed radioactivity was assumed to be 80% (Hosein *et al.* 1967).

Statistical analysis

Percentage absorption values were converted to logarithms before statistical analysis and the results reconverted as antilogarithms to recover the original units (Cook *et al.* 1969). Paired *t* tests were used to compare absorption from any two test meals within the same study by determining whether mean log absorption ratios differed significantly from zero.

RESULTS

Organoleptic studies

Chocolate drink powders fortified with ferric pyrophosphate and reconstituted with cold, hot (80°) and boiling water or milk were judged to be acceptable with respect to colour and taste. Similarly chocolate drink powders fortified with ferrous fumarate and reconstituted with cold or hot (80°) milk or water were acceptable. The ferrous fumarate-fortified products reconstituted with boiling water or milk, however, changed colour from red/brown to an unacceptable grey.

Fe absorption studies

In the first two studies, we investigated the effect of processing (vacuum drying) on the relative absorption of three different Fe sources from a chocolate drink powder. In the first study, the Fe sources (ferrous fumarate, ferrous sulphate and ferric pyrophosphate) were added to the chocolate drink powder just before consumption and thus received no processing. In the second study, the same Fe sources were added to the ingredients of the chocolate drink powder and were subjected to all the processing steps during the manufacture of the product.

The results of the first study are shown in Table 3. When added to the chocolate drink powder just before consumption, mean absorption was 3·31% for ferrous fumarate, 2·82% for ferrous sulphate, and 2·11% for ferric pyrophosphate. None of these differences was significant ($P > 0·10$). Moreover, there appeared to be no significant effect of processing on the absorption of ferrous sulphate which averaged 3·52% when added before processing and 2·82% when added to the final product ($P > 0·30$). The only pair of test meals from which absorption differed significantly was ferric pyrophosphate added after processing (2·11%) and ferrous sulphate added before processing (3·52%, $P = 0·008$). It should be noted that the sensitivity of the comparison between these meals was enhanced because they were given on consecutive days rather than separated by a 2-week interval. The variance of absorption ratios is usually appreciably lower when tests are performed simultaneously rather than sequentially. Compared with ferrous sulphate (RA = 1·00), the

Table 3. *Study 1. Absorption of iron-fortification compounds added to a chocolate drink powder immediately before consumption by adult subjects**

Subject no.	Sex	Age (years)	Packed cell volume	Serum ferritin (ng/ml)	Fe absorption (% dose): Ferrous fumarate (A)	Ferrous sulphate (B)	Ferric pyrophosphate (C)	Ferrous sulphate (heat processed) (D)	Relative absorption†: A:B	C:B	D:B
1	♂	22	0·50	200	1·31	1·36	2·05	3·53	0·96	1·51	2·60
2	♂	22	0·43	130	1·12	0·90	1·26	1·58	1·24	1·40	1·76
3	♂	24	0·49	76	0·78	1·66	1·05	1·75	0·47	0·63	1·05
4	♀	21	0·42	74	4·33	2·46	3·87	2·33	1·76	1·57	0·95
5	♀	35	0·44	45	5·43	1·15	1·33	1·66	4·72	1·15	1·44
6	♂	22	0·44	44	2·51	2·21	1·83	4·15	1·14	0·82	1·88
7	♂	22	0·47	31	2·81	3·02	1·75	3·00	0·93	0·58	0·99
8	♀	21	0·41	20	1·67	1·92	2·01	6·02	0·87	1·04	3·14
9	♀	28	0·39	17	12·60	11·63	2·72	7·71	1·08	0·22	0·66
10	♀	33	0·37	11	39·17	36·96	7·17	13·52	1·06	0·19	0·37
Mean‡		25	0·44	45	3·31	2·82	2·11	3·52	1·17	0·75	1·25
− 1 S.E.					2·26	1·96	1·76	2·81	0·97	0·59	1·02
+ 1 S.E.					4·83	4·05	2·53	4·42	1·41	0·95	1·53

* For details, see p. 000.
† Absorption relative to ferrous sulphate added after processing.
‡ Geometric mean except for age and packed cell volume.

h by adult subjects

Table 4. *Study 2. Absorption of iron compounds added to a chocolate drink powder during manufacture and subjected to processing, ~~by~~ adult-subjects**

Subject no.	Sex	Age (years)	Packed cell volume	Serum ferritin (ng/ml)	Fe absorption (% dose): Ferrous fumarate (A)	Ferrous sulphate (B)	Ferric pyrophosphate (C)	Reference dose (D)	Relative absorption†: A:B	C:B	D:B
1	♂	22	0·44	106	1·73	1·08	0·50	16·41	1·60	0·46	15·19
2	♀	31	0·39	83	2·03	0·93	0·07	19·13	2·18	0·07	20·56
3	♀	20	0·39	43	3·01	2·42	0·30	14·21	1·24	0·12	5·87
4	♀	29	0·41	31	1·56	1·42	0·35	31·28	1·09	0·24	22·02
5	♂	25	0·46	25	11·23	4·91	0·65	41·42	2·29	0·13	8·45
6	♀	29	0·43	16	5·20	4·41	0·31	101·63	1·17	0·07	23·04
7	♂	24	0·43	11	2·28	0·23	0·61	18·96	9·91	2·69	82·43
8	♀	24	0·39	9	67·23	24·93	6·35	73·73	2·69	0·25	2·95
9	♀	21	0·39	5	21·30	13·60	1·58	24·06	1·56	0·11	1·76
Mean‡		25	0·41	24	5·27	2·62	0·55	30·01	2·01	0·21	11·45
−1 S.E.					3·41	1·62	0·37	23·86	1·61	0·14	7·74
+1 S.E.					8·15	4·24	0·83	37·75	2·52	0·31	16·96

* For details, see p. 000.
† Absorption relative to ferrous sulphate.
‡ Geometric mean except for age and packed cell volume.

Table 5. *Study 3. Effect of ascorbic acid (25 mg) on absorption by human subjects of native iron or ferrous fumarate added to a chocolate drink powder**

Subject no.	Sex	Age (years)	Packed cell volume	Serum ferritin (ng/ml)	Fe absorption (% dose): No ascorbic acid: Native Fe (A)	No ascorbic acid: Ferrous fumarate (B)	With ascorbic acid: Native Fe (C)	With ascorbic acid: Ferrous fumarate (D)	Absorption ratio: B:A	C:A	D:B	D:C
1	♂	38	0·45	209	1·00	1·12	1·21	0·96	1·12	1·21	0·85	0·79
2	♂	39	0·43	185	0·12	1·30	0·92	0·47	10·83	7·66	0·36	0·51
3	♀	29	0·47	63	2·28	5·42	2·55	2·77	2·37	1·11	0·51	1·09
4	♀	22	0·41	41	1·17	1·20	2·20	1·86	1·02	1·88	1·55	0·86
5	♀	27	0·44	40	1·36	1·90	1·10	1·07	1·39	0·80	0·56	0·97
6	♀	24	0·38	34	2·52	2·31	2·60	1·93	0·91	1·03	0·83	0·74
7	♀	28	0·40	27	2·56	3·18	1·15	1·52	1·24	0·44	0·47	1·32
8	♀	22	0·43	26	1·00	1·28	5·16	4·02	1·28	5·16	3·14	0·78
9	♀	32	0·37	14	9·10	13·08	6·98	3·15	1·43	0·76	0·24	0·45
10	♀	20	0·42	6	3·00	11·78	9·06	7·45	3·92	3·02	0·63	0·82
11	♀	21	0·37	3	8·23	23·08	8·72	16·07	2·80	1·05	0·69	1·84
Mean†		27	0·42	31	1·80	3·35	2·71	2·33	1·86	1·51	0·70	0·86
−1 S.E.					1·26	2·41	2·09	1·73	1·49	1·16	0·56	0·76
+1 S.E.					2·56	4·66	3·52	3·14	2·33	1·96	0·86	0·97

* For details, see p. 000.

† Geometric mean except age and packed cell volume.

Table 6. *Study 4. Effect of ascorbic acid (100 mg) on absorption by human subjects of native iron or ferrous fumarate added to a synthetic liquid formula meal**

					Fe absorption (% dose)							
					No ascorbic acid		With ascorbic acid					
Subject no.	Sex	Age (years)	Packed cell volume	Serum ferritin (ng/ml)	Native Fe (A)	Ferrous fumarate (B)	Native Fe (C)	Ferrous fumarate (D)	Absorption ratio B:A	C:A	D:B	D:C
1	♀	28	0·40	103	4·77	4·58	2·15	2·13	0·96	0·45	0·47	0·99
2	♀	40	0·40	91	7·05	10·81	9·96	11·36	1·53	~~6·61~~ 1·41	~~4·74~~ 1·05	1·18 1·14
3	♀	28	0·42	78	0·60	1·16	4·18	5·30	1·93	6·97	4·56	1·27
4	♀	29	0·41	37	6·78	15·07	14·68	13·57	2·22	2·17	0·90	0·92
5	♀	32	0·41	22	3·25	5·26	6·16	7·03	1·62	1·90	1·34	1·14
6	♀	37	0·39	13	26·35	23·38	33·32	31·31	0·88	1·26	1·34	0·94
7	♀	38	0·36	12	2·36	4·17	9·43	13·17	1·77	4·00	3·16	1·40
8	♀	35	0·44	9	9·92	15·25	46·57	51·25	1·54	4·69	3·36	1·10
Mean†		33	0·40	31	4·78	7·14	10·21	11·26	1·49	2·14	1·58	1·10
−1 s.e.					3·23	5·07	7·11	7·91	1·33	1·57	1·20	1·05
+1 s.e.					7·09	10·07	14·66	16·02	1·67	2·90	2·07	1·16

1·41 1·05 1·14

* For details, see p. 000.

† Geometric mean except age and packed cell volume.

RA of ferrous fumarate not subjected to processing was 1·17 and the RA for ferric pyrophospyhate was 0·75.

In contrast to these small differences in absorption for the Fe compounds not subjected to processing, marked differences in their absorption occurred when they were added to the ingredients of the chocolate drink powder before processing (Table 4). Mean absorption was 5·27% for ferrous fumarate, 2·62% for ferrous sulphate and 0·55% for ferric pyrophosphate. The absorption of ferric pyrophosphate was < 1% in all but two subjects, and in none of the subjects was the absorption of ferrous sulphate or ferric pyrophosphate higher than that of ferrous fumarate. The mean absorption of ferrous fumarate was significantly greater than ferrous sulphate ($P = 0{\cdot}016$) and ferric pyrophosphate ($P < 0{\cdot}00001$). The RA of ferrous fumarate subjected to processing was 2·01 whereas that for ferric pyrophosphate was only 0·21. Reference dose absorption averaged 30% in these relatively-Fe-depleted subjects (mean serum ferritin 24 μg/l). This represents an eleven-fold inhibiting effect of chocolate milk powder on absorption of a comparable dose of ferrous sulphate.

The third study was performed to evaluate the influence of ascorbic acid on Fe absorption from the chocolate drink powder and to calculate the contribution of the absorbed ferrous fumarate to the Fe requirements of the subjects. In eleven subjects, absorption of the native Fe from the non-Fe-fortified milk drink ranged from 0·12 to 9·1%, with a geometric mean of 1·8% (Table 5). Surprisingly, mean absorption increased appreciably to 3·35% when the powdered formula was fortified with ferrous fumarate (absorption ratio 1·86; $P = 0{\cdot}022$). The second pair of test meals were given specifically to examine the effect of fortifying the product with ascorbic acid. The ascorbic acid content of the chocolate powder without and with added ferrous fumarate was 26 and 25 mg serving respectively. Nearly identical mean absorption values of 2·71 and 2·33% were observed and these did not differ significantly from values obtained without added ascorbic acid ($P > 0{\cdot}10$), although the absorption of the native Fe was increased by 50%.

The absorption of Fe from the liquid formula meal to which the two isotopes had been added simultaneously was 4·78% for the native Fe and 7·14% for the ferrous fumarate (Table 6). These values were significantly different ($P < 0{\cdot}05$). The addition of ascorbic acid increased the absorption of the native Fe to 10·21% ($P < 0{\cdot}05$) and that of the ferrous fumarate to 11·26%. This latter increase was not significant ($P > 0{\cdot}05$), neither was the difference in absorption between the two tags.

DISCUSSION

The Fe compounds of high bioavailability, such as ferrous sulphate and ferrous gluconate, cause unacceptable colour and flavour changes in many food vehicles. Less bioavailable, but more inert Fe sources, such as ferric pyrophosphate and certain forms of elemental Fe have thus been commonly used to fortify foods such as infant cereals and chocolate drink powders. Although these Fe compounds are less well absorbed than ferrous sulphate, they cause no organoleptic problems.

Recently, two alternative Fe compounds were proposed for the fortification of infant cereals (Hurrell *et al.* 1989*a*). These compounds, ferrous fumarate and ferrous succinate, were shown to be as well absorbed as ferrous sulphate but, unlike ferrous sulphate, did not cause organoleptic problems. These findings prompted the present re-assessment of Fe compounds used to fortify chocolate drink powders. From an organoleptic viewpoint, ferrous fumarate was judged to be an acceptable Fe fortificant for chocolate drink powders provided that the powder was mixed with cold milk for consumption, as in the present study, or was mixed with hot water or milk up to a temperature of 80°. Adding the ferrous

fumarate-fortified powder to boiling water or milk resulted in the typical red/brown colour of the product being transformed into an unacceptable grey colour. This latter finding would unfortunately exclude the use of ferrous fumarate in commercially sold chocolate drink powders.

The most unexpected finding from the present study, however, was the marked effect which processing (vacuum drying) had on the relative absorption of the different Fe compounds. When the Fe compounds were added to the chocolate drink immediately before consumption, thus avoiding processing, the RA values (relative to unprocessed ferrous sulphate = 1·00) were 1·17 for ferrous fumarate and 0·75 for ferric pyrophosphate. The RA value for ferrous fumarate was similar to that obtained in previous studies, although that for ferric pyrophosphate was somewhat higher than previously reported (Hurrell *et al.* 1989*a*). These small non-significant differences in RA of the unprocessed Fe compounds became highly significant differences after they had been processed with the chocolate drink powder. After processing, the Fe from ferrous fumarate was ten times better absorbed than that from ferric pyrophosphate and twice as well absorbed as that from ferrous sulphate, whose absorption changed little on processing (Table 3).

The influence of heat sterilization on the relative bioavailability (RBV) of Fe compounds in rats has already been studied to some extent in liquid products. In agreement with our own results, these studies have shown that the RBV of ferrous sulphate is unchanged by heat processing (Theuer *et al.* 1971, 1973; Wood *et al.* 1978). In contrast to our findings, however, the RBV of ferric pyrophosphate and other insoluble Fe compounds was shown to increase with processing. Theuer *et al.* (1971, 1973), using the haemoglobin repletion test in rats, determined the RBV of Fe salts in liquid soya-bean and milk-based infant formulas. In both types of formula heat sterilization increased the RBV of ferric pyrophosphate. The increase in RBV was from 39 to 93 in the soya-bean formula and from 78 to 125 in the milk formula. Wood *et al.* (1978) similarly found that heat treatment of a chicken diet as a liquid slurry increased the RBV of ferric pyrophosphate to chicks from 7 to 90. On the other hand, in human studies, the baking of bread seemed to have little positive effect on the RA of ferric orthophosphate (Cook *et al.* 1973), or of carbonyl Fe (Hallberg *et al.* 1986). Different methods of heat processing may, therefore, have opposite effects on the bioavailability of an Fe compound. This must be due to the influence of the processing method on the solubility of the food Fe in the gastrointestinal tract.

The Fe in the chocolate drink powder processed with ferrous fumarate was unusual as it did not appear to enter the common Fe pool. This is indicated by the finding that after processing the absorption from ferrous fumarate is almost double that of the native Fe from the chocolate drink powder (Table 5) and double that of ferrous sulphate (Table 4), and that it is not increased by the addition of ascorbic acid (Table 5). These findings are analogous to the behaviour of haem-Fe and NaFeEDTA (McPhail *et al.* 1985), two Fe compounds which do not enter the common pool. The absorption of Fe from these compounds is greater than the absorption of pool Fe in foods that strongly inhibit Fe absorption. It is believed that the Fe remains bound to the chelate rather than exchanging freely with all ingested Fe.

We first thought that a new complex had been formed from ferrous fumarate and the chocolate drink powder ingredients during processing. However, in the liquid formula meal, the unprocessed ferrous fumarate was also significantly better absorbed than the native meal Fe (Table 6) and, although ascorbic acid increased Fe absorption by about 60%, this increase was not statistically significant and was not as great as the increase in native Fe absorption on the addition of ascorbic acid. It seems, therefore, that ferrous fumarate, whether processed or not, does not completely enter the common Fe pool.

The addition of ascorbic acid to the non-Fe-fortified chocolate drink powder increased

mean Fe absorption from 1·8 to 2·7%, an increase of 50% (not significant). Each serving of chocolate drink contained 0·5 mg Fe and either 8·6 or 26·4 mg ascorbic acid (Table 1). The enhancing effect of ascorbic acid on Fe absorption depends on the amount of ascorbic acid added, the quantity of Fe present, and the amount and nature of the absorption inhibitors present in the meal. In a semi-synthetic liquid formula meal identical to that used in our studies, except for the addition of Ca, phosphorus and 4·1 mg Fe, Cook & Monsen (1977) reported an increase in absorption of 65% on addition of 25 mg ascorbic acid and a four-fold increase on the addition of 100 mg ascorbic acid. The same quantity of ascorbic acid (100 mg) added to a farina–milk meal fortified with 3 mg Fe as ferrous sulphate, ferric orthophosphate or electrolytic Fe also increased Fe absorption 2·5–4-fold (Forbes *et al.* 1989), whereas similar quantities of ascorbic acid added to infant formula or infant cereals have resulted in up to ten-fold increases in Fe absorption (Derman *et al.* 1980). Based on these results, the effect of 100 mg ascorbic acid on ferrous fumarate absorption from the synthetic liquid formula seems particularly low, although the modest increase in absorption of the native Fe from the chocolate drink powder on addition of 25 mg ascorbic acid would seem to be more or less as expected considering the inhibitors of Fe absorption present in the product (casein and calcium from milk, phytate from malt and polyphenols from cocoa).

Our studies indicate that ferrous fumarate is a useful Fe compound for the fortification of chocolate drink powders, and that the amount of Fe absorbed from a chocolate drink powder fortified with this compound would be likely to have a significant impact on Fe balance in children and adolescents. It could be included in school lunch programmes, for instance, and reconstituted with cold or warm milk or water. Mean absorption values of 5·27% were observed in subjects with an average serum ferritin of 24 μg/l (Table 4) and 3·35% in subjects with a mean ferritin of 31 μg/l (Table 5). These serum ferritin levels are higher than in school-aged children, indicating that even higher absorption levels will occur in this age-group. Based on a reference dose absorption of 40%, which is commonly taken to represent individuals with borderline Fe deficiency, absorption of 7% from the fortified drink would be predicted. Even this may be an underestimate of the absorption that would occur in Fe-deficient children. For example, absorption in six subjects with serum ferritin levels below 15 μg/l ranged from 2·3 to 67·2% in the present study with a composite mean of 9·5% (Table 4). This level is encouraging in view of the fact that milk is commonly regarded as inhibitory to Fe absorption and one might also expect the polyphenols in the cocoa and the phytate in the malt to inhibit absorption. Our study suggests that in the absence of food, one glass of chocolate milk drink will furnish approximately 0·5 mg absorbable Fe/serving. Two glasses/d, therefore, would cover a substantial part of the Fe requirement of school-aged children (1·0 mg absorbable Fe/d; National Academy of Sciences, 1980) and would be likely to have a significant impact on Fe nutrition.

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SERUM TRANSFERRIN RECEPTOR FOR THE DETECTION OF IRON DEFICIENCY IN PREGNANCY

Marisa T. Carriaga, M.D.*, Barry S. Skikne, M.D.,
Brent Finley, M.D., Bonnie Cutler, M.N., James D. Cook, M.D.

Division of Hematology, Department of Medicine and Department of Gynecology and Obstetrics, Kansas University Medical Center, Kansas City, Kansas 66103, USA.

*Present address: Department of Pathology, Georgetown University, Washington DC

Address reprint requests to James D. Cook, M.D., Division of Hematology, Kansas University Medical Center, 39th & Rainbow, Kansas City, Kansas 66103, USA.

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Running head: Serum transferrin receptor in pregnancy

SUMMARY

Measurements of circulating transferrin receptor provide a sensitive quantitative index of tissue iron deficiency in otherwise healthy subjects. The present investigation was undertaken to examine the diagnostic utility of this new iron parameter in pregnancy. A battery of iron-related measurements, including serum transferrin receptor levels, was performed in 176 women in third trimester pregnancy who were attending a university prenatal clinic. The mean receptor level of 5.96±2.37 mg/L (±1SD) did not differ significantly from levels in non-pregnant individuals and the frequency distributions were likewise comparable. Elevations in serum receptor above 8.5 mg/L occurred only in women with depleted iron stores defined by serum ferritin levels. Abnormal levels were found in 11 out of 13 women with overt iron deficiency anemia. Our findings indicate that serum receptor levels are not influenced by pregnancy *per se* and are a sensitive index of iron deficiency. By combining serum receptor and serum ferritin measurements, the entire spectrum of iron status in pregnancy can be assessed.

Key terms: Iron status; Serum transferrin receptor; Iron deficiency anemia

INTRODUCTION

All women in their childbearing years are at risk of developing iron deficiency, but pregnant women are especially vulnerable. The heightened iron requirements due to the growing fetus and expanding maternal red cell mass often outstrip the available supply of iron from the diet and iron stores. The prevalence of anemia during pregnancy exceeds 50% in many countries (1) and is considered to be a sensitive index of the iron and nutritional status of a population. Although the consequences of iron deficiency during pregnancy have been long debated, there is recent evidence that even mild anemia or hypoxia increases the rate of premature delivery and perinatal mortality (2-5).

While iron deficiency occurs commonly in pregnant women, the optimal method for its detection is still debated. Anemia has been traditionally used as an index of iron lack, but physiological alterations in plasma volume and red cell mass during pregnancy diminish the reliability of hemoglobin or hematocrit determinations. Some of the established measurements of iron status such as the serum iron and transferrin levels are altered by pregnancy independently of iron status, while others are not sensitive to iron deficiency of recent onset.

The mechanism by which iron gains entry into cells has been defined more clearly in recent years (6-8). Diferric transferrin binds to a specific glycoprotein receptor on the cell surface. A coated pit is then formed which invaginates to form a vesicle termed an

endosome. When the pH of the endosome falls, the iron is released into the cytoplasm and the iron-free transferrin bound to its receptor is returned to the cell surface and released. The receptor density is greatest in the erythroid marrow and placenta, tissues that have the highest iron requirement. When iron supply is curtailed, transferrin receptor synthesis is upregulated, allowing a cell to compete more effectively for diferric transferrin. Transferrin receptor has recently been detected in serum and the concentration shown to be elevated in iron deficiency and hemolytic anemia (9-12). Phlebotomy studies in healthy subjects have demonstrated that the serum receptor is an early and sensitive laboratory index of tissue iron deficiency (13). A progressive rise in serum receptor levels during gestation has been reported, and attributed to a placental origin rather than iron deficiency (14). The present investigation was undertaken to examine in greater detail the utility of serum receptor measurements for detecting iron deficiency in pregnancy.

MATERIALS & METHODS

Subjects. Measurements of serum transferrin receptor and iron status were performed in 176 women who were in the third trimester of their pregnancy and attending prenatal clinics at the Kansas University Medical Center. Their ages ranged from 15 to 42 years. Women with multiple gestations were excluded from the study. Sampling was delayed until the third trimester because the prevalence of iron deficiency increases during the latter part of gestation and because the physiological expansion

in plasma volume has usually reached a plateau by week 28. Sampling was weighted to emphasize the first and last months of the third trimester: 52 women were in week 28-32, 107 in week 36-40, 5 in week 26-28 and 12 in week 33-35. Gestational age was determined antenatally by sonography in over 85% of women and by clinical examination in the remainder. The protocol for this investigation was approved by the Human Subjects Committee at the University of Kansas Medical Center. Written, informed consent was obtained from each subject prior to their participation in the study.

Laboratory Methods. Blood samples were obtained by venipuncture at the time of the clinic visit without regard to prior food intake. Blood was drawn into trace metal free vacutainer tubes and the serum was stored at -20°C until the day of assay. Blood was also collected into separate tubes containing dried EDTA for automated red cell and erythrocyte protoporphyrin measurements. Serum transferrin receptor determinations were performed using a two-site enzyme-linked immunosorbent assay (ELISA) as described previously (11). This assay was established using monoclonal antibodies prepared against transferrin-saturated receptor purified from human placenta. The between-assay variability measured over a 12-month period was less than ±3%. Six standards with concentrations ranging from 0.5 to 50 ng/ml and all unknowns were assayed in triplicate. All receptor values obtained during the course of the study were corrected to the average of 2 quality control sera that were assayed in each microtitre plate.

Automated blood counts were performed by an automated cell counter (Ortho Diagnostic Systems, ELT8/WS, Westwood, MA) which provided measurements of hemoglobin, mean corpuscular volume (MCV), and red cell mean index (RCMI). Erythrocyte protoporphyrin levels were measured directly with a hematofluorometer (ZPP meter, Model 205, Aviv Biochemical Inc., Lakewood, NJ) using whole blood controls provided by the manufacturer. Triplicate measurements were performed within 4 days of blood collection. Serum ferritin was measured by a two-site ELISA established with monoclonal antibodies (15). Serum iron was measured by a coulometric method (Ferrochem II, ESA, Bedford, MA) as described previously (16). Total iron binding capacity (TIBC) was measured by a magnesium carbonate adsorption technique (17). Transferrin saturation was calculated by expressing the serum iron as a percentage of the TIBC.

RESULTS

Depleted iron stores were detected in 44.3% of women with a moderate increase from 32.6% to 51.4% from the first to the last month of the third trimester (Table 1). Based on WHO criteria, the frequency of anemia was 19.9% in the total sample with a slight decrease in prevalence between the first and last month. In the total sample of 176 women, serum receptor levels averaged 5.96±2.37 mg/L (±1SD). This did not differ significantly from the mean of 5.63±1.42 mg/L observed in 82 normal male and female volunteers reported previously ($p>0.10$) (11). However, 10.2% of the pregnant women

had values above the upper cut-off level of 8.5 mg/L. The frequency distribution of serum receptor levels in the pregnant women was similar to that observed in 174 normal subjects (mean ±1SD, 5.73±1.45 mg/L) who had participated in a prior study (Figure 1) (18).

The serum receptor increased significantly from 5.36±2.67 mg/L in the first month to 6.20±2.04 mg/L in the third month of the last trimester (p=0.015). When women with depleted iron stores as defined by a serum ferritin <12 ug/L were excluded, the serum receptor was also lower in early third trimester (4.68 ± 1.12 mg/L) as compared to late third trimester (5.29 ± 1.44 mg/L). This 12% difference was also statistically significant (p=0.018).

To determine whether an elevated serum receptor level occurs independently of iron deficiency, we examined serum ferritin levels in those women with an elevated serum receptor level (Figure 2). Of 18 such women, 15 had a serum ferritin <12 µg/L indicating absent iron stores. The remaining 3 women had serum ferritin levels <16 µg/L. None of the remaining 63 women with a serum ferritin <12 µg/L had elevated receptor levels. This finding indicates that pregnancy *per se* does not influence receptor levels significantly and that the serum receptor is a valid index of iron deficiency in pregnancy.

Overt iron deficiency was relatively uncommon in our pregnant women because the majority were taking iron supplements regularly. Since the prevalence of iron deficiency increases during gestation, we compared the iron measurements in early and late third trimester (Table 2). The mean hemoglobin concentration remained constant and the serum ferritin decreased only slightly. Non-fasting serum iron levels were constant, but the TIBC increased significantly. The erythrocyte protoporphyrin, RCMI and MCV all changed in the direction of iron deficiency, as did the transferrin receptor level.

The laboratory definition of iron deficiency anemia in late pregnancy has never been firmly established. We did not consider it appropriate in the context of the present investigation to perform bone marrow examinations to diagnose iron deficiency. Overt iron deficiency anemia was therefore defined using multiple laboratory criteria. These criteria included anemia (hemoglobin <110 g/L), and at least two of the following: MCV ≤80 fl, RCMI >4.0, or erythrocyte protoporphyrin >1.77 µmol/L RBC. Of the 13 women who satisfied these criteria, the serum receptor level was elevated in all but two (Figure 3).

DISCUSSION

The importance of mild anemia during pregnancy has been long debated. Many believe that the anemia is due mainly to hemodilution and therefore represents no risk

for the mother or fetus. However, recent studies indicate that anemia is associated with prematurity. For example, Lieberman and coworkers (5) reported a significant increase in prematurity at hematocrit levels below 38% and concluded that a decrease in hematocrit by a single point was associated with a 24% increase in the risk of prematurity. Their finding is in keeping with an earlier prospective study demonstrating an increase in the prevalence of low birth weight, premature delivery, and perinatal mortality in women with a hemoglobin concentration <104 g/L prior to 24 weeks of gestation (3). The hypoxia associated with smoking (2) or residence at higher altitude (4) is also believed to result in an increased rate of premature birth. Even mild iron deficiency as defined by serum ferritin levels <10 ug/L were reported to be associated with premature labor (19,20). These reports offer compelling reasons for identifying iron deficiency and prescribing iron for its correction.

The unequivocal detection of iron deficiency in pregnancy is difficult. Earlier studies relied heavily on measurements of serum iron, TIBC, and transferrin saturation, but these are of limited value in pregnancy. The serum iron is low because of the high placental uptake of iron and the TIBC increases even in iron replete women due to hormonal effects (21). The serum ferritin, a sensitive clinical index of iron deficiency, falls dramatically during the second and third trimester (22,23) and as a result, a normal ferritin value is of greater value in excluding iron deficiency than a reduced level is in detecting it. The mean ferritin in our composite sample was 14.3 ug/L, far below the mean in non-pregnant women of 25-30 ug/L. Nearly one-half of our

pregnant women had values <12 ug/L in late third trimester indicating absent iron stores, but not necessarily iron deficient erythropoiesis. Because the ferritin level is abnormally low in the majority of pregnant women, other laboratory indices of iron deficiency are required.

Red cell parameters such as MCV, RCMI, and erythrocyte protoporphyrin are potentially more reliable than iron transport measurements for identifying iron deficient erythropoiesis because of their greater specificity and stability. However, their utility is diminished in pregnancy because values change only slowly with the onset of iron deficiency and frequently occur too late in gestation to be diagnostically useful. Microcytosis is a sensitive index of iron deficiency, but its value is limited by the physiological increase in MCV which occurs during pregnancy (24). Heterogeneity in red cell size (RCMI) was found to be no more sensitive than the MCV in a recent study in 331 pregnant women, indicating that it also has limitations (25). The erythrocyte protoporphyrin is not available in most hospital laboratories.

The serum transferrin receptor is a sensitive measure of tissue iron supply. When normal subjects were phlebotomized repeatedly to induce varying degrees of iron deficiency there was little change in receptor levels during storage iron depletion, but a precise quantitative relationship was subsequently observed between receptor levels and the deficit in tissue iron (13). The serum receptor was more sensitive to tissue iron deficiency then either the erythrocyte protoporphyrin or MCV in that study. In

pregnancy however, a progressive rise in serum receptor levels after 20 weeks of gestation was reported and attributed to a placental origin rather than iron deficiency (14). However, in the present study, we did not find an elevation in serum receptor levels in third trimester in the absence of iron deficiency. We observed a slight 16% increase in receptor values between early and late third trimester, but this change was far less than reported previously. In addition, the mean receptor levels in our study were similar to those in non-pregnant controls. The modest increase in serum receptor during the last trimester paralleled changes in other red cell parameters. Thus, significant correlations were observed with erythrocyte protoporphyrin and MCV (Figure 4) indicating that the increase in serum receptor was due to iron deficiency. In keeping with prior phlebotomy studies (13) serum receptor levels were as reliable in identifying iron deficiency as a combination of laboratory parameters (Figure 3).

In countries where the prevalence of iron deficiency in pregnancy is low and where most women are given iron supplements prophylactically, serum transferrin receptor measurements cannot be recommended for routine screening for iron deficiency. Their major value in this setting will be to confirm iron deficiency in women with anemia and/or low serum ferritin levels. Serum receptor levels will also be useful in defining more precisely the relationship between mild iron deficiency and pregnancy outcome.

Transferrin receptor measurements will be valuable in epidemiological studies in pregnant women in developing countries where the prevalence of anemia may exceed 50%. The cause of anemia in pregnancy in Third World countries is less certain and infection is often considered to be an important factor. Unlike conventional red cell measurements which are affected similarly by iron deficiency and infection, serum receptor levels are increased only rarely in patients with the anemia of chronic disease (26). Several laboratory indices of iron status such as MCV, RCMI, and erythrocyte protoporphyrin require costly electronic equipment and prompt processing of the blood specimen. On the other hand, serum receptor measurements require only a few µl of serum and can be performed on capillary specimens that have been stored in the frozen state. By coupling serum receptor with serum ferritin measurements, the entire spectrum of iron status during pregnancy can be assessed with the same methodology. The wider application of receptor measurements in areas where the prevalence of anemia in pregnancy is high and its cause uncertain will assist in better defining the true prevalence of iron deficiency and identifying the most effective measures for its control.

Because of the relatively small number of women with overt iron deficiency anemia in our study, and the lack of definitive criteria of iron deficiency anemia in pregnancy, further studies are required to define the exact sensitivity and specificity of serum receptor measurements for detecting iron deficiency anemia. When multiple laboratory criteria were used for this diagnosis, falsely normal receptor levels were observed in

only 2 of 13 women. Most iron parameters changed in the direction of iron deficiency between the first and last month of third trimester, but more significant changes occurred in serum receptor and automated red cell parameters. Studies in a more iron deficient population of pregnant women in whom iron deficiency is defined by hematological response to iron will assist in further defining the reliability of receptor measurements. Our present results indicate that the serum receptor will assume major importance in the detection of iron deficiency anemia in pregnancy.

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Table 1. Number of women in third trimester with storage iron depletion or anemia

	Early	Late	Composite
Gestational age (weeks)	28-32	36-40	26-40
Number of women (%)			
Storage depletion*	17 (32.7)	55 (51.4)	78 (44.3)
Anemia+	13 (25.0)	20 (18.7)	35 (19.9)
Total	52	107	176

* serum ferritin <12 µg/L
+ Hgb <110 g/L

Table 2. Iron status measurements during the third trimester. (Mean±1SD)

	EARLY	LATE	COMPOSITE
Gestational age (weeks)	28-32	36-40	26-40
Number of subjects	52	107	176
Hgb (g/l)	115 ± 10.4	118 ± 10.3	117 ± 10.1
Serum ferritin (µg/L)+	15.2 (7.5-31.0)	12.6 (6.3-25.2)	14.3 (7.3-27.8)
Serum iron (µmol/L)	13.2 ± 8.3	12.3 ± 7.0	12.7 ± 7.3
TIBC (µmol/L)	93 ± 14	100 ± 13*	97 ± 14
Transferrin saturation (%)	14.7 ± 9.7	12.7 ± 7.3	13.5 ± 8.2
Erythrocyte protoporphyrin (µmol/L RBC)	1.48 ± 0.60	1.78 ± 0.76*	1.71 ± 0.72
RCMI	1.48 ± 2.57	2.18 ± 2.23**	1.96 ± 2.34
MCV (fl)	92.3 ± 6.8	90.2 ± 6.4**	91.0 ± 6.6
Serum transferrin receptor (mg/L)	5.36 ± 2.67	6.21 ± 2.04**	5.96 ± 2.37

+ analyzed as logarithms
* $p<0.05$ (early vs. late)
** $p<0.01$

LEGENDS

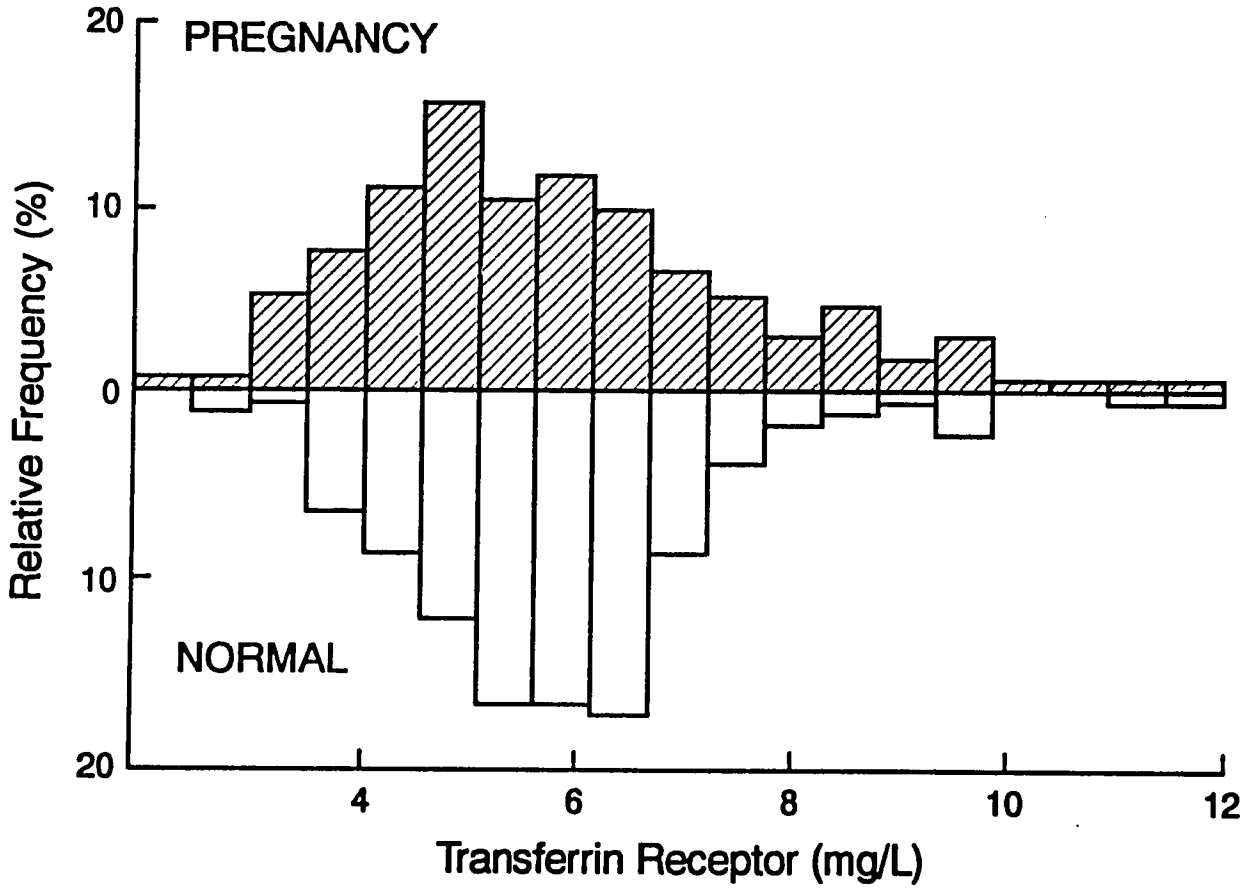
Figure 1. Frequency distributions of transferrin receptor levels in third trimester pregnancy and in healthy control subjects (18).

Figure 2. Relationship between serum ferritin and transferrin receptor levels in third trimester pregnancy. The serum ferritin values are plotted on a logarithmic scale. The interrupted lines depict the cut-off levels for serum ferritin (12 μg/L) and transferrin receptor (8.5 mg/L).

Figure 3. Transferrin receptor levels in 13 women with laboratory evidence of overt iron deficiency anemia (see text). The shaded area depicts the normal range for transferrin receptor. All but two women had elevated receptor levels.

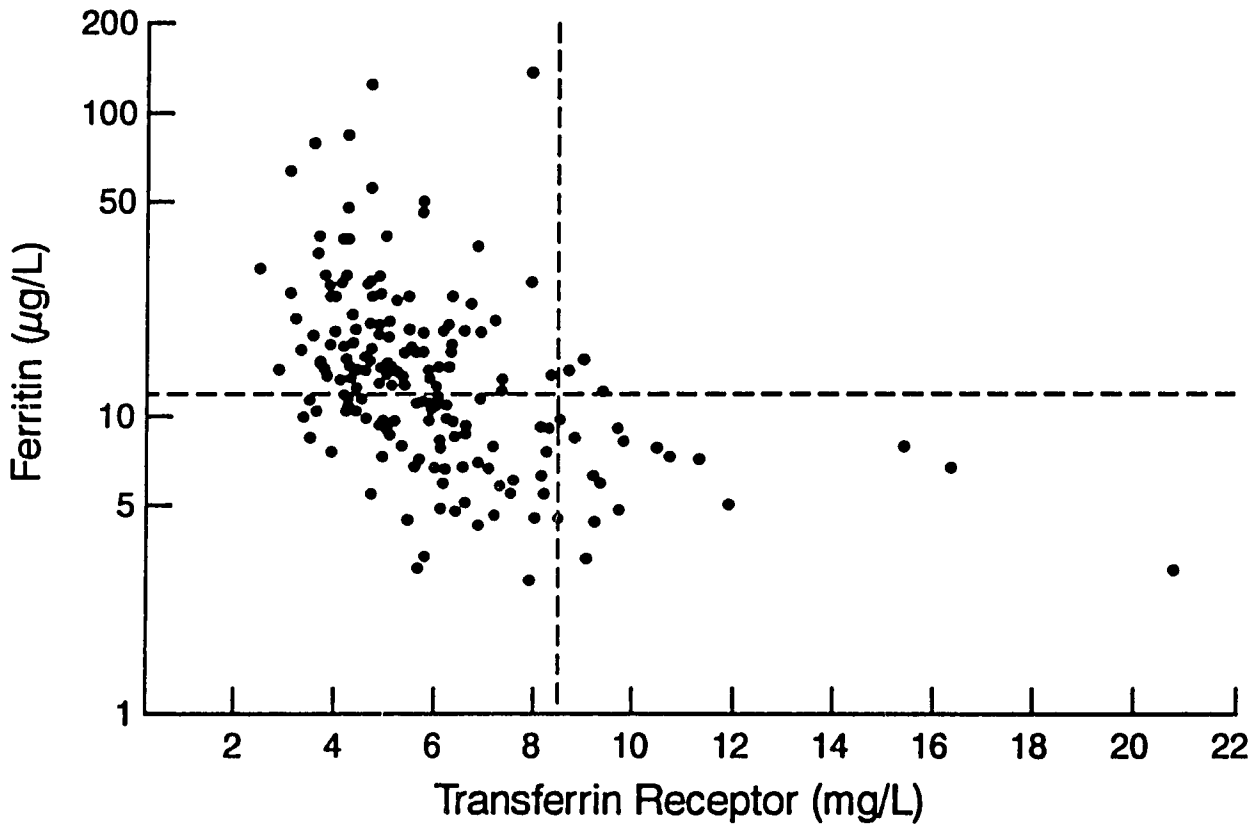
Figure 4. Relationship between mean corpuscular value (MCV) and transferrin receptor levels is shown in the upper panel ($r = -0.641$, $p<0.001$) and between free erythrocyte protoporphyrin (FEP) and transferrin receptor in the lower panel (correlation coefficient = 0.565, $p<0.001$).

Figure 1


PREGNANCY
NORMAL
Relative Frequency (%)
20
10
0
10
20
4
6
8
10
12
Transferrin Receptor (mg/L)

107

Figure 2


200
100
50
10
5
1
Ferritin (μg/L)
2
4
6
8
10
12
14
16
18
20
22
Transferrin Receptor (mg/L)

108

Figure 3

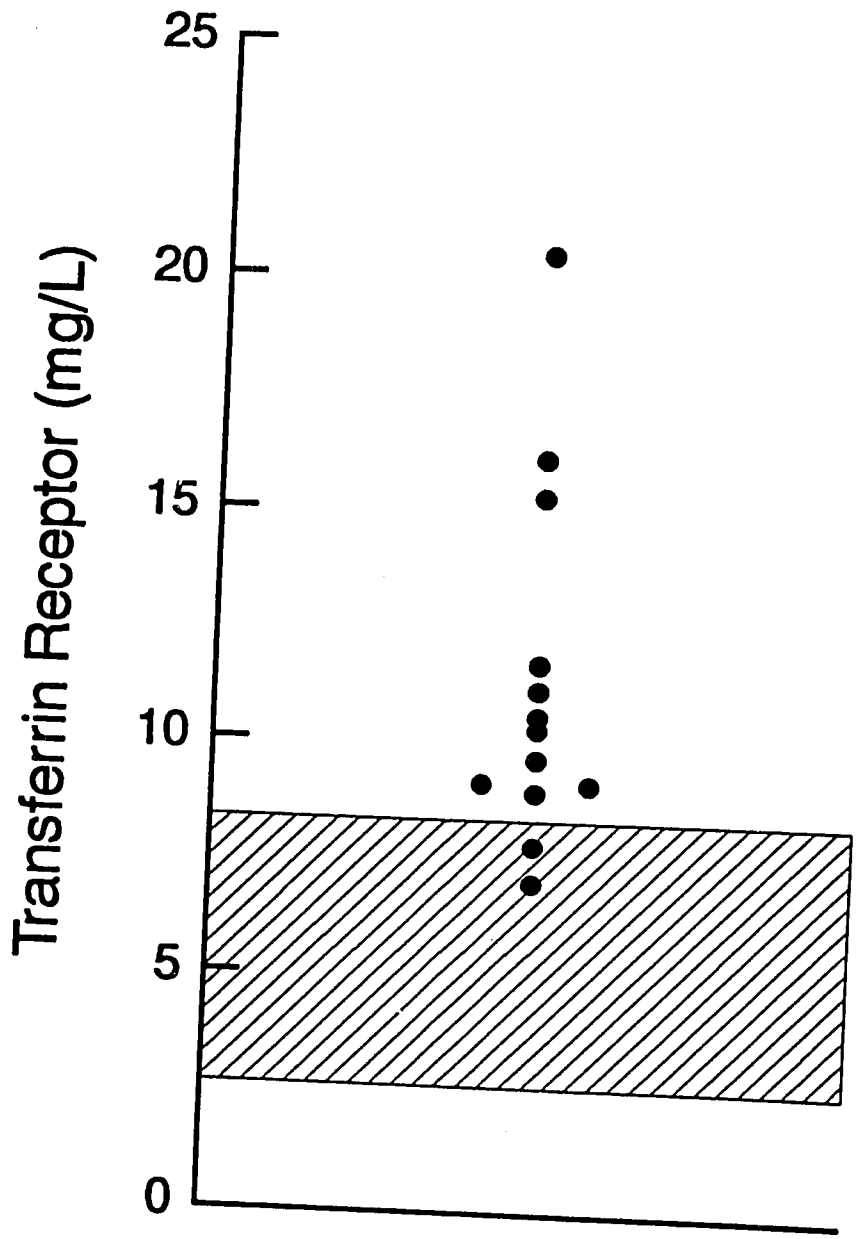
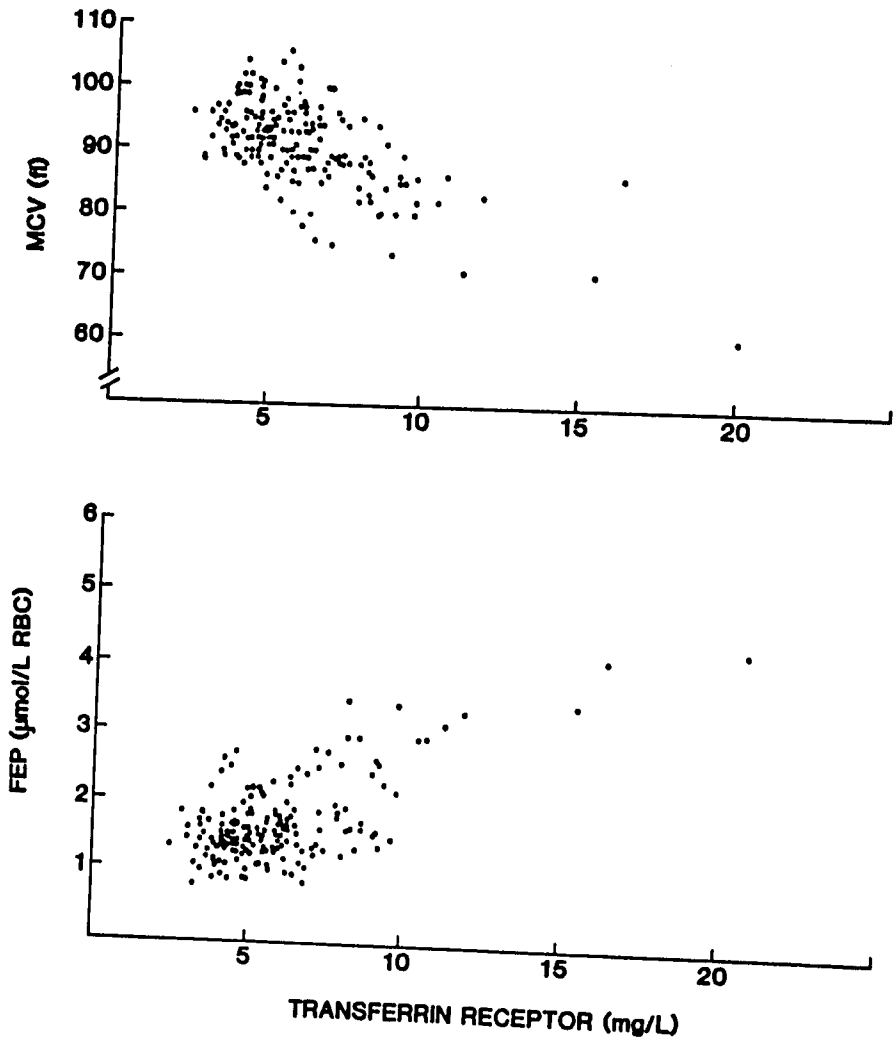


Figure 4


110
100
90
80
70
60
MCV (fl)
5
10
15
20
6
5
4
3
2
1
FEP (µmol/L RBC)
5
10
15
20
TRANSFERRIN RECEPTOR (mg/L)

SERUM TRANSFERRIN RECEPTOR DISTINGUISHES THE ANEMIA OF CHRONIC DISEASE FROM IRON DEFICIENCY ANEMIA

Beverly J. Ferguson, M.D., Barry S. Skikne, M.D.,
Karen M. Simpson, M.D.*, Roy D. Baynes, M.D., James D. Cook, M.D.

Divisions of Hematology and Allergy, Immunology, and Rheumatology*, Department of Medicine, Kansas University Medical Center, Kansas City, Kansas 66103, USA.

Address reprint requests to James D. Cook, M.D., Division of Hematology, Kansas University Medical Center, 39th & Rainbow, Kansas City, Kansas 66103, USA.

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Running head: Serum transferrin receptor in the anemia of chronic disease

Abbreviations: ELISA = enzyme-linked immunoassay; MCV = mean corpuscular volume; RCMI = red cell mean index; TIBC = total iron-binding capacity

ABSTRACT

Recent studies have shown that the serum transferrin receptor is a sensitive quantitative measure of tissue iron deficiency. The present study was undertaken to determine its ability to distinguish iron deficiency anemia from the anemia of chronic inflammation and to identify iron deficiency in patients with liver disease. The mean transferrin receptor level in 17 normal controls was 5.36±0.82 mg/L as compared to 13.91±4.63 mg/L in 17 patients with iron deficiency anemia ($p<0.001$). The mean serum receptor level was normal in all 20 patients with acute infection including 5 with acute hepatitis and was also normal in 9 of 11 anemic patients with chronic liver disease. Receptor levels were within the normal range in all but 4 of 41 patients with anemia of chronic disease. We conclude that unlike serum ferritin levels, which are disproportionately elevated in relation to iron stores in patients with inflammation or liver disease, the serum transferrin receptor level is not affected by these disorders and is therefore a reliable laboratory index of iron deficiency anemia.

INTRODUCTION

In patients with laboratory evidence of iron deficient erythropoiesis, the identification of true iron deficiency as the underlying cause is important because of its prompt response to therapy and the clue it may provide to potentially serious gastrointestinal disorders. The distinction between iron deficiency anemia and the anemia that accompanies infection, inflammation, or malignancy, commonly termed anemia of chronic disease, is frequently difficult. Conventional laboratory indices of iron status such as serum iron, TIBC, transferrin saturation, red cell size heterogeneity, MCV, and erythrocyte protoporphyrin do not always adequately distinguish these common causes of anemia. The serum ferritin identifies iron deficiency when the level is below 15-25 µg/L, and excludes it when the level is elevated above 100 µg/L. However, in many anemic patients with laboratory evidence of iron deficient erythropoiesis, the serum ferritin falls in the diagnostic grey zone between 20-100 µg/L. Because the absence of stainable iron on bone marrow examination is generally regarded as the only reliable index of true iron deficiency in this situation, marrow examinations are required to identify iron deficiency despite the expense, inconvenience, and discomfort to the patient.

Reports from several laboratories indicate that the serum transferrin receptor level is significantly increased in patients with iron deficiency anemia.[1-4] The effect of inflammation or liver disease on this new laboratory index of iron status is unknown. If the receptor levels remain within the normal range in these patients, the assay may prove diagnostically useful both in distinguishing true iron deficiency from anemia of chronic

disease and in detecting iron deficiency in patients with acute infection or chronic liver disease. The present study was undertaken to assess the utility of the serum transferrin receptor level in distinguishing the anemia of chronic disease from iron deficiency anemia.

MATERIAL AND METHODS

Patients. Studies were performed in 56 patients with inflammation, 15 patients with liver disease, 17 patients with iron deficiency anemia, and 17 healthy volunteer subjects. Patients were excluded if they had a malignancy, renal insufficiency as defined by a serum creatinine >2 mg/dl, or a history of blood transfusion within the preceding month. Patients with acute or chronic inflammation had an erythrocyte sedimentation rate >30 mm/hr or a C-reactive protein >1.8 units, or both. The 15 patients with acute inflammation had clinical and laboratory evidence of acute bacterial infection of at least 48 hr duration and a WBC >10.5 x 10^9/L. This group included 6 patients with bacterial pneumonia and 5 with acute pyelonephritis. The 41 patients with chronic inflammation were all anemic as defined by hemoglobin concentration <120 g/L in women and <130 g/L in men. In addition to anemia, all patients had a minimum six month history of an inflammatory illness and a serum ferritin level >50 µg/L. The presence of storage iron was confirmed by bone marrow examination in 17 of these patients. The specific diagnoses included chronic bacterial infection (21), rheumatoid arthritis (12), collagen vascular disease (4), and inflammatory bowel disease (4).

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Patients with liver disease included five with acute viral hepatitis based on a compatible history and clinical presentation, positive serological markers, and an SGOT >200 mg/dl. The diagnosis of chronic liver disease in the remaining 10 patients was based on clinical history, physical signs and a serum bilirubin >1.5 mg/100 dL or SGOT >40 mg/dL. The diagnosis was confirmed by liver biopsy in 3 of these patients. Eight had evidence of ethanol-related liver disease. All patients with chronic liver disease were anemic as previously defined.

Studies were performed in 17 healthy volunteer subjects and an equal number of patients with iron deficiency anemia. The diagnosis of iron deficiency anemia was based on hemoglobin criteria described above and a serum ferritin level <15 µg/L. Patients with severe iron deficiency anemia as defined by hemoglobin concentration <80 g/L in men and <70 g/L in women were excluded in order to obtain a similar degree of anemia to that occurring in the anemia of chronic disease group.

Laboratory Measurements. Complete blood counts were performed using an automated cell counter (Ortho Diagnostics, ELT.8/WS, Westwood, MA) which provided measurements of hemoglobin, MCV and RCMI. Serum iron and TIBC were performed by methods recommended by the International Committee for Standardization in Hematology.[5,6] Serum erythropoietin was measured by enzyme-linked immunoassay (ELISA) according to the instructions supplied by the manufacturer (Clinigen Erythropoietin EIA, Amgen Diagnostics, Thousand Oaks, CA). Serum ferritin was

measured with an ELISA using monoclonal antibodies specific for L-type ferritin.[7] Serum transferrin receptor was measured by a similar ELISA technique using monoclonal antibody reagents.[3] This assay has a sensitivity of 0.5 μg/L and a variability between assays of <3%.

Statistical Analysis. Data are expressed as mean ± standard deviation for each group. A comparison of laboratory results in the various patient groups was based on analysis of variance. Identification of significant difference between groups was based on Scheffe's *post hoc* test.[8]

RESULTS

The degree of anemia in patients with iron deficiency and chronic inflammation was similar (Table 1). Compared with controls, the MCV was significantly decreased only in patients with iron deficiency anemia ($p<0.0001$) and patients with chronic inflammation ($p=0.017$). The RCMI was higher in patients with iron deficiency anemia but did not differ significantly from controls in the other groups. The serum iron level was significantly lower than controls in iron deficiency and in both acute ($p=0.004$) and chronic ($p=0.0006$) inflammation, but did not differ in the remaining study groups. The TIBC was significantly reduced in patients with chronic inflammation ($p=0.005$) and chronic liver disease ($p=0.0002$), but did not differ from controls in the remaining groups. The serum ferritin was significantly elevated in all groups ($p<0.0002$) except patients with iron deficiency.

The mean serum transferrin receptor level was 5.36±0.82 mg/L in normal subjects (range 3.8-7.2 mg/L) which was similar to the mean of 5.63±1.42 mg/L observed previously in 82 normal subjects.[3] In iron deficiency, the mean serum transferrin receptor level was sharply increased to 13.91±4.63 mg/L, and all but two patients with iron deficiency had values above the upper limit of 8.5 mg/L[3] (Figure 1). All patients with acute infection and acute hepatitis had serum receptor levels within the normal range. The mean transferrin receptor level of 5.65±1.91 mg/L in patients with chronic inflammation was nearly identical to that in normal controls although four patients in this group had a slight increase in serum receptor levels. Two of the 10 patients with chronic liver disease also had slight elevations in serum receptor but the mean level of 5.98±2.06 mg/L did not differ significantly from controls.

In patients with iron deficiency a significant correlation was observed between the decrement in circulating hemoglobin concentration and the elevation in serum transferrin receptor ($r=0.80$, $p<0.001$). In iron deficient patients with mild anemia as defined by a hemoglobin concentration ≤25 g/L below normal, the mean receptor level was 10.57 mg/l whereas a higher mean value of 16.86 mg/L was observed in patients with moderate anemia as defined by a hemoglobin concentration 25-50 g/L below normal (Figure 2). In contrast, the mean serum transferrin receptor level was virtually identical in patients with chronic inflammation who had mild (5.61 mg/L) and moderate (5.71 mg/L) anemia.

Serum erythropoietin levels were measured in patients with iron deficiency and the anemia of chronic disease in an attempt to explain the lower receptor levels in those with the anemia of chronic disease. The mean erythropoietin level was 28.8±10.8 mU/ml in patients with mild iron deficiency anemia and 94.1±39.4 mU/ml in those with moderate iron deficiency anemia ($p<0.001$). In the patients with chronic inflammation, the mean erythropoietin level was 24.7±25.7 mU/ml in those with mild anemia and 30.8±26.3 mU/ml in those with moderate anemia ($p>0.05$). Thus, the rise in erythropoietin levels seen in the iron deficiency anemia patients with more severe anemia did not occur in the patients with the anemia of chronic disease.

DISCUSSION

The mechanisms by which cells acquire iron from their environment has been the subject of intense investigation in recent years. The major uptake occurs by receptor-mediated endocytosis of diferric transferrin. Diferric transferrin binds to its specific receptor on the cell surface followed by internalization of the ligand complex within an endocytic vesicle. When the vesicle becomes acidified, iron is released from transferrin to the cytosol while the apotransferrin-receptor complex is returned to the cell surface for further recycling.[9-11] The focal point of this process is the transferrin receptor, a protein with two identical polypeptide chains each weighing 90 kDa and containing 760 amino acid residues. These chains are coupled by a pair of disulfide bonds to form a 180 kDa molecule, most of which is located external to the cell surface.[12] Clinical interest in the transferrin receptor

has been stimulated by studies from several laboratories showing that small concentrations can be detected in human serum using sensitive immunoassay techniques. This material has been recently characterized biochemically and shown to be a truncated form of the intact protein consisting of a monomeric fragment of the extracellular domain.[13]

Organ distribution of intact transferrin receptor reflects the iron requirement of individual tissues. The highest content of transferrin receptor is found in the erythroid marrow and in the placenta which is used as a source for isolation and purification of the protein. In patients with complete bone marrow ablation, the serum receptor levels fall to about 50% of basal indicating that nonerythroid tissue sources contribute significantly to circulating levels.[4,14] The most dramatic elevations in serum receptor occur in patients with an expanded erythroid marrow. A high correlation has been demonstrated in patients with various hematologic disorders between serum transferrin receptor levels and total erythroid precursor mass as measured ferrokinetically.[4] Apart from patients with hemolysis or ineffective erythropoiesis, the only other disorder associated with a significant elevation in serum receptor is iron deficiency. This elevation apparently reflects the increased density of surface transferrin receptor on iron deprived cells. Studies in which iron deficiency has been induced in healthy subjects by serial phlebotomies have demonstrated that the serum transferrin receptor is a sensitive and highly quantitative guide to early tissue iron deficiency.[15] In advanced iron deficiency, the mean serum receptor increases to approximately 3-times the normal mean. Serum transferrin receptor

measurements offer an important advantage over more established red cell parameters of iron deficiency, such as MCV or erythrocyte protoporphyrin, because the serum receptor level increases earlier in evolving iron deficiency.[15]

With the exception of serum ferritin and TIBC, laboratory measurements of iron status are similarly affected in iron deficiency and in chronic inflammation. Because the serum ferritin is disproportionately increased in patients with inflammation or liver disease, a marrow examination is frequently required to diagnose iron deficiency in these patients. The present study was undertaken to evaluate the effect of inflammation and liver disease on the serum transferrin receptor level and demonstrated that it remains normal in most patients. This agrees with animal studies showing a decrease in serum receptor levels with acute inflammation.[16] The most important finding was in patients with the anemia of chronic disease; all but 4 of these 41 patients had serum transferrin receptor levels within the normal range. Latent iron deficiency was excluded in 3 of these 4 patients who consented to a bone marrow examination. Mild hemolysis might have accounted for the increased receptor levels in these patients, but their reticulocyte counts were not elevated and erythroid marrow cellularity was judged to be normal in those who had a bone marrow examination.

Despite extensive study over several decades, the pathophysiology of the anemia which occurs in chronic inflammation is still not completely understood. Impaired iron supply to the erythroid precursors due to a block in the reutilization of iron from senescent red cells,

reduced erythropoietin stimulation and shortened red cell life span have all been proposed as mechanisms responsible for anemia of chronic disease.[17,18] The present study did not address any evidence relative to hemolysis. There was an element of iron deficient erythropoiesis in our patients with chronic inflammation, but it was less marked than in patients with iron deficiency and a similar degree of anemia. For example, the MCV, RCMI, and transferrin saturation in patients with anemia of chronic disease differed from normal but to a lesser extent than with iron deficiency (Table 1). Immunoassay measurements demonstrated that the major reason was due to reduced erythropoietin stimulation similar to prior observations in anemia of chronic disease.[19-21] Thus, the normal transferrin receptor level in the anemia of chronic disease is due predominantly to diminished erythropoiesis.

An important question not addressed by the present study is whether the serum transferrin receptor level can identify iron deficiency when it occurs in patients with acute or chronic inflammation. We attempted to exclude patients with combined iron deficiency and inflammation in our study by requiring that all patients with anemia of chronic disease have ferritin levels above 50 µg/L. It is difficult to find patients with coexisting iron deficiency and chronic inflammation, but it is likely that the serum transferrin receptor increases when iron deficiency develops in these patients. Further studies on this point are needed.

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TABLE 1

Comparison of iron status parameters between patient groups (Mean ± SD)

	Hemoglobin (g/L)	MCV (fl)	RCMI	Serum Iron (µg/dl)	TIBC (µg/dl)	Saturation (%)	Ferritin* (µg/L)	Transferrin Receptor (mg/L)
Normal controls (17)	143±12	93±3	0.34±0.66	75±28	377±67	20±8	43(23-80)	5.36±0.82
Iron deficiency anemia (17)	95±12	74±8	6.20±4.27	21±15	428±76	5±4	7(4-12)	13.91±4.63
Acute infection (15)	139±12	88±5	2.25±1.96	32±23	302±120	14±15	252(103-613)	5.11±1.42
Anemia of chronic disease (41)	102±12	84±7	3.13±3.05	35±25	257±87	14±11	220(86-559)	5.65±1.91
Acute hepatitis (5)	144±8	94±4	1.35±1.51	121±72	400±70	33±24	2438(1071-5552)	4.80±1.19
Chronic liver disease with anemia (11)	111±13	97±9	2.68±1.51	52±32	193±80	28±19	280(116-677)	5.98±2.06

* Geometric mean (±SD)

LEGENDS

Figure 1. Serum transferrin receptor levels in normal subjects and patients with iron deficiency anemia, acute inflammation, anemia of chronic inflammation, acute hepatitis and chronic liver disease. The horizontal line indicates the upper normal range.

Figure 2. Transferrin receptor levels in anemia of chronic disease (•) and iron deficiency anemia (∘) at mild and moderate degrees of anemia. The horizontal dashed line indicates the limit of the normal range.

Figure 1

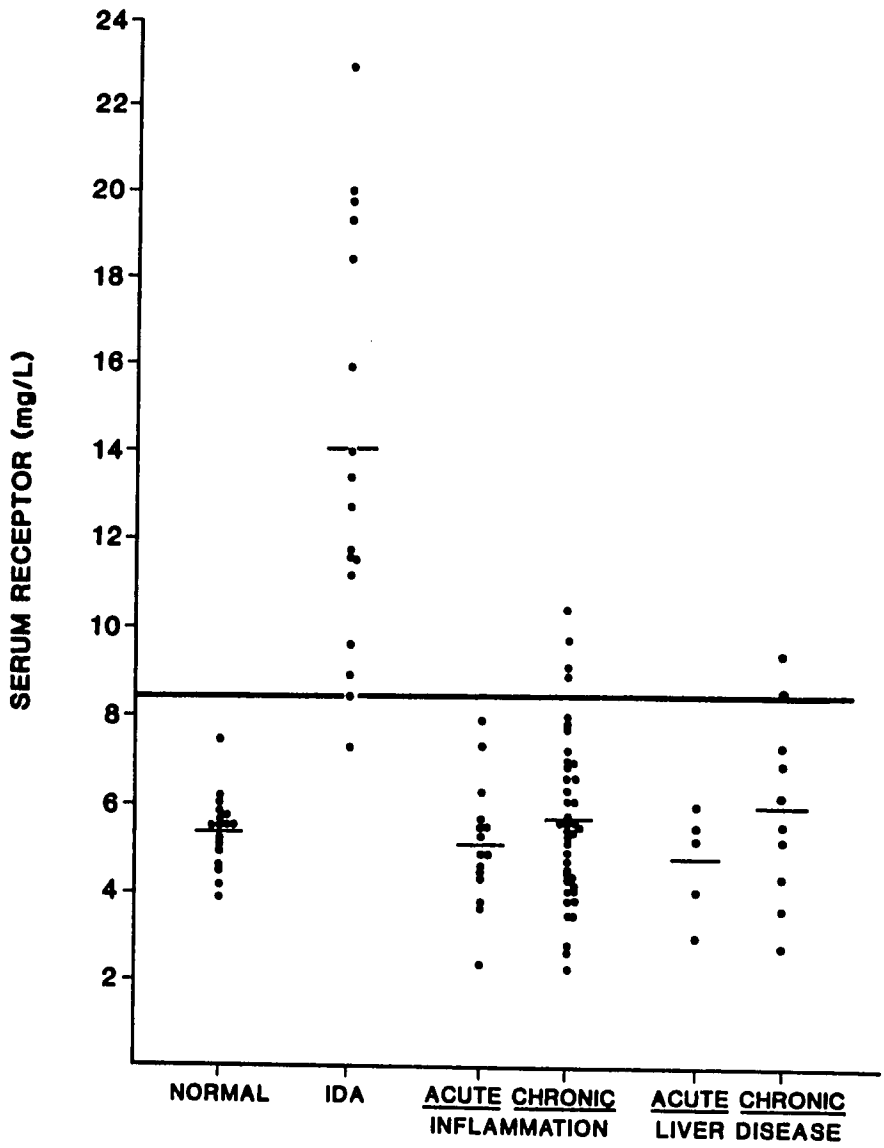
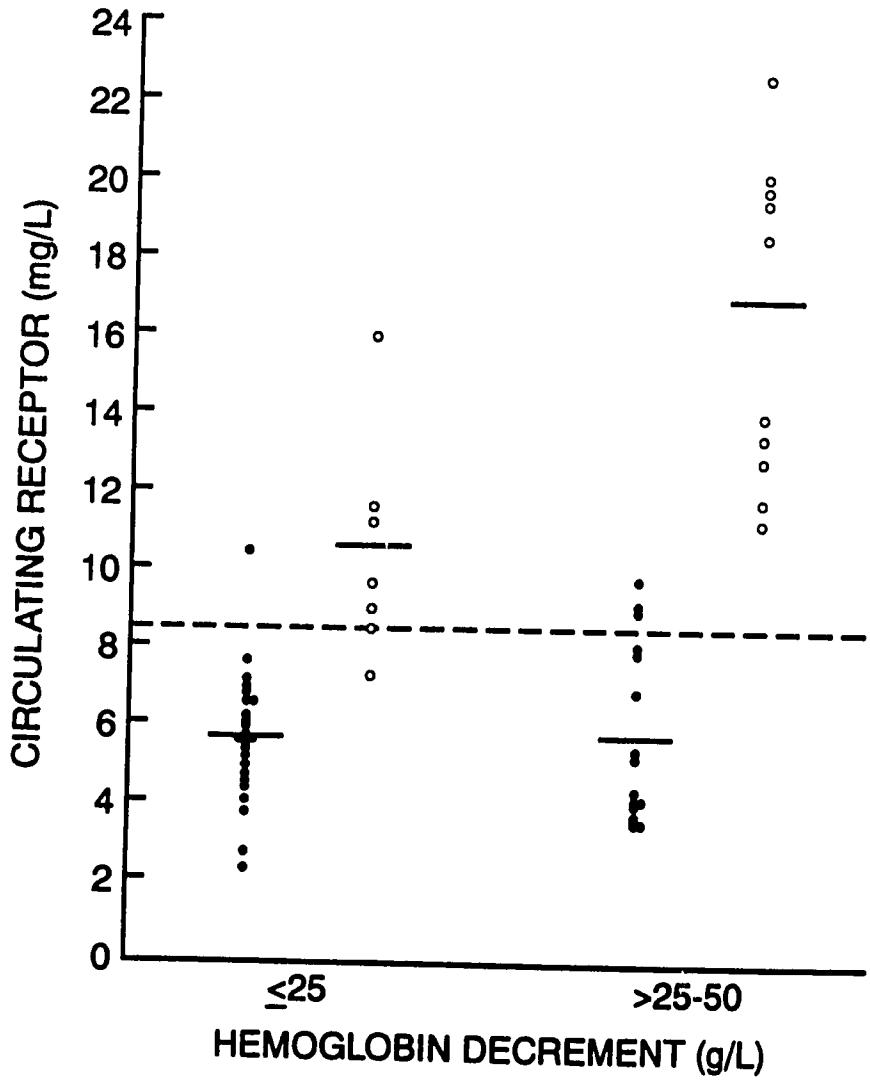

SERUM RECEPTOR (mg/L)
24
22
20
18
16
14
12
10
8
6
4
2
NORMAL
IDA
ACUTE
CHRONIC
INFLAMMATION
ACUTE
CHRONIC
LIVER DISEASE

Figure 2


CIRCULATING RECEPTOR (mg/L)
24
22
20
18
16
14
12
10
8
6
4
2
0
≤25
>25-50
HEMOGLOBIN DECREMENT (g/L)

APPENDIX B

APPENDIX 1

EVALUATION OF A NOVEL DELAYED RELEASE FORMULATION FOR IRON SUPPLEMENTATION IN PREGNANCY

W. K. Simmons
K. C. Bingham

June 1991

INTRODUCTION

Iron deficiency anaemia is a nutritional disorder that is prevalent worldwide, especially among pregnant and lactating women and young children (1-2). In Jamaica, studies during the last two decades have consistently demonstrated a high prevalence of iron deficiency anaemia in physiologically vulnerable groups (3-6). These studies found that among pregnant women between 45.0% and 61.6% were anaemic, using the WHO criterion of haemoglobin levels below 11.0 g/dl (4,6, 7). The most recent survey reported that 52% of pregnant women had haemoglobin levels less than 11.0 g/dl and were thus anaemic by international criteria (6). The situation is much the same in the rest of the Caribbean (8-9).

In an effort to decrease the prevalence of anaemia in Jamaica, the Government has implemented the four approaches recommended by WHO, i.e. fortification, supplementation, nutrition education and public health measures, with varying success.

The supplementation programme strives to supply iron or iron/folate tablets to all pregnant women already in ante-natal clinics. However, the programme has been plagued with problems. Among these is the problem of compliance due to the gastrointestinal side effects (nausea, epigastric discomfort and vomiting) that are attributed to the oral preparations. The efficacy of supplementation therefore becomes questionable.

To overcome gastrointestinal side effects while concomitantly improving iron nutrition, a gastric delivery system (GDS) formulation was recently developed in Europe. This is an orally

administered hydrocolloid iron capsule designed for the controlled release of its iron (10-11).

The GDS iron was evaluated by an absorption and a side effect study in the U.S.A. Evaluation of the efficacy of the preparation showed that absorption of GDS iron was two to four times higher than that of ferrous sulphate and that gastrointestinal side effects were reduced (12).

It was decided to conduct a study to address compliance, gastrointestinal side effects and efficacy of GDS iron in pregnancy in maternal and child health (MCH) clinics in a population where iron deficiency is a problem, over a period of 12 weeks.

In this paper the methodology is given with summarized results regarding efficacy, compliance and side effects. The detailed results of the hematology will be given in a subsequent paper. A second study on side effects is the subject of a third paper.

GENERAL OBJECTIVES

The primary objective of the study was to determine the effects of the two forms of supplementation on various haematological indicators. Haemoglobin levels are regarded as important but other important measures that should be positively influenced by iron supplementation are serum iron, free erythrocyte protoporphyrin, serum ferritin and serum transferrin receptor levels. We

sought to determine effects over each of the two six-week periods of the study.

Secondary objectives were to compare the three treatment groups for differences in compliance and side effects. The relationships of compliance to hemoglobin gain and to side effects were also examined. It had been hypothesized that compliance would correlate positively with both hemoglobin gain and with the prevalence of specific side effects in the two supplementation groups.

METHODOLOGY

The study was conducted in eight MCH centres in Kingston and St. Andrew, and Spanish Town, Jamaica, West Indies. Clinics were chosen that did not presently have iron tablets for distribution to pregnant women. Field work was carried out primarily by two teams, under the direction of two field supervisors. An additional team was hired for the Spanish Town Health Centre. At all the centres phlebotomies were performed by the registered nurses there. Subjects were recruited for the study on the basis that they fulfilled the following criteria:

Age	16-35 years
Gestational age	14-22 weeks
Haemoglobin	8-11 g/dl
Absence of Sickle Cell Disease (Haemoglobinopathies SS and SC)	

Screening for sickle cell disease could not be done at the health centres, but patients who were later found to have these haemoglobinopathies were automatically cropped from the study. Age, gestational age and haemoglobin were readily ascertained: age and gestational age simply by asking, and haemoglobin by the Hemocue system (13). Providing the patient fulfilled the age and gestational age criteria, 3 mls of blood was collected by venipuncture in freeze dried EDTA. With the needle still in the vein, the haemoglobin was quickly determined (45-60 seconds), and if it fell within the desired range, a second sample (clotted) was taken for biochemical determinations.

Samples were kept in an ice box (approximately 4°C), and then transferred to the Iron Nutrition Monitoring Laboratory, at the Caribbean Food and Nutrition Institute, Kingston, Jamaica.

On being recruited, each patient was randomly assigned to one of three treatment groups:

GROUP A	GROUP B	GROUP C
Placebo (1 tablet/day)	GDS Iron 50 mg elemental iron (1 tablet/day	Ferrous Sulphate 100 mg elemental iron (2 tablets/day
Folate 400 mg	Folate 400 mg	Folate 400 mg

and a questionnaire labelled "Questionnaire A" administered. This questionnaire explored socio-economic, maternal and health information.

The experiment was not 'double-blind' since the pills had different formats. Each patient was given two weeks' supply of tablets, plus an additional two or four tablets, depending on the treatment group, and asked to return at two week intervals for additional supplies. At each two weekly visit, she was required to return the box received at the previous visit so that the number of tablets remaining could be checked as a measure of compliance. Another questionnaire "Questionnaire B", designed to seek information about the patient's behaviour concerning treatment, i.e. level of compliance and the side effects of the treatment, was administered at each two weekly visit.

The experiment lasted twelve weeks in all. At the six and twelve week visits, blood was again taken and all the haematological and biochemical determinations repeated. If, at any point, haemoglobin fell below 7 g/dl the patient was dropped from the study, referred to a physician, and encouraged to begin or continue iron therapy.

At the end of three months supplementation, each patient was given sufficient iron tablets to continue therapy until delivery.

Blood collected in health centres was delivered to the laboratory within three hours of collection. Serum was separated from the clotted sample and stored at -20°C for subsequent analyses. FEP was measured on the EDTA blood and the remainder of that sample taken to the Sickle Cell Research Laboratory, Kingston, Jamaica, for the other haematological determinations and haemoglo-

bin electrophoresis to be done to determine any haemoglobinopathies.

The Haemoglobin level (Hb), Packed Cell Volume (PCV), Red Blood Cell Count (RBC), White Blood Cell Count (WBC), Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH), Mean Corpuscular Haemoglobin Concentration (MCHC), and the Red Cell Diameter Width (RDW) were estimated on the Coulter counter.

CHARACTERISTICS OF THE SAMPLE

376 ante-natals satisfied criteria and were admitted to the study. Of these, 325 completed at least 6 weeks of supplementation but only 275 actually completed the entire 12 weeks; and 266 completed all six 'B' questionnaires. This represents a drop-out rate of 14% at 6 weeks and a further 13% between 6 and 12 weeks.

Analysis of the main factors responsible for this 27% attrition was difficult as our field workers were in most cases unable to locate patients who did not keep their appointments. They were, however, able to ascertain from some of the patients why they dropped out. Lack of interest, premature deliveries and migration were the most prevalent reasons given. There was no difference noted among the three treatment groups regarding reasons for dropping out.

As can be seen in Table 1, most of the respondents (66.2%) were young adults, 24 years and under.

Table 1: Ages of Antenatal Sample.

Age group (yrs.)	N	% of sample
< 20	120	31.9
20 - 24	129	34.3
25 - 29	76	20.2
30 - 34	35	9.3
>= 35	4	1.1
Missing	12	3.2
Total	376	100.0

69% of the women were unemployed and those employed were distributed among a few marginal work types with domestic work being the more prevalent (9.7%), followed by factory work (8.1%) and higglering (5.9%).

To ensure that no patient with sickle cell disease was included in the study, haemoglobin electrophoresis was performed on all EDTA-blood taken at the first visit. The genotypes for antenatals comprising the total sample are displayed in Table 2.

Table 2: Genotypes of Antenatals.

Haemoglobinopathy	N	%
AA (normal)	305	81.1
AS	47	12.5
AC	11	2.9
Missing	13	3.5
Total	376	100.0

Two patients with 'SC' genotype were excluded from the study. Other haemoglobin chain variants observed were:

α Chain variants	(1 case)
β Chain variants	(2 cases)
Hb Lepore	(1 case)
β Thalassaemia	(6 cases)

The distribution of ante-natals among the three treatment groups was as follows:

Placebo	-	120	FeSO4	-	122
G.D.S.	-	131	Missing	-	3

One way analysis of variance and chi-squared tests confirmed that all three groups were equivalent in age, gestational age, anthropometry, social background and educational levels. However, the ante-natals in the placebo group had significantly higher haemoglobin levels than those in the GDS iron and $FeSO_4$ groups. This initial lack of equivalence in Hb's between the three groups was disappointing but not regarded as catastrophic: first, the GDS iron and FeSO4 groups were initially equivalent on all measured variables; and, second, comparisons between groups were to be based on Hb gains and not on absolute Hb levels. Initial Hb levels are provided in Table 3.

HAEMATOLOGY

Initially, the antenatals in the placebo group had higher haemoglobin than those in the GDS group ($t = 4.33$, $p<0.01$) and the $FeSO_4$ group ($t = 3.12$, $p<0.01$). There was, however, no difference between the two supplemented groups.

Table 3: Haemoglobin Levels at 0, 6 and 12 Weeks by Treatment Group

		Placebo	GDS	FeSO4
Baseline	Mean	10.5	9.9	10.0
	SD	.93	1.05	1.16
	N	119	129	119
At 6 weeks	Mean	10.0	10.3	10.5
	SD	1.09	1.01	1.02
	N	101	107	96
At 12 weeks	Mean	9.9	10.8	10.9
	SD	1.13	1.13	1.21
	N	89	89	85

Six weeks of supplementation had a very clear positive effect on the haemoglobin levels in both supplemented groups. In the GDS group there was a rise of 0.39 g/dl and in the FeSO4 group the increase was 0.46 g/dl. Meanwhile in the Placebo group there was a decline of 0.48 g/dl. All three changes are highly significant ($p<.01$). During the second six weeks of the study similar improvements in the supplemented groups occurred and there was a continued small but significant decline in Hb levels in the Placebo Group. Table 4 shows haemoglobin gains.

Table 4: Haemoglobin Changes by Treatment Group.

	Placebo	GDS	FeSO4
0 - 6 weeks	-0.48	0.39	0.46
(N)	(101)	(107)	(96)
6 - 12 weeks	-0.18	0.54	0.34
(N)	(84)	(83)	(81)
0 - 12 weeks	-0.64	0.88	0.83
(N)	(89)	(89)	(85)

Examination of a priori contrasts in one way analyses of variance revealed:

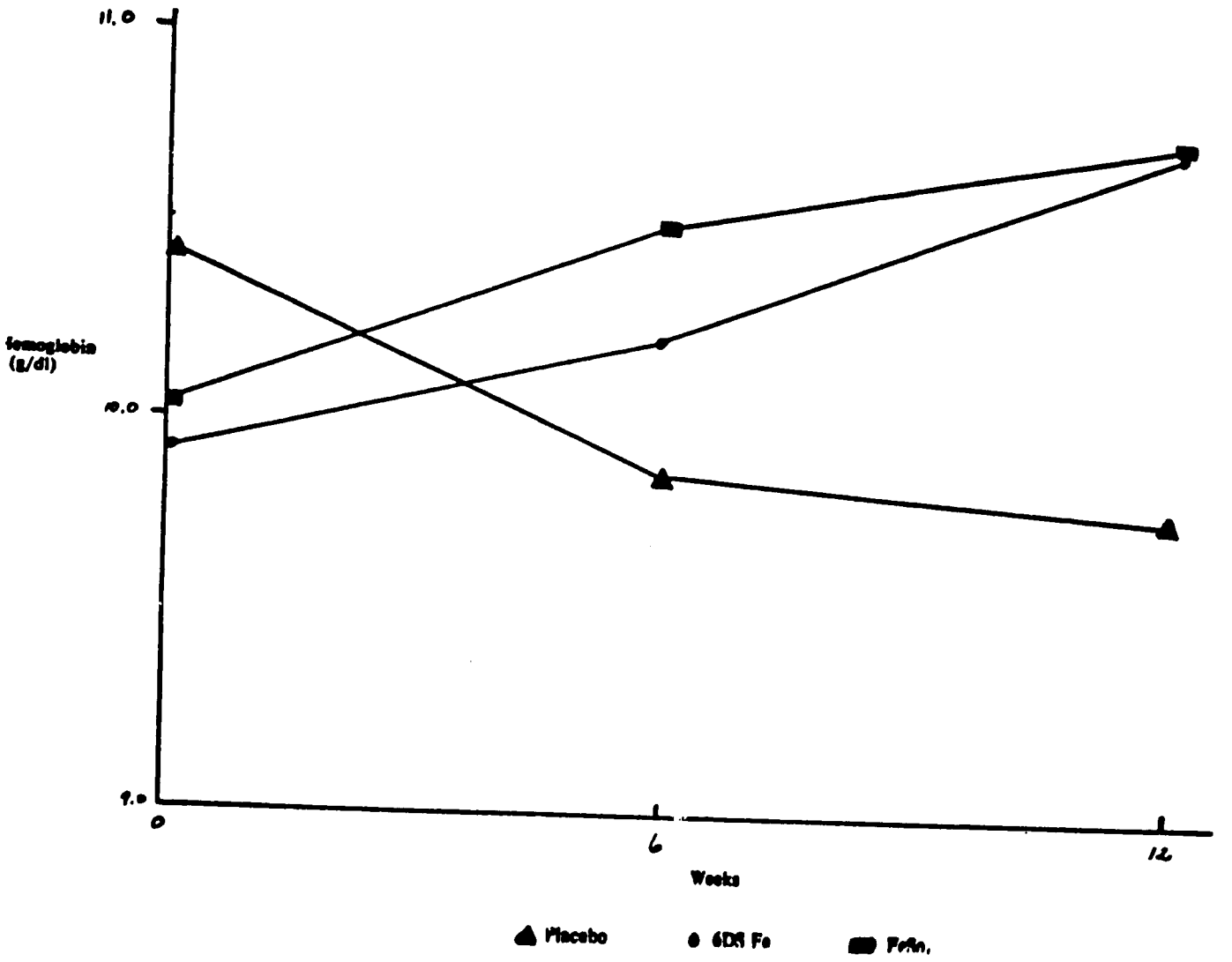
* highly significant ($p<.01$) differences between each iron group and the Placebo Group;

* no significant difference between the two iron groups.

These results apply over each time period 0-6, 6-12 and 0-12 weeks.

Overall each supplemented group experienced a rise of close to 1g/dl over the 12 week period while the Hb level in the Placebo group declined by approximately 0.6 g/dl. All three Hb values for each of the three groups are depicted graphically in Figure 1. It should be noted that attrition rates are similar (23%, 30% and 28% for Placebo, GDS and FeSO4 respectively -- see Table 5).

Figure 1: Hemoglobin Changes in 12 Weeks



COMPLIANCE

In order to comply, antenatals needed, first, to return for their pill supplies and, second, to actually consume the pills. Our evidence of compliance (as well as of side effects) is based upon the completed 'B' questionnaires.

Three research questions regarding compliance are of particular interest:

(a) Was there any difference in attrition rates between treatment groups?

(b) Was there any difference in compliance (pills consumed) between treatment groups?

(c) Was compliance positively related to haemoglobin gain?

Attrition rates.

We now examine whether there was any detectable difference in drop-out rates between the three treatment groups. Table 5 provides the necessary information. The number of B questionnaires completed at each stage is taken as an indicator. (The numbers of C questionnaires at 0, 6 and 12 weeks give slightly different, but similar, results.)

Table 5: Initial Sample Size and Numbers of 'B' Questionnaires Completed.

	Placebo	GDS	FeSO4	Total
Initially	121	132	123	376
At 2 weeks	111	121	116	348
At 4 weeks	107	118	108	333
At 6 weeks	105	115	105	325
At 8 weeks	102	112	102	316
At 10 weeks	98	109	96	303
At 12 weeks	93	93	89	275
Drop-out rate over 12 weeks	23%	30%	28%	27%

Evidently, the attrition rate was slightly higher in the two supplemented groups but a chi-squared test fails to demonstrate a significant effect.

Compliance measures.

Compliance over each two-week period covered by a 'B' questionnaire was assessed by two methods:

(a) by counting the number of pills returned

compliance = proportion of pills taken;

(b) by asking "how many days ...?"

compliance = proportion of days pills taken.

Each biweekly compliance measure ranges from 0 to 1. Whereas the pill count should be a more valid indicator of compliance, in cases where a pill box was not returned the second approach was used to provide an estimate. Thus the preferred measure is a

composite one. (A fuller discussion of the various methods used to estimate compliance will appear as a subsequent report.)

Compliance data was averaged to provide three indicators corresponding to weeks 1-6, 7-12 and, overall, 1-12, as shown in Table 6 below.

Table 6: Compliance by Treatment Group

Weeks		Mean	S.D.	N
1-6	Placebo	.93	.10	101
	GDS	.91	.13	111
	FeSO4	.95	.08	105
7-12	Placebo	.95	.08	92
	GDS	.94	.09	93
	FeSO4	.95	.11	89
1-12	Placebo	.94	.07	86
	HBS	.93	.09	89
	FeSO4	.95	.08	89

Compliance values were fairly high throughout with few significant differences between groups. During the first six weeks the compliance in the FeSO4 group was significantly higher ($p<.05$) than that in the GDS group. This was the only difference which was significant. Overall it was found that 59% of antenatals had compliance exceeding 0.95 and 22% had perfect reported compliance.

Compliance and haemoglobin gain.

We had hypothesized that highly complying subjects would achieve higher Hb gains in the two iron supplementation groups. A satisfactory correlation would serve to validate the compliance measure. The surprising results appear in Table 7.

Table 7: Correlations between Compliance and Hb Gain by Treatment Group, over the Full 12 Weeks.

	Placebo	GDS	FeSo4
r	-.03	0.05	-.04
N	80	85	82

The lack of correlation in the supplementation groups raises serious questions about the measure of compliance. Were antenatals dishonest in their responses to questionnaire items regarding the number of pills taken? Further doubts are raised by the facts that some placebo group subjects achieved gains comparable to those in the two iron groups. See Table 8 below. It appears that, whatever they reported, many subjects who were provided with iron pills just did not take them.

Table 8: Hb Gains over 12 Weeks in the Three Treatment Groups.

	Number of Subjects		
	Placebo gp	HBS gp	FeSO4 gp
less than -1	27 30.3%	4 4.5%	4 4.7%
-1 to 0	37 41.6%	17 19.1%	16 18.8%
0 to 1	23 25.9%	29 32.6%	29 34.1%
1 to 2	0 0%	22 24.7%	25 29.5%
greater than or equal to 2	2 1.1%	17 19.1%	11 22.9%
N	89	89	85

A stepwise multiple regression analysis was performed to determine the relative importance of several independent variables on haemoglobin gain. Treatment group was the most influential variable, as expected, yielding a multiple R of 0.52 and accounting for 27% of the explained variance. At this point in the analysis the following partial correlations emerged between independent variables and haemoglobin gain:

age	-0.151	education	0.048
gestation pd.	0.143	compliance	0.031
weight	-0.074	height	0.015

Once age and gestation period had been entered, the stepwise analysis was discontinued having explained 30% of the variance.

One must conclude that compliance, as measured in this study, was not a factor which influenced haemoglobin gains. We are unable to determine the extent to which each of the following explanations contributed to this unexpected result.

1. Compliance was not measured reliably.
2. Compliance values were too skewed and too high.
3. Medical conditions, treatments and dietary fluctuations, for which data was not collected, affected the results.

Also, there was no relationship between ferritin levels and compliance.

SIDE EFFECTS

At each administrataion of a 'B' questionnaire, at two weekly intervals, each antenatal was asked to report the occurrence of 'side effects'. A simple yes/no question elicited this information:

"Have you had any of the following problems since your last visit?"

No attempt was made to ascertain the strength or frequency of side effects during each two-week period.

The substudy of side effects sought to answer three major questions:

(a) Was there any difference between reported side effects between the three treatment groups

(i) over the first two weeks,

(ii) over the full twelve week period?

(b) In general, was there any trend (increasing or decreasing) over the twelve-week period; and were the same patterns observed in each treatment group?

(c) Was there a positive correlation between compliance and prevalence of side effects?

Each question was examined by looking at the various side effects independently, in turn.

Further, it was of interest to determine which symptoms were problematic in the two iron groups, but less so in the placebo group.

Table 9 shows the level of side effects reported over the first two weeks. The most frequently reported symptom was abdominal pain, followed by nausea, vaginal itch, heartburn and constipation. Fewer persons (<1%) complained of vomiting, diarrhoea or loss of appetite.

Table 9: Numbers of Antenatals in Each Treatment Group Reporting Side Effects at Two Weeks.

	Control (N=111)	GDS (N=121)	FeSO4 (N=116)
Diarrhoea	4	3	4
Vomiting	6	10	9
Loss of appetite	6	12	11
Nausea	16	23	26
Rash	0	4	3
Heartburn	14	18	14
Vaginal itch	15	16	21
Rectal itch	4	3	5
Abdominal pain	33	36	39
Constipation	14	10	17
Ankle swelling	4	6	7

While chi-squared tests on these frequencies do not reveal any significant differences between groups, there does appear to be a slight tendency for vomiting, loss of appetite and nausea to be more prevalent in the two iron groups than in the control group. Fewer side effects occurred in the placebo group and the other two groups were similar (totals 116, 141 and 156, respectively).

The same five symptoms were also most prevalent over the full twelve-week period (see Table 10). There is no indication that these are consequences of supplementation since they are also the most prevalent symptoms in the control group.

Table 10: Percentage of each Treatment Group Reporting Side Effects over Twelve Week Period.

	Control (N=112)	GDS (N=123)	FeSO4 (N=116)	Chi-squared
Diarrhoea	8.9	13.0	18.1	4.16
Vomiting	11.6	17.9	16.4	1.91
Loss of appetite	17.0	22.0	17.2	1.23
Nausea	32.1	35.0	37.9	0.84
Rash	3.6	6.5	6.1	1.13
Heartburn	33.9	42.3	33.6	2.49
Vaginal itch	29.5	27.6	31.9	0.52
Rectal itch	9.8	13.8	11.2	0.95
Abdominal pain	51.8	56.9	60.3	1.72
Constipation	27.7	25.2	25.0	0.26
Ankle swelling	20.5	25.2	19.0	1.49

Once again no chi-squared value proved to be significant. These results, therefore, fail to demonstrate <u>any</u> significant adverse effects of supplementation either at two weeks or over the twelve-week period. Furthermore, the experimental treatment, GDS iron, certainly appears no worse than conventional FeSO4 with regard to side effects.

Trends in side effects.

To determine whether side effects tended to decrease after the first two weeks the prevalence over the first two weeks was compared to the mean prevalence over all six administrations of the 'B' questionnaire, for each side effect. Results appear in Table 11. To compensate for attrition all frequencies have been adjusted so that they are appropriate for a standardized 'complete' sample of 376. The chi-squared test was used to compare the distribution

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over time with the rectangular distribution, with same mean. The direction of trend, if any, was then determined by inspection.

Table 11: Adjusted Frequencies of Side Effects by Q. Administration, Sample Size 376.

	Administration						Mean	Chi-
	1	2	3	4	5	6	Prev	Sqd.
Diarrhoea	12	15	8	15	10	18	13.0	5.23
Vomiting	27	26	9	14	10	8	15.7	23.82**
Loss of appetite	31	27	9	13	9	16	17.5	25.11**
Nausea	70	61	35	42	40	26	45.7	30.07**
Rash	8	0	6	7	1	7	4.8	12.18*
Heartburn	50	63	56	64	55	60	58.0	2.45
Vaginal itch	56	62	43	42	30	32	44.2	18.40**
Rectal itch	13	23	15	19	6	8	14.0	26.86**
Abdominal pain	117	108	82	84	71	89	91.8	16.28**
Constipation	44	40	29	26	31	38	34.7	7.13
Ankle swelling	18	19	23	27	38	47	28.7	23.21**

* sig. at 5% level (5df)
** sig. at 1% level (5df)

Decreasing trends can be seen in the cases of vomiting, appetite loss, nausea, vaginal itch and abdominal pain. Ankle swelling is the only symptom which increases.

Compliance and side effects.

Many subjects have high compliance and no reported side effects. Hence, correlations between compliance and side efects tend to be negative and very much affected by small numbers of unusual cases. For example, in the GDS group, over the first two weeks, compliance and reported diarrhoea (yes/no) yielded a point-biserial correlation of -.23 ($p<.01$), yet only three subjects out

of 121 mentioned this problem. Over the full twelve weeks similar correlations also seem to be spurious. It seems, therefore, that these two variables -- compliance and count of a particular reported side effect -- have such low variability that correlations are meaningless.

DISCUSSION

Iron deficiency anaemia in pregnancy has been shown to be a public health problem in most Caribbean countries. The prevalence of this anaemia varies from 11.0 to 70.0 percent of pregnant women in the various Caribbean countries (14).

A study conducted in eight Caribbean countries indicated that a major problem was side effects and non-compliance in taking iron tablets. Complaints included epigastric discomfort, nausea and vomiting (15).

In our ante-natal clinics iron therapy takes the form of approximately 50 mg elemental iron administered two to three times daily, depending on the patients haemoglobin level. It is debatable whether the iron should be given on an empty stomach or with meals. If the iron is taken on a empty stomach, the absorption increases but also the possibility of side effects increases. In the present study we recommended that the tablets be taken after meals to reduce the possibility of low compliance because of side effects, arising from taking the tablets on an empty stomach.

Attempts have been made to improve absorption while also reducing gastrointestinal irritation. One approach has been to use a slow release preparation. The epigastric discomfort therefore should be reduced. In the past there were three types of such preparations:

1. A tablet that is gradually eroded.
2. An iron salt which is packed in channels within the insoluble matrix.
3. A capsule containing coated granules that dissolve at different rates.

Since iron absorption is most active in the proximal duodenum, it could be expected that such preparations would have a reduced absorption as compared to iron preparations that dissolve rapidly. Some studies confirm this problem (16-17), but good clinical responses have also been noted using these preparations (18-20). The problem with these preparations, however, is their increased cost. It was this problem that led to the development of the Gastric Delivery System (GDS).

A double-blind placebo-controlled trial was performed in the U.S.A. to compare the gastrointestinal side effects associated with 50 mg iron daily given either as GDS iron or conventional ferrous sulphate. The latter was associated with a significantly higher frequency of nausea and anorexia, whereas there was no significant difference in reported side effects between patients receiving GDS and placebo. The study indicated that a single GDS capsule daily

provides the same amount of absorbed iron as conventional ferrous sulphate given three times daily, and does not produce gastro-intestinal side effects (10).

The present study was designed to evaluate the GDS iron in a clinic setting in Jamaica. In most concise form, the results appear in Table 12. Evidently, the absorption of the GDS iron was superior to that of the ferrous sulphate and side effects and compliance were similar.

Table 12: Summary of Results over 12 Weeks.

	Placebo	GDS	FeSO4
Supplementation (mg)	0	50	100
Hb gains (g/dl)	-0.64	0.88	0.83
Average compliance	0.94	0.93	0.95*
Side effects per person	4.83	6.01	5.25*
Attrition	23%	30%	28%*

* Non-significant difference

To evaluate the effectiveness of a treatment, clinicians and researchers must determine whether their patients adhere to therapeutic recommendations. Methods of assessing compliance with medications prescribed for various illnesses all have inherent limitations. Pill counts are cumbersome, require strict control, and are generally used only in basic research. Techniques more easily employed by clinicians, such as direct inquiry or monitoring treatment outcomes, fail to identify many patients who do not take their medications as prescribed. It does appear, from the present

study, that the accurate measurement of compliance is more important, for research purposes, than the emphasis on high compliance. Indeed a higher variability in compliance scores would better enable the complex relationships among variables to be unraveled.

As was noted in the results, many different side effects were questioned on each ante-natal when she returned at two, four, six, eight and twelve-week intervals. There were no side effects noted in either the GDS iron group or ferrous sulphate group, that did not appear in the placebo group. Some side effects did diminish after a four-week period but also diminished in all three groups. There are two possible explanations for these results. They are:

1. By starting to question the pregnant women about side effects at two weeks they had forgotten about these side effects, if the side effects presented themselves early; or

2. Pregnant women were already experiencing discomfort due to the pregnancy and did not notice side effects from the iron therapy, if they did exist.

A small follow-up study has been designed to investigate shorter term side effects as well as to attempt to measure their intensity. If this fails to reveal significant differences between treatment groups then one will be tempted to conclude that a greater concern exists regarding side effects of iron supplements than is warranted and that the effects of pregnancy tend to swamp those due to iron pills.

If a new iron preparation such as GDS is being promoted in the health service or in a country, cost is extremely important. The projected cost of one GDS iron tablet is approximately equal to that of two ferrous sulphate tablets. This is in contrast to other slow release preparations such as Fe FOL which cost nearly four times as much for similar efficacy. (Table 13).

Table 13: Costs of Various Types of Supplementation.

	FeSO4	Fe FOL	GDS
Number of tablets	2	1	1
Amount of iron (mg)	120	47	50
Cost/day (US cents)	0.52	1.96	0.52

The results of the hematology are encouraging. With the convenience of one pill per day and at no extra cost, GDS deserves further consideration as an alternative to conventional FeSO4.

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ANNEX 1

RESEARCH TEAM

Mr. Keith Bingham
Computer Specialist
University of the West Indies
Statistician and Analyst on the Project

Dr. J.D. Cook
Phillips Professor of Medicine
Kansas University Medical Centre
Haematology Consultant

Mrs. Jean Jackson
HBS Iron Project
Sociologist
Field Supervisor

Mrs. Maria Jackson
Ministry of Health
Government of Jamaica
Nutritionist
Field Supervisor

Dr. Sam Kahn
Senior Nutrition Advisor
USAID
Washington, D.C.

Dr. A.W. Patterson
Director
Caribbean Food and Nutrition Institute
Obstetrics and Gynaecology, Public Health Consultant

Dr. W.K. Simmons
Caribbean Food and Nutrition Institute
Public Health Nutritionist
Principal Investigator

Mrs. Marjorie Thomas
HBS Iron Project
Biochemist
Responsible for all haematological and biochemical determinations on the project

APPENDIX 2

DRAFT

A PROJECT PROPOSAL

A PARTNERSHIP FOR IMPROVING IRON NUTRITION IN GRENADA

THE FORTIFICATION OF WHEAT FLOUR (Using The Nutribusiness Model)

Collaborative Project With

THE GOVERNMENT OF GRENADA

the

CARIBBEAN AGRO INDUSTRIES Ltd.

and

THE CARIBBEAN FOOD AND NUTRITION INSTITUTE

Caribbean Food and Nutrition Institute
Kingston 7, Jamaica

Pan American Health Organization
Pan American Sanitary Bureau, Regional
Office of the World Health Organization

1991

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EXECUTIVE SUMMARY

Previous studies have shown that anaemia is a public health problem in Grenada. An islandwide anaemia survey of that country was conducted in 1985-86. The results of the study showed that anaemia and iron deficiency were serious problems throughout the entire population. The prevalence of deficient plasma ferritin levels indicating no iron reserves was as high as 62% in some population groups.

Iron supplements or iron fortified foods have been found to be cost-effective in raising haemoglobin levels of iron deficient populations resulting in increased productivity.

Studies have shown that wheat flour and wheat products are the principal contributors to energy and protein among most Caribbean populations. In the case of Grenada, wheat flour is the staple food for the population and would thus be a logical choice as a vehicle for iron fortification.

In Grenada, wheat is imported and is milled locally at a central location. Baking flour which is used in the baking trade, and for home use, is fortified with iron and B-complex vitamins. Thirty-seven milligrams of iron per kilogram of baking flour is used. The counter flour which is sold for home use and mainly in rural areas in Grenada is not fortified with iron or B-complex vitamins. The ratio of sales of baking flour to counter flour is 55:45. This means that 45% of the wheat flour (counter), which is the main source of wheat flour for home consumption, is not fortified with iron or B-complex vitamins.

The purpose of this study will be to fortify all wheat flour in Grenada with a suitable type and level of iron, evaluate the impact of the fortification programme and institute an anaemia monitoring system. This will mean adding 44mg of iron per kilogram of counter flour. The type of iron added will be ferrous sulphate.

The project will use the nutribusiness strategy. This strategy promotes indigenous private sector enterprises in food processing, thus enabling these enterprises to produce higher quality foods. It is a partnership among private enterprise and governmental organizations. Nutribusiness links nutrition and the private sector to address well-defined nutritional needs of the population.

The project will last for three years and will be evaluated by sensitive hematological parameters. The objective of the study is to significantly reduce iron deficiency in the entire Grenadian population. The evaluation will be based on analyses of blood samples and questionnaire responses obtained from randomly chosen households and from antenatals attending clinics in depressed areas. Government statistics relating to health and productivity will also be used to determine trends.

This proposal seeks funding of US$56,500 to start the study.

I. INTRODUCTION

Existing data indicate that anaemia exists in all countries of the English-speaking Caribbean. It is most common in pregnant and lactating women, and in pre-school children, with country prevalences (using WHO criteria) between 27-100% and 11% and 76%, respectively.

Severe anaemia, with a haemoglobin concentration in the blood below 8 g/dl, is found in about 6% of pregnant and lactating women and 11% of pre-school children in some of the countries.

There are three major causes of anaemia in the Caribbean:

1. Poor nutrition;
2. Specific parasitic infestations; and
3. Haemoglobinopathies.

All three causes are important from a public health point of view, and all three may interact.

The major (dietary) nutritional cause of anaemia is iron deficiency, probably due to a low absorption in the intestine and an inadequate dietary intake. A dietary deficiency of folate is sometimes also responsible. Changes in food habits of some sections of the population are associated in some instances with a deficiency of vitamin B_{12}. This appears to be currently a minor problem in the English-speaking Caribbean, but it could increase.

Hookworm and whipworm (trichuris) are the two main parasites associated with anaemia. Infestations of hookworm are isolated in certain areas of the population. The major haemoglobinopathy causing anaemia is sickle-cell disease [1-11].

In 1972, a nutrition survey was conducted in the village of La Poterie in Grenada. Anaemia was found to be a problem in all ages studied in the survey. Thirty-eight percent of pre-school age children, 65% of school-age children, 22% of adult males, and 49% percent of non-pregnant, non-lactating females were found to be anaemic by the World Health Organization (WHO) standards. Low serum iron and folates were found in the pre-school age children and adult females [12].

Another study conducted in 1976 indicated that 51% of ante-natals were anaemic, according to suggested WHO haemoglobin standards. Eighteen point five percent of pre-school age children had haemoglobin levels below 10.5% g/dl [13]. The WHO recommended haemoglobin standard for pre-school age children is 11.0 g/dl [4].

Most recent data obtained from health clinics showed that of the 400 women assessed prenatally, 234 (58.5%) had haemoglobin levels below 11 g/dl in 1983, and in 1984, of the 479 women tested, 353 (73.7%) had low haemoglobin levels, i.e. below 11 g/dl [15].

The Government of Grenada is concerned about the poor performance of its students on the CXC Examination. Questions have been raised about the possibility

of iron deficiency contributing to the poor school performance of Grenadian school children.

In 1985, two females died of post-partum haemorrhage, which could be caused from anaemia. Because of the fear that anaemia could be a major public health problem in Grenada, the Government of Grenada requested that the Caribbean Food and Nutrition Institute (CFNI) study the problem of anaemia in Grenada and develop a programme for its control.

As a first step, a population survey of iron status was planned. The variables to be studied were demographic, social, dietary, parasitic and haematological. The blood tests were haemoglobin, hematocrit, sickling, and serum ferritin. Qualitative counts of parasites in stool samples were done by CAREC. The dietary data concentrated on frequency of consumption on iron-rich food, enhancers and inhibitors on iron absorption, and possible vehicles for iron fortification.

The sample was statistically representative, with sub-samples of pregnant and lactating women. The study was conducted in November 1985.

In summary, the findings were that anaemia was widely prevalent in all ages and gender groups ranging from a low of 14% in young men to a high of 74% in pregnant women. The next most vulnerable groups were children aged 0-5 years (with a peak of 12-24 months) and lactating women. Serum ferritin results demonstrated an almost universal lack of iron stores, pointing to iron deficiency as a significant aetiological factor. SS haemoglobinopathy was found to be present in

0.5% of the sample, and 15% had an AS genotype. Hookworm was found in 6% of the stool samples. Although other parasites were found, the load was not considered to be sufficient to be a cause of the anaemia demonstrated [16].

The qualitative pattern of the diet was satisfactory, enhancers of iron absorption were consumed often and inhibitors rarely. However, quantitative data on the diet were not collected.

In June 1986, a workshop on the Grenada Anaemia Survey was convened to discuss the results of the survey and to develop a strategy for the control of anaemia in Grenada [17].

The participants at the workshop were divided into five working groups and were requested to work out a simple, workable strategy. The groups recommended specific procedures to improve supply management, supplementation programmes, haemoglobin screening, education, and fortification. It was recommended that all flour be fortified and that the effectiveness of such a programme be evaluated by the initial blood samples and the subsequent monitoring of blood samples every six months, up to two years. [7]

After the workshop, considerable work was done on the control of anaemia in pregnant women and pre-school age children. In pre-school children regular administration of iron is difficult. In pregnant women supplementation maintains the iron status but does not correct the initial deficiency. However, to reach the entire

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population other approaches must be considered. The fortification of wheat flour in Grenada should reach this objective.

Wheat flour has been fortified with iron in numerous countries around the world. Some countries have had these fortification programmes for as long as forty years. However, the fortification of wheat flour with iron has never been evaluated on a national scale.

The nutribusiness concept could be the vehicle to ensure a constructive and lasting programme. Nutribusiness links nutrition and the private sector to address well-defined nutritional needs of populations. Operationally, nutribusiness provides quality foods and food products at affordable prices through private-sector approaches. It uses environmentally regenerative production, storage, and distribution techniques and avoids pollution and inappropriate or excessive use of energy and other natural resources. Nutribusiness thus ensures optimal nutritional quality, safety, and acceptability of foods provided through commercial channels and in keeping with local cultural and environmental imperatives. The nutribusiness model addresses "well defined nutritional needs and populations". When nutritional needs are specified and target populations carefully defined, it is possible to examine how needs can be met (Annex 1).

The programme will be implemented by Caribbean Agro Industries Ltd. which is the flour mill in Grenada. Guidance and suggested levels of fortification will be provided by the Government of Grenada. The project will be evaluated by Caribbean

Agro Industries Ltd, The Government of Grenada and the Caribbean Food and Nutrition Institute (Annex 2).

Once such a programme is implemented and evaluated, it is believed that the prevalence of iron deficiency anaemia in Grenada should be reduced. It is also believed that the methodology and experience gained in Grenada would be applicable and could be used in other Caribbean countries and, possibly, in other parts of the world. A description of Grenada can be seen in Annex 3.

HOPEFUL MAJOR BREAKTHROUGHS

The following initiatives and evaluations will be attempted for the first time in this project:

First Time

1) A Government, a private sector company and an international agency will collaborate on an iron nutrition project through the Nutribusiness Approach.

First Time

2) The fortification of wheat flour with iron will be evaluated on a national scale.

First Time

3) The plasma transferrin receptor will be used to evaluate a programme on a national scale (see section VI).

First Time

4) A cost/benefit analysis will be conducted after fortifying wheat flour with iron.

II. FOOD FORTIFICATION

The fortification of foods - the addition of selected nutrients to foods in order to improve their nutritive value - is a commonly used strategy for improving or maintaining the nutritional status of a population by increasing the daily intake of specific nutrients. The technology for adding nutrients to foods is well developed and has been used in many countries for many years. Primary examples are the addition of iodine to salt, vitamin A to fats and sugar, and vitamin C to juices. The most widely used food fortification technology is for the addition of B-vitamins and iron to cereal foods such as flour, bread, meals, and pasta. The fortification of wheat flour with vitamins and iron is presently being used in more than 20 developed and developing countries. Also, in at least 12 countries that do not produce their own flour, the imported flour is required to be fortified with vitamins and minerals. The fortification of foods has been shown to be a simple, effective and relatively inexpensive way of introducing nutrients, such as bioavailable iron, into the daily diet.

The technology for the fortification of cereal food, such as wheat flour with iron, involves two major components:

1. The installation and operation of the equipment for adding the nutrient;
   a. in reasonable amounts
   b. on a fairly regular basis
2. Consumption by those in the lower economic levels, the ones most in need of the nutrients. Since cereal foods are the primary staple food for so many

of the world's population, especially in developing countries, they are the most frequently selected vehicles for fortification.

II.1 Wheat Flour Fortification in the English-speaking Caribbean

Wheat flour and wheat products are the principal foodstuffs consumed in the Caribbean, except Guyana. As can be seen in Figure 1, this wheat and wheat flour is imported from West Germany, the United States, Canada and Puerto Rico. Some of the pre-ground wheat flour imported into the Caribbean is fortified with vitamins and iron.

Most wheat flour imported into the Caribbean and ground locally is fortified with vitamins and iron except in Suriname. These wheat products are fortified at the following levels:

1.	Thiamine	-	4.4	-	5.5	mg/kg
2.	Riboflavin	-	2.6	-	3.3	mg/kg
3.	Niacin	-	35.0	-	46.0	mg/kg
4.	Iron			-	28.0 36.0	mg/kg
5.	Calcium	-	1.1	-	1.4	mg/kg

Fortification with calcium is optional and is usually not done.

The iron added in both pre-ground as well as locally ground wheat is in the form of either ferrous sulphate or reduced iron. Ferrous sulphate has a higher bioavailability than does reduced iron of a large particle size. The reduced iron size used is 44 mu in size. Studies have shown that this large particle size reduced iron is

absorbed to a limited size. Since wheat provides 17 and 24% of available energy and 18 to 30% of protein for those Caribbean countries from which information is available, this produce needs special mention in relation to fortification with iron [18].

II.2 Present Fortification Programme in Grenada

In the case of Grenada, wheat flour is the staple food of the population. The amount of wheat flour produced and available on a per capita basis can be seen in Table 1. This is the logical choice as a vehicle for an iron fortification programme.

In Grenada, presently 99% of wheat flour used is milled locally at the central location. Both baking flour and counter flour are produced. Baking flour, which is used in the baking trade and some home use, is fortified with iron, thiamine, riboflavin and niacin. The iron is added at 37.0 mg/kg of baking flour and is in the form of ferrous sulphate. Forty-five percent of the flour is counter flour. This means that 45% of the wheat flour (counter flour) which is the main source of wheat flour for home consumption and probably the flour purchased by the lower socio-economic group, is not fortified with iron or B-complex vitamins.

Both baking flour and counter flour are sold in 100 lb. bags to bakeries and supermarkets. The supermarkets usually divide both wheat flours in 2, 8, 10 or 15 lb. bags. A two-pound bag of baking flour costs EC$1.66, and a two-pound bag of counter flour sells for EC$1.56. Baking flour is also sold in five-pound labelled packets.

II.3 Change in Iron Fortification Programme in Grenada

As part of an iron fortification programme of wheat flour in Grenada, specifications will be established and plans developed with the Grenadian Caribbean Agro

Industries Limited (The flour mill), to ensure that the form of iron added to all wheat flour produced in the country be ferrous sulphate.

The wheat flour component of the fortification programme can be viewed both as:

1. An upgrade of the nutritional value: the change in iron form used in a food already part of an intervention; and
2. As a new nutrition intervention involving the fortification of counter flour with ferrous sulphate or electrolytic reduced iron and B-vitamins.

This intervention will make iron fortified flour available to people who have not had access to fortified flour.

About 55% of the wheat flour used in the country is baking flour and is produced at the Caribbean Agro Industries Limited in St. George's, Grenada. The type of flour currently produced is used in commercial bakeries to produce breads of all types and is available for some home use. The flour is fortified with B-vitamins and iron by use of a specially prepared premix which is added to the flour using typical food fortification technology. This premix contains enzymes and oxidation materials in addition to the vitamins and iron. These ingredients are not usually included in standard premixes. The form of iron used in the premix is ferrous sulphate and is added at the level of 37 mg/kg of baking flour.

Although precise costs are not available at this time, preliminary calculations suggest a slight increase in cost for adding the premix to the counter flour. The producer has agreed to absorb the increase in the cost as part of his effort for improving the nutritional value of the flour.

The remaining 45% of the flour used in Grenada is "counter flour". This flour traditionally is used by the majority of the population for general home use and probably used by the lower socio-economic group, since it is less expensive than the baking flour. Available information indicates that this flour is not fortified with any nutrients, including iron, so that the general population which consumes counter flour have not been recipients of any added iron in their daily diet through the use of counter flour. The counter flour should be fortified with B-vitamins and iron. Ferrous sulphate could be considered as the iron source of choice. A premix for this use will be much less complicated, ingredient-wise, than that used for the bread flour. It is expected that this premix also will undergo a long holding time in the mill before use, but no problems are anticipated with the use of ferrous sulphate (which is the standard for comparison) which is available from producers of premises. The cost to fortify wheat flour with B-vitamins and iron (ferrous sulphate) to current U.S. standards is about US$0.90 per tonne. The iron level for this fortification would be 44 mg/kg of flour.

In review, the iron source contained in the premix, used to fortify the wheat flour produced in the country, should be ferrous sulphate. The required iron level in all wheat flours should be determined regardless of iron source used.

A programme for the iron fortification of counter flour will involve the addition of iron such as ferrous sulphate in the mill at the time of production. The iron will be blended into a premix for ease and accuracy of addition to the meal. A level of 44 mg per kilogram of counter flour is suggested. Ferrous sulphate will be used.

The project will be self-sustaining in that Caribbean Agro Industries Ltd. has agreed to continue the fortification of wheat flour with B-complex vitamins and iron after the initial project has been completed.

III. OBJECTIVES OF THE STUDY

The general aim of the project is to significantly reduce iron deficiency in the entire Grenadian population through wheat flour fortification. To this end we will need to have all appropriate monitoring devices in place so that the benefits of the improved fortification can be observed. Such benefits may relate not only to health effects but also to worker productivity and academic achievement. Measures in the latter areas will of necessity be crude but their importance is such that they cannot be overlooked. Additionally, it will be necessary to look more closely at the high risk group: antenatals living in depressed areas.

Specific Objectives

1. To evaluate the short term impact of the change in fortification on iron status, general health and productivity of the entire population by:
   a) A survey of households which are a representative of the entire population.
      1) Hematology
      2) Food Consumption patterns
      3) Socio-economic parameters
   b) Analysis of Government statistics.
      1) Health Parameters
      2) Productivity
      3) Educational achievement

2. To evaluate the impact of the change in fortification on the detailed hematology of pregnant women attending clinics in depressed areas.
   1) Hematology
   2) Socio economic parameters
3. To improve the country wide surveillance system for monitoring anaemia and its effects. This system will have the following features:
   a) It will identify appropriate indices of productivity that are routinely part of Government statistics.
   b) It will identify appropriate health indicators that are or should be a part of Government statistics.
   c) It will ensure that appropriate observations are taken in the hospitals and clinics and transmitted to the health authorities.
   d) It will include a sub-system for the quality control of wheat flour through random sampling in food retail outlets activities and bakeries.

IV. METHODOLOGY

IV.1 Impact of Iron Fortification

The evaluation of the impact of the change of iron fortification of wheat flour derives from several sources. The first is the improved iron status of a random sample of households and after the institution of the change in fortification. The second relates to the measurement of maternal mortality and morbidity. The third

relates to productivity and academic attainment data collected by the establishment of an improved surveillance system. These changes will be evaluated for three years and some of the parameters will be continually measured and evaluated after the initial three year period of the project.

Household Survey

The population of Grenada is approximately 97,495 persons. A 1.02 percent sample will be 1,000 persons. If we assume approximately four persons per family, 250 families will be chosen for the study. However, to allow for families moving during the study 300 families will be chosen from a random sample of households chosen by enumeration districts (ED's) from the entire population. A single well-structured pre-tested questionnaire will be administered to the same households at the start of the project. The questionnaire will be administered by trained interviewers. It will focus on age, sex and employment status of household members and purchasing and consumption patterns.

The questionnaire will contain questions regarding the length of time flour is held at home before consumption. This would be important to ascertain whether the fortified flour is used within the designated shelf life. Qualitative food consumption data will be collected on each of the households. This will be done by asking questions on which food items are eaten rarely, once or twice weekly, three to seven times per week, or more than once per day. This should include some measurement of meat, fish, poultry, legumes, coffee, tea and citrus fruits and or other vitamin C rich foods. A larder survey may be conducted on a sub-sample of households.

A finger prick blood sample will be taken and the haemoglobin, plasma ferritin, and the transferrin receptor levels would be determined from each family member except pre-school age children under six months of age.

The same questionnaire including information on food consumption and finger prick blood samples will be collected at the eighteenth and thirty-six month on the same households.

Pregnant Women

The Government of Grenada has recently completed a study identifying Economically Depressed Communities in Grenada. A sample of 200 pregnant women that are attending antenatal clinics will be chosen from these depressed communities (See Annex 4). Only those pregnant women who enter the clinics at 16-20 weeks of pregnancy will be studied. Any woman with sickle cell disease will be excluded from the study. A questionnaire will be administered which will include name, age, and gestational period.

A blood sample will be taken and a hemoglobin, plasma ferritin and plasma transferrin receptor level determined. The blood samples will be taken at the beginning of the study and will be repeated at 18 and 36 months on ante-natals attending the same ante-natal clinics.

Other Parameters

The hospital system consists of main multidisciplinary hospital (General Hospital) in the capital (240 beds), a smaller general hospital in a rural area (40 beds) and a general hospital in Carriacou (32 beds).

Information will be collected on a yearly basis on mortality due to anaemia. Also in each of the three hospitals the number of patients admitted with a diagnosis

of anaemia will be recorded. The cost of treating these anaemic patients particularly patients admitted who are pregnant, will be recorded. This will include the cost of maintaining the bed, blood transfusions, etc. This information will be collected on a yearly basis.

Information will also be collected on a yearly basis to include passes in the CXC examination, low weight per age of pre-school age children, infant mortality rate, banana production, cocoa production, nutmeg production and gross national product.

V. ANALYSIS

V.1 Laboratory

The blood samples will be analysed at the Clinical Laboratory in Grenada and the Iron Nutrition Monitoring Laboratory at CFNI, Jamaica. The Department of Haematology, Kansas Medical Centre, Kansas City, U.S.A., will serve as a reference laboratory. A finger prick sample of blood will be collected from each person at the start. The sample will be taken in a plastic disposable blood collection device by using a disposable spring loader lancet. Twenty micro-litres of blood will be taken by Hemocue micro-cuvette and the micro-cuvette put into the Hemocue Photometer. The haemoglobin will be determined by the Hemocue method [20]. The whole blood sample will be kept cold and sent to the Central Hospital Laboratory, where it will be spun at 2,000 r.p.m. for 10 minutes. The plasma will be removed and frozen. The frozen plasma samples will be hand-carried to CFNI, Jamaica.

The plasma ferritin and the transferrin receptor level samples will be determined by the ELISA [21-22].

V.2 Statistical Analysis

1. Simple graphical analyses of trends in various Government statistics for productivity, educational attainment and health. These will include analysis of retrospective data where possible.
2. Simple graphical analysis of trends in various haematological measures as applied to the various sub population groups:

   0-5 years, 6-4 (M), 6-14 (F), 15-44 (M),

   45 and over (M), 45 and over (F), antenatals.

   This will be supplemented by ANOVAs to compare measures before and after the change in fortification.
3. An appropriate technique for statistical analysis of quality control data (flour) is yet to be worked out.
4. Data on flour consumption will be related to haematological parameters using correlational techniques.

VI. HEMATOLOGY

Estimating the severity of mild iron deficiency assumes greater importance in population studies. Earlier surveys relied on single cut-off levels of laboratory parameters to distinguish normal from iron-deficient segments of a population. In the evaluation of the Health and Nutrition Examination Survey (HANES II) in the United States, combinations of measurements were used to define prevalence of impaired

iron status or iron deficiency anaemia. (23) Another recent approach is to use algorithms to estimate body iron quantitatively in each sampled individual. (24) Because of the close correspondence between the serum ferritin and body iron stores, this calculation is reliable in otherwise healthy individuals if residual iron stores are present. At the other extreme in patients with overt anaemia, the deficit in body iron can be estimated quantitatively from the decrease in circulating haemoglobin concentration. Between these limits, estimating mild deficits in functional iron is more difficult. In previous report, combinations of the serum ferritin, transferrin saturation, and erythrocyte protoporphyrin were used to estimate body iron between the occurrence of storage depletion and the development of anaemia (24).

A recent study has shown that the serum reception level is a reliable index of early tissue iron deficiency. Of all the measurements examined, the serum receptor was the most reliable quantitative guide to a deficiency in the functional iron compartment. Therefore, the iron status of a population could be fully assessed by using serum ferritin as a measure of iron status, serum receptor as a measure of mild tissue iron deficiency, and haemoglobin concentration as a measure of advanced iron deficiency (25).

This could be a major methodological breakthrough. This will be the first time that the receptor level has been used on a national scale. Hopefully such a sensitive indicator as the receptor level could be shown to reduce the time necessary to evaluate an iron nutrition project.

The haemoglobin, ferritin and receptor levels can be estimated on a finger prick sample of blood which will greatly facilitate the conduct of the study. Also all these measurements will be conducted in Grenada and Jamaica and will be the first time that these two sensitive hematological indicators will be used in developing countries.

VII. SURVEILLANCE

A surveillance system will be designed and left in place after the fortification of counter flour has been evaluated. The present surveillance system consists of estimating the haemoglobin level on pregnant women. This system will continue. From 1985-1988 hemoglobin levels were collected on pre-school age children but was discontinued because of a lack of hemoglobinometers. Data will continually be collected on maternal mortality and morbidity, low birth weight infants, weight per age on infants and pre-school age children, etc. Quality control will be conducted on both counter and baking flour by Caribbean Agro Industries and the Produce Chemist Laboratory. (See
Section X. Quality Assurance).

VIII. ECONOMIC JUSTIFICATION OF FORTIFICATION

The end results in food fortification programmes are the improvement in iron status of the target population, increased productivity and reduced adverse consequences arising from poor iron status. Food fortification is justified when these positive attributes could be traced to the programme.

Approximately 10% of iron contained in wheat flour is absorbed. Thus 10% of the known level of iron added per unit wheat flour consumed is expected to enhance the iron status which in turn would enhance work output. The literature suggests worker output of between one and two percent is associated with a one percent

increase in the Hb status. Among populations with severe iron deficiency anaemia, supplementation has resulted in increases in Hb status of 25 percent and over (26). Under such conditions, worker productivity could increase 25-50%.

A series of economically unquantified adverse consequences are associated with poor iron status. These include maternal mortality and morbidity, poor growth and development, impaired immune system, reduced learning capacity and lassitude.

At the rate of 44 mg per kg of iron added to the wheat flour and an average per capita consumption of 0.15 kg flour per person per day, 0.66 mg or iron is expected to be absorbed. This represents 66% of the iron retention requirement of adult males and post-menopausal females. Since approximately 55% of the flour consumed is fortified at 37 mg/kg with iron whose bio-availability is suspect, an additional contribution of less than 33% is anticipated from the change. This additional contribution from a single fortified food source could have a significant impact on the haemoglobin status and thus on worker productivity. The cost of the supplementation estimated at $0.90 per tonne is negligible while the benefits could be great. Levin reports studies in which the benefit cost ratios of iron fortification were in the order of 5-8 in Indonesia, 30-47 in Kenya and 49-79 in Mexico (26). The fortification of wheat flour in Grenada is intuitively justifiable. This study will test the economic justification and other benefits of the proposed changes.

10 % absorption

IX. MONITORING

The importance of establishing the efficiency of an iron fortification programme cannot be over-emphasized. It is seldom possible to measure the impact of a fortification programme once it is implemented, and this is particularly true with respect to iron fortification. In contrast to fortification with nutrients such as niacin, thiamine, or riboflavin, where the objective is to prevent the occurrence of isolated deficiencies, it is feasible to alter body iron stores of an entire population through dietary manipulation.

The customary approach to monitoring efficiency is to conduct a pilot study, but there are several drawbacks to this. During a preliminary trial, the supply of a fortified food is usually ensured whereas, this is not necessarily the case when implemented on a national scale. Another important limitation of preliminary iron trial is that because of the inhibiting effect of the diet on iron absorption, changes in iron status in a population produced by iron fortification occur very slowly, often only after several years. Pilot studies, therefore, delay the implementation of a fortification programme and are very costly because of the long duration of the trial.

It is therefore planned to start the study on a national scale and carefully monitor the population by using very sensitive haematological indices.

X. QUALITY ASSURANCE

During stage one and throughout an iron fortification programme, quality assurance activities must be carried out to verify the iron content of the fortified

flours. Sampling and testing for iron content and sorting out effect should be carried out routinely at the mills during the production of the products. The in-plant quality testing should be the responsibility of and carried out by the companies, since they have the laboratory facilities and good technical personnel with which to do this. This will be conducted by Caribbean Agro Industries Ltd.

Samples of products should be drawn periodically from the market shelf by designated government representatives and analyzed for iron content. This is needed to verify both the presence of added iron and in the proper amount. These samples will be analyzed at the Produce Chemist Laboratory, St. George's, Grenada. This level of quality assurance activity is necessary to ensure that there is a given level of iron intake each day based on expected consumption. A successful intervention can be accomplished only when the iron is present in the food on a continuing basis in the correct amount. Anything less than this could lead to unsatisfactory results due to lack of proper control rather than a failure of the intervention itself.

XI. SAFETY OF FORTIFICATION

The possible adverse effect of adding large amounts of iron to the diet in certain segments of a population has been discussed from time to time and deserves comment. In reviewing studies in which possible adverse effects of iron have been reported, it is important to define the route of iron administration. When large doses

of Imferon (iron dextran) were given by deep subcutaneous injection to new born infants, an increased frequency or fatal septicemia was noted, but it was not clear, whether this was due to iron or due to the intramuscular injection. There is some published evidence that the frequency of malaria may increase when full doses of therapeutic oral iron are administered to a susceptible population.

On the other hand, it must be stressed that no adverse effects have yet been demonstrated when conventional levels of iron fortification have been implemented in a population. Homozygotes for the iron loading gene, i.e. **idiopathic hemochromatosis**, have been demonstrated to occur in 1:300-500 American whites but this genetic disorder has never been clearly identified in a black individual. Countries such as Sweden are continuing to fortify the diet at a level that provides approximately 40% of dietary iron intake without demonstrable adverse effects. Therefore, there seems to be little basis for concerns for a fortification programme that will double the intake of dietary iron even in iron replete individuals. Nevertheless, because of the lack of information on the maximal consumption of wheat flour by older children and adults, it is desirable to establish consumption patterns of this food vehicle throughout the population.

XII. TRAINING

Preliminary discussions have taken place for training members of the Grenadian Food and Nutrition Council. This training will probably consist of the Systems

Analyst, U.W.I., Mona, Jamaica, in developing a training course for the computer analyst and members of the council attending training courses in the U.S.A. on Training for Project Managers. The CFNI Public Health Nutritionist will train all Public Health Nurses on the use of the Hemocue for determining the haemoglobin level and the latest techniques for taking blood samples.

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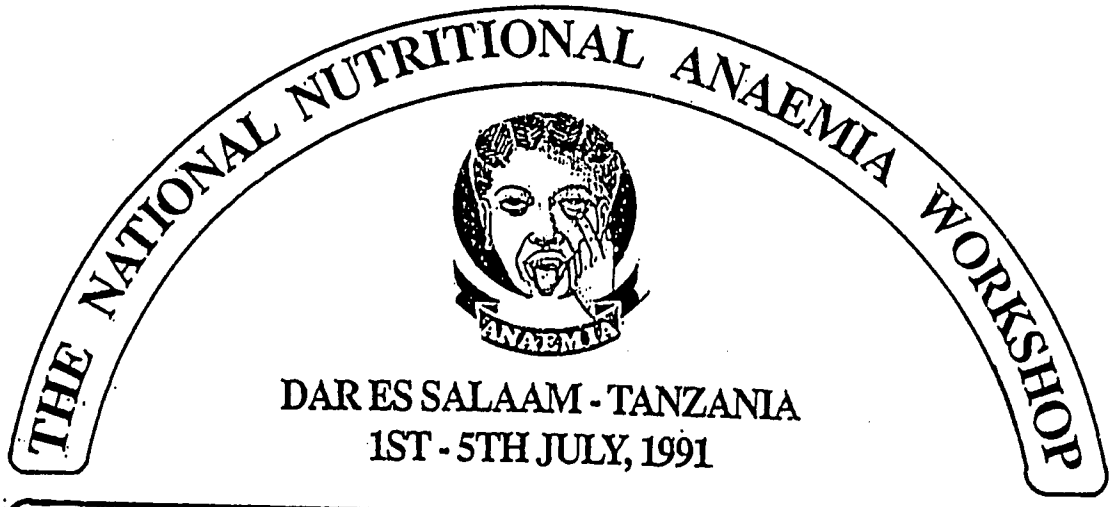
APPENDIX 3



PROGRAMME

1ST - 5TH JULY 1991

DAR ES SALAAM

TANZANIA

WORKSHOP ORGANIZING COMMITTEE

1.	DR. F. P. KAVISHE	COORDINATOR
2.	Dr. B. MDUMA	CHAIRPERSON
3.	DR. S. KIMBOKA	SECRETARY
4.	DR. S. R. TATALA	MEMBER
5.	MR. W. LORRI	MEMBER
6.	MRS. M. G. MATERU	MEMBER
7.	MS. V. LYAMUYA	MEMBER
8.	MRS. M. SILA	MEMBER
9.	MS. Z. LUKMANJI	MEMBER

Acknowledgement for Assistance:

To all those who have helped laboriously with the organizing of the workshop.

These include, participants of TFNC project of Nutritional Anaemia, Members of National Nutritional Anaemia Consultative Group (NNCG) and other TFNC staff.

WORKSHOP OBJECTIVES AND BACKGROUND

OBJECTIVES:

1. To make situation analysis of nutritional anaemia in Tanzania

2. To exchange information and experiences from within and outside Tanzania on prevention and control measures.

3. To discuss the draft proposal for a Five Year National Programme for the Control of Nutritional Anaemia in Tanzania.

4. To work out final guidelines on the strategies for the control of nutritional anaemia in Tanzania

BACKGROUND

What is anaemia?

Anaemia is a condition characterized by DECREASE OF HAEMOGLOBIN in the body to below the normal range. The decrease develops when the rate of normal Red Blood Cell production by the bone marrow fails to keep pace with their destruction or with any losses from haemorrhage.

Nutritional Anaemia is anaemia, the underlying cause of which is failure of the bone marrow to obtain sufficient nutrients which it requires for normal RBC production and maturation, hence the term "nutritional" anaemia. Important examples of nutritional anaemias are the microcytic and megaloblastic anaemias (due to iron and folic acid deficiencies respectively).

Nutritional Anaemia is a wide spread health problem, most vulnerable groups for which are women and children. In the developing countries at least half of the non-pregnant women and nearly two thirds of pregnant women have haemoglobin levels below those established by the WHO as indicative of anaemia.

The overall proportion of anaemic women is highest in Asia and Oceania, followed in descending order by Africa and Latin America. Accurate information on prevalence of anaemia in pre-school children is not available but figures of 20-50% have frequently been mentioned. The total figure of anaemic individuals, according to WHO estimates, may be 500 million and perhaps more.

National figures for nutritional anaemia in Tanzania are yet to be worked out but surveys so far done in various parts of the country reveal significantly high prevalence rates. National estimates of malnutrition for different population groups have indicated that 32% of the 7,580,000 examined had anaemia, pregnant women and underfives being the most affected.

Also anaemia 1,200,000 pregnant women and 1,980,000 underfives examined, 80% and 45% were anaemic respectively. Anaemia was found in 25% of the 4,400,000 school children and other adults examined. (1986-87 survey).

Anaemia survey carried out in the Isles in January/February 1990 which covered 24 Party Branches (equivalent of villages) of Unguja and Pemba among pregnant and lactating women and underfives revealed anaemia prevalences of:

- 31.6% in pregnant women
- 42.5% in lactating women
- 56.2% in underfives

Anaemia is an important cause of morbidity and, when severe, mortality. The morbid condition reduces work capacity with adverse effects on productivity and earnings. Maternal anaemia contributes to the oftenly noted high maternal mortality rate, abortions, intrauterine growth retardation, low birth weight and increased perinatal mortality. Childhood anaemia is associated with apathy, inactivity and significant loss of cognitive abilities.

Interventions for the control of nutritional anaemia include supplementation (with iron and folic acid), the target group of highest priority being women during pregnancy and early lactation. Other approaches, mostly of long term, are needed to correct the high incidences of deficiency states among the general population.

One of these, is food fortification with a suitable iron compound. The other is change of feeding habits and dietary modification to improve iron bioavailability (by increase in the intake of haem iron obtainable from foods of animal origin, increase in the vitamin C intake to favour iron absorption and by reducing inhibitors of iron absorption in the diet). Intestinal parasite (hookworm, schistosoma) and malaria controls are other important measures of long term significance.

In general it is only through a mix of approaches and strategies that the problem of nutritional anaemia can be tackled with some success. The National Workshops on Nutritional Anaemia, therefore, has been organised for that purpose - to deliberate on and lay down strategies for the control of nutritional anaemia in the country.

Sunday 30th June, 1991
Arrival and Registration at
Kurasini T.E.C.

V. Lyamuya
V. Mambosho

M O N D A Y 1ST J U L Y, 1991

TIME	EVENTS	RESPONSIBLE
08.30- 09.00	Registration continues	V. Lyamuya. V. Mambosho
	SESSION I: OPENING AND INTRODUCTORY STATEMENTS	Chairperson:Mr.Temalilwa Rapporteur:Mr. Mselle/ Mr. Kweba
09.00- 09.15	Opening Remarks	Dr. F. P. Kavishe Managing Director, TFNC
09.15- 09.30	World Bank Statement	Dr. Ted Greiner World Bank Representative
09.30- 09.45	WHO Statement,	Dr. E. A. Duale WHO Representative
09.45- 10.00	UNICEF Statement:	Ms. L.Van der Assum UNICEF Representative
10.00- 10.10	Welcoming Address	Dr. J. Temba Board Chairman-TFNC
10.10- 10.30	Opening Address	Prof. P. Sarungi Hon. Minister for Health
10.30- 11.00	T E A / C O F F E E	
	SESSION II: AN OVERVIEW OF THE ANAEMIA PROBLEM	Chairperson: M. Mehari Rapporteur:Mr. Lorri/ Dr. Mberesero
11.00- 11.15	Public Health significance of Nutritional Anaemia	Dr. F. P. Kavishe
11.15- 11.30	Anaemia in the African countries	Dr. T.N.Maletnlema
11.30- 11.45	An Overview of the present situation of anaemia in Tanzania	Dr. S. Mnyika

Monday Contd

TIME	EVENTS	RESPONSIBLE
11.45- 12.00	Anaemia prevalence among pregnant women in Tanzania rural	Dr. S. Tatala
12.00- 12.15	Malaria and Anaemia Situation in Tanzania	Dr. Mutabingwa
12.15- 12.30	Prevalence and severity of Nutritional Anaemia as seen at Muhimbili Medical Centre	Prof. Magessa
12.30- 12.45	Schistosomiasis and Hookworm in Rutamba, Lindi District	Dr. F. Nguma
12.45- 13.00	Anaemia in pregnancy at the MMC	Dr. S. Massawe
13.00- 13.15	Discussion	
13.15- 14.30	L U N C H B R E A K	
		Chairperson: Dr.Kavishe Rapporteur: Mr Jonathan Dr. Tatala
14.30- 14.45	The prevalence of Anaemia in Eight Villages in Rural Tanzania	Dr. A.B.M.Swai
14.45- 15.00	Hookworm and Anaemia in pregnancy (is the Haemocult test of any significance) + TDI in pregnancy anaemia	Prof. M. Kaisi
15.00- 15.15	Case management of Anaemia in children at Ifakara	Dr. B.M. Mboya
15.15- 15.30	The Blood picture in Nutritional Anaemia	Prof. R. Lema
15.30- 16.00	Anaemis Situation and control Kigoma Region	Dr. Mbaruku
16.00- 16.15	Discussion	
16.15- 16.45	T E A / B R E A K	

TUESDAY, 2ND JULY, 1991

TIME	EVENTS	RESPONSIBLE
	SESSION III: REGIONAL ANAEMIA CONTROL	**Chairperson: Prof.Kaisi** **Rapporteur: Mr. Basheke/ Mr. Magonya**
08.30 - 08.45	Proposal for programme awareness in Tanga region	Dr. Mberesero
08.45 - 09.15	DSM, Coast, Morogoro Regions	RMO's
09 15 - 09.45	Mwanza, Kagera, Mara Regions	RMO's
09.45 - 10.15	Shinyanga, Dodoma, Singida Regions	RMO's
10.15 - 10.45	Tabora, Kigoma, Rukwa Regions	RMO's
10.40- 11.15	T E A / C O F F E E	
	SESSION III: Contd.	
11.15 - 11.45	Iringa, Mbeya, Ruvuma	RMO's
11.45 - 12.15	Lindi, Mtwara, Zanzibar	RMO's
12.15 - 12.45	Kilimanjaro, Arusha, Tanga,Rombo District (DMO)	RMO's
12.45 - 13.00	Discussions	
13.00- 14.00	L U N C H B R E A K	
	SESSION IV ANAEMIA CONTROL STRATEGIES	**Chaiprson: Dr.Malangalila** **Rapporteur:Mr. Basheke/ Mr. Magonya**
14.00 - 14.15	Strategies for the control of Nutritional Anaemia.	Prof. M. G. Medhin
14.15 - 14.30	Health Policy Implications of Anaemia in Tanzania	Dr. F. Mrisho
14.30 - 14.45	Agricultural Policy Issues on the control of Nutritional Anaemia	Mr. S.M. Jonathan
14.45 - 15.00	Safe Motherhood and place of of Anaemia in MCH Cards	Dr. A. Mzige
15.00 - 15.15	Lactating women a risk group for Iron deficiency	Dr. Ted Greiner
15.15 - 15.30	Recommended dietary allowances for Iron in different countries	Mrs. M. Materu
15.30 - 15.45	Iron Bioavailability	Dr. A.Mosha/W.Lorri
15.45 - 16.00	Infatile anaemia: its relatioship with malaria and nutrition factors	Dr. Van Meus
16.00 - 16.15	Discussions	
16.15- 16.45	S O F T D R I N K S	

W E D N E S D A Y 3RD J U L Y, 1 9 9 1

TIME	EVENTS	RESPONSIBLE
	SESSION V: ANAEMIA CONTROL STRATEGIES (Continued)	**CHAIRPERSON:Dr.F. Mrisho** **Rapporteur:Mr.Ruhiye/ Dr. Mbaruku**
08.30 - 08.45	Food Fortification with Iron	Ms. Z. Lukmanji
08.45 - 09.00	Iron Supplementation	Dr. Cook
09.00 - 09.15	Compliance to iron supplementation during pregnancy	Ms. Lotta Ekstrom
09.15 - 19.30	Promotion of Horticultural products (Vit. C, folic acid and iron rich foods)	Mr. Mselle
09.30 - 09.45	Pregnant women and Anaemia control	Dr. K Lugeye
09.45 - 10.00	Nutritiona Anaemia among pregnant and Lactating women an alternative approach	Dr. Kayombo
10.00 - 10.30	Anaemia control in the context of programme for women and children Community Based	Dr. B. Ljungqvist
10.30 - 11.00	Discussion	
11.00- 11.30	T E A / B R E A K	
	SESSION VI: DRAFT PROPOSAL FOR THE PRESENTATION OF THE DRAFT	**Chairperson:Dr.S.Massawe** **Rapporteur:Mr. Kweba/ Mrs Missano**
11.30- 13.00	Presentation of the Draft	Dr. S. Kimboka
13.00 - 14.30	L U N C H B R E A K	
14.30 - 16.00	**Draft Proposal Discussion**	**Groups**
16.00 - 16.30	S O F T D R I N K S	

THURSDAY, 4TH, JULY, 1991

TIME	EVENTS	RESPONSIBLE
	SESSION VII: ANAEMIA CONTROL PROGRAMME DRAFT DISCUSSION	**Chairperson:Dr.Massawe** **Rapporteur:RMO-Lindi/ Dr. Mduma**
08.30- 12.00	Working groups for control program Draft Discussions	Dr. S. Kimboka
12.00- 13.30	L U N C H B R E A K	
13.30- 16.00	Group work continued and concluded	
16.00- 16.30	T E A / C O F F E E	

F R I D A Y 5TH, J U L Y, 1 9 9 1

	SESSION VII: PRESENTATION OF DRAFT	**Chairperson: Prof. Mehari** **Rapporteur: Dr. Sanga/ Dr. Massawe**
08.30- 10.00	Presentation of the Report	Dr. S. Kimboka
10.00- 10.30	**T E A / C O F F E E**	
	SESSION IX: REVIEW OF GROUP REPORTS FORMULATION OF RECOMMENDATIONS	**Chairperson: Prof. Mehari** **Rapporteur: Dr. Sanga/ Dr. Massawe**
10.30- 12.00	Summary of Group Report and Recommendations	Dr. S. Kimboka
12.00- 13.30	**L U N C H B R E A K**	
	SESSION X: ADOPTION THE OF REPORT AND RECOMMENDATIONS	**Chairperson: Dr. Mzige** **Rapporteur: Dr. Tatala/ Dr. Mduma**
13.30- 2.30	Adoption of the Report	
	SESSION XI: CLOSURE OF THE WORKSHOP	
2.30 - 3.0-	Closing Remarks	Dr. F. P. Kavishe
	Closing Speech	Minister, Community Development, Women Affairs and Children

WORKSHOP ORGANIZING COMMITTEE

1.	DR. F. P. KAVISHE	COORDINATOR
2.	Dr. B. MDUMA	CHAIRPERSON
3.	DR. S. KIMBOKA	SECRETARY
4.	DR. S. R. TATALA	MEMBER
5.	MR. W. LORRI	MEMBER
6.	MRS. M. G. MATERU	MEMBER
7.	MS. V. LYAMUYA	MEMBER
8.	MRS. M. SILA	MEMBER
9.	MS. Z. LUKMANJI	MEMBER

Acknowledgement for Assistance:

To all those who have helped laboriously with the organizing of the workshop.

These include, participants of TFNC project of Nutritional Anaemia, Members of National Nutritional Anaemia Consultative Group (NNCG) and other TFNC staff.

POLICY IMPLICATIONS OF NUTRITIONAL ANAEMIA

By Dr. F. Mrisho,
National Family Planning Programme-
Ministry of Health

Abstract

Various policy implications are examined bearing in mind that a national control programme is envisaged

Policy issues considered include:

- o Methodology for screening and the resource implications of the chosen methodology
- o Control strategy considering
  - preferential targeting
  - haematenics usage
  - specific interventions e.g. malaria control, and iron fortification
- o Identificationof gaps in knowledge and setting research priorities
- o Resource implications of the programme

Lastly the author suggests the need for an explicity policy for anaemia control.

STUDY TO ASSESS PREVALENCE OF ANAEMIA AND FACTORS ASSOCIATED WITH NON-COMPLIANCE OF HAEMATINICS IN PREGNANT MOTHERS IN RURAL POPULATION

Dr. S. TATALA,

TANZANIA FOOD AND NUTRITION CENTRE

Abstract

Two surveys were carried out sumultaneously in health facilities in Dodoma, Iringa and Mbeya region.

- to assess the prevalence of anaemia amongst
- identify factors in relation to their compliance of haematinics

Data was collected from the annual reports in MCH clinics visited and by interviewing pregnant mothers. The results indicate that the prevalence of anaemia, using 8.5g d/l cut off point for Hb levels, ranged from 3%-24.3%.

- In relation to haematinics supplementation, factors identified for non-compliance by pregnant women included:-

- inadequate supply of haematinics with EDP, kits, hence they were used more as medicine than supplements

- Unpleasant side effects.

Majority of the mothers interviewed seem to be aware of anaemia and complications associated with it.

PLANNING AND EXECUTION OF ANAEMIA CONTROL STRATEGIES

Mehari Gebre-Medhin, MD,
Department of Paediatrics, University Hospital, Uppsala, Sweden

Abstract

The first step in developing a control strategy is to carry out a situation analysis. It will be necessary to establish a minimal data base for initial decision making and to identify information gaps that will have to be filled as the control programme evolves. Information required will include the epidemiology of anaemia, including age and sex distribution, geographic distribution, and main causes. A firm grasp of the management structure of primary health care, community organization, and availability of health staff and facilities is another important prerequisite. It will be necessary to identify the main dietary sources of iron as well as ascorbic acid, other food sources that will either enhance or inhibit iron absorption, and cultural practices, dietary or otherwise, that may have a bearing on the occurence of anaemia.

Anaemia control strategies build on four basic principles: supplementation with medicinal iron, public education to increase dietary intake, the control of infection and the fortification of a staple food with iron.

The presentation, will discuss these general objectives and their components. These strategies must focus on the most vulnerable groups and concentrate in the first instance on the reduction of severe anaemia. WOrld wide experience suggests thaht the prime target groups will be women in the second half of pregnancy and preschool children.

ANAEMIA CONTROL WITHIN THE CONTEXT OF COMMUNITY-BASED PROGRAMMES FOR WOMEN ADN CHILDREN IN TANZANIA

By: B. Ljungqvist
Programme Officer, UNICEF, Tanzania

Abstract

Anaemia is one of the major problems affecting the health and survival of women and children in Tanzania. Increased transmission of malaria during recent years seems to be the single most important reason for the increase in the number of reported severe anaemia in early infancy and pregnancy, but the problem is evidently caused by dietary as well as by disease factors. Effective control of anaemia mortality and morbidity will thus have to include medical as well as dietary measures, and the magnitude of the problem makes it imperative to carefully consider the cost-effectiveness of alternative approaches.

UNICEF has, together with WHO and other agencies, assisted the Government of Tanzania to develop and initiate community-based approaches to PHC and child survival and development with emphasis on social mobilization and participation. The apparent success of these approaches in terms of improved child growth, immunization coverage, child survival rates and generally improved public awareness and participation is very encouraging. Experiences acquired are now used as a basis for the Tanzania National Plan of Action for child survival, protection and development for the 1990's. The same principles are adopted for the forthcoming 1992-1996 UNICEF country support programme for Tanzania.

The presentation will summarize the main elements of this emerging community-based approach to programmes for women and children. These includes advocacy, information, education and other mobilization activities together with direct support of essential inputs (medicines and other supplies and equipment, transport, etc) and services. A tentative proposal for how various anaemia control measures would fit into this general framework will be presented together with some preliminary cost estimates.

INFANTILE ANAEMIA AND ITS RELATIONSHIP WITH MALARIA AND NUTRITIONAL FACTORS

A summary of data from Tanzania, and a report of a population sdtudy in 3 villages in Kilimanjaro region

By: A.H.J. Van Meurs,
Paediatrician K.C.M.C., Moshi

Abstract

Severe anaemia in small children is a major cause of hospital admission throughout the country, and it seems to be on the increase. The case fatality rate is around 10%, and often blood transfusion is necessary, a hazardous treatment in these times of HIV infections.

75-80% of cases occur within the first year of life. In Kilimanjaro region cases come solely from the malarious lowlands and not from the higher slopes of the maountain, where malaria is virtually absent. There is a seasonal influence coinciding with the end of the malaria season.

A study in 1989 of 450 children under 5 of age, in 3 villages of the slopes of East Kilimanjaro, from 1200 to 1400m. altitude showed that basic parameters of health were quite adequate here (water supply, housing, distance to clinic, educational level etc).

Only 1.3% of the children were marasmic (W/A < 60%), and 16% had W/A 60-80%. The mean Haematocrit was 36 Vo 1%. Only 1.5% had a positive blood slide for malaria parasites.

The diet, as estimated from a 24 hours recall interviews showed that 13% had a diet of low iron availability, and 30% of intermediate availability.

These data show that in the absence of malaria, anaemia is nonexistent inspite of marginal iron intake in children < 5 years.

IMPROVING MATERNAL AND CHILD SURVIVAL THROUGH THE APPRAISAL AND CONTROL OF ANAEMIA IN KIGOMA REGION

By: Dr. G. Mbaruku, Medical Officer
Regional Hospital

Abstract

Anaemia is an important cause of morbidity and mortality in both children under the age of 3 years and pregnant women in Kigoma Region. Data from MCH clinics districts indicate that about 35 per cent of pregnant women attending antenatal clinics have haemoglobins below 8.5 gm/dl while about 50 per cent of children attending MCH clinics have haemoglobins below 7.8 gm/dl.

Severe anaemia is the number one killer of infants and young children aged 6 months and up to 4 years in our community. In 1989, out of a total number of 368 underfives deaths in the Regional Hospital, 196 (53.2%) were due to anaemia alone. While out of 12 maternal deaths, 8 (66%) were due to causes related to anaemia. In the districts corresponding mortality figures for anaemia were 59% for children and 61% for pregnant women.

A study is proposed with the hypothesis that malnutrition and malaria are the main causes of anaemia in underfives and pregnant women in Kigoma Region. The study would involve both a base line survey to assess the prevalence of anaemia and Knowledge Attitude and Practice Study in 100 villages in all four districts.

The results which will be disseminated to health planners, community, Ministry of Health and donor agencies, will form the basis of further interventions in future.

PROPOSAL FOR A PROGRAMME OF ANAEMIA AWARENESS IN TANGA REGION

By: Mberesero B.S.
Bombo Regional Hospital

Abstract

Nutritional anaemias have been the number one cause of antenatal and hospital admissions in Bombo hospital. The situation is the same in the district hospitals and health centres. Anaemia is noticed more amongst the mothers of low social economic status than those of the higher economic status.

Main causes of Anaemia in Tanga region are:

- lack of adequate nutrition
- recurrent infections - mainly malaria
- closely spaced births

Anaemia is present in all groups of population but is most prevalent in pregnant women and poses a bigger threat to their life and health.

The maternal mortality rate in Bombo hospital, is amongst the highest in the country; 80% of the deaths are due to haemorrhage mainly in already anaemic mothers.

One would expect that spread of education up to primary school level which Tanzania has achieved would have improved people's food habits, but this has not happened. People still eat what they are accustomed to eat, even when it has very low nutritional value. This is because many of them are not aware of the association between good nutrition and anaemia.

The proposed regional programme of Anaemia Awareness intends to solve the problem of anaemia and its associated complications in pregnant women.

ASSESSMENT OF A PROGRAMME FOR PREVENTION AND MANAGEMENT OF ANAEMIA IN PREGNANCY AT PHC LEVEL IN DAR ES SALAAM

A prospective controlled Intervention Study

By: Dr. S. N. Massawe

Abstract

Anaemia in pregnancy is very prevalent in Dar es salaam. It is an important cause of maternal morbidity and, is currently the number one cause of maternal mortality.

The proposed study has the following main objectives:-

- To determine the prevalence and causes of Anaemia in Pregnancy in Dar es salaam, and its relation to maternal characteristics.

- To define existing problems with the provision of prophylaxis for anaemia at MCH clinics.

- To determine the effect of intervention aimed at reducing the incidence of severe morbidity due to anaemia in pregnancy in the study clinic compared to that in controlled clinic.

The planned interventions include:

- Community based health education
- Educational workshop for health workers
- provision of screening facilities
- provision of prophylactic drugs
- continuing health education at AN clinic.

NUTRITIONAL ANAEMIA AMONG THE PREGNANT AND LACTATING WOMEN: AN ALTERNATIVE APPROACH TO MANAGEMENT

By: Mr. E.J. Kayombo
Traditional Medicine Research Unit,
Muhimbili Medical Centre-Dar es salaam

Abstract

Anaemia is defined as an iron deficiency. The causes, spread and its health repercussion particularly to pregnant and lactating women are well documented. Various measures have been taken to curb the problem. The measures include educational health campaign, maternal child health care programmes and literacy education. Despite the various measures taken, the reduction of anaemia is not significant according to various document literature.

There are several factors which account for such slow reduction of anaemia problem in the country. The important ones are: firstly, some of the programmes were hit and run (health educational programmes), some were sporadic (e.g. encironmental sanitation campaigns). Effective programmes need to be continuous and should have an inbuilt self evaluation mechanism. Secondly, emphasis had been on home economics and nutritional education. Home economics and nutritional education are important but the programme should include improved agricultural activities in places with adequate land; and off farm income generating in areas which have inadequate land. These will ensure regular supply of food, rich in iron. Thirdly, the programmes targeted to child bearing women have neglected the socio-structures (role of man, traditional birth attendants and traditional leader), which influence as to what the pregnant or lactating women whould eat.

Programmes which aim to tackle the problem should be user-oriented and have multidimensional approach. Designing such programmes need an understanding of the community's life style and of their basic needs.

The user-oriented approach to reduce the problem of anaemia calls for health personnel who is well versed with socio-cultural and related health aspects.

PREVALENCE AND SEVERITY OF NUTRITIONAL ANAEMIAS AS SEEN AT MUHIMBILI MEDICAL CENTRE (MCC) DAR ES SALAAM DURING A 2 YEAR PERIOD

By: Dr. Mmari M.P.W; Dr. Magesa PM
Department of Haematology and Blood Transfusion
Muhimbili Medical Centre

Abstract

Anaemia continues to be among the major causes of morbidity and mortality in the medical wards at Muhimbili Medical Centre. During the period January 1989 to December 1990 it accounted for 18.3 percent of all admissions into these wards. Detailed laboratory investigations and actual classification of these anaemias remains far from adequate since 93.8 percent of the anaemic patients left the hospital with a clinical title of unspecified anaemias. Among the 266 patients investigated, anaemia due to nutritional deficiency appeared most frequently (49.6%) that any other aetiological factors. Many female patients (54.5%) especially those aged below 30 years were found to be iron deficient. Folate/B_{12} deficiency was the second commonest cause of the nutritional anaemias (28.8%) affecting female of the childbearing age (15-30 years).

The severity of the nutritional deficiency anaemias was equally marked at diagnosis in both sex at various age groups with the majority (56.9%) presenting with haemoglobin concentrations below 60 g/l.

The current situation has not changed much from that reported by Nhonoli in the same hospital seventeen years ago. We need to ask ourselves why.

IS THERE AN ALTERNATIVE TO BLOOD TRANSFUSION? A CLINICAL CASE MANAGEMENT STUDY IN CHILDREN WITH ANAEMIA AT IFAKARA HOSPITAL

Dr. D. M. Mboya, Dr. B. Holzer
St. Francis designated district hospital/STIFL, Ifakara

Abstract

A randomized intervention study was undertaken in 110 children admitted for various reasons but all with anaemia. Standards treatment, of anaemia prior, to the occurence of HIV infection, was the transfusing of a few units of whole blood.

The blind alternative case management strategy was a consequent malaria prophylaxis with chloroquine 5 mg/kg body weight and folic acid. All children were followed for 3 months by home visits.

The average PCV reached comparable levels after 2 months in the malaria prophylaxis group compared to the transfusion group.

As a hospital based management strategy, it is therefore proposed that a 2 month chloroquine prophylaxis including folic acid as an effective case management strategy as whole blood transfusion; the former with possible resource savings as well as a reduced risk of HIV infection.

POLICY ISSUES ON THE CONTROL OF NUTRITIONAL ANAEMIA IN TANZANIA

By: Mr. S.M. Jonathan, Development Studies Institute,
Sokoine University of Agriculture, Morogoro

Abstract

The paper identifies the risk groups as women and children for reasons of patriarchy. Society has accorded status to men whose privileges have included taking more and better foods.

Dodoma, Morogoro, Mtwara, Lindi, Tanga, Arusha and Coast regions constitute the risk zones for nutritional anaemia.

Seasonality is also important; the rainy season is the busiest time of the year yet by then food stocks have dwindled. In addition it is a time of vulnerability to endemic diseases such as malaria, and those associated with worms.

The paper recommends ways of integrating nutrition into agriculture and rural development. An important aspect of this is the identification of crops that are rich in proteins and minerals within specific localities. The role of NGOs in identifying and assessing village level resources is very important. An important strategy should involve the formation of grassroot organizations and groups that will mobilize the affected people.

Grassroots groups and organizations are better placed to deal with cultural norms that constrain women and children's access to good food. There are practices which need to be discarded. The forbidding of rabbit and antelop meat in Kilosa district, for example, should be a target among other things.

PREGNANT WOMEN AND ANAEMIA:
A CASE IN MOROGORO URBAN AND SELECTED VILLAGES WITHIN THE DISTRICT

By: Kalunde Lugeye

Abstract

Anaemia in pregnant women is a serious and prevalent public health problem. WHO estimates that one half the pregnant women in the world are affected and three of every five pregnant women in developing countries. Because not all women receive prenatal care and not all prenatal programmes analyze blood or report their findings, no one knows the exact extent of maternal anaemia. But observers agree it is widespread. Pregnant women are particularly susceptible to anaemia due to increased requirements for the developing fetus, bodily losses throughout reproductive years, dietary factors, parasitic infections and other diseases.

The aim of the present study is to examine the extent of maternal anaemia, contributing factors, types of problems causes by the condition and how pregnant mothers will be helped in Morogoro Urban and selected villages within the District.

THE EFFECT OF PROPHYLACTIC ONCE WEEKLY CHLOROQUINE AND DAILY PROGUANIL ON MATERNAL MALARIA PARASITAEMIAS AND HAEMOGLOBIN LEVELS IN MUHEZA, TANZANIA

By: T.K. Mutabingwa and L.N. Malle
National Institute for Medical Research,
Amani Centre Tanga

Abstract

The suspected prophylactic failure of chloroquine during pregnancy, necessitated the assessment of daily proguanil as an alternative. Pregnant women, mostly primiparae and secundiparae in their first or early second trimester are randomly given either 300 mg base chloroquine (CQ) once weekly or 200 mg daily proguanil (PROG). Depending on the timing of the women's visits monitoring variables are collected viz: blood smears once fortinightly, capillary blood for haemoglobin estimation once every eight weeks together with 2mls of urine and blood for serology and drug assays.

Between October 1988 and June 1989, 302 women had been enrolled of whom 132 delivered (68 on PROG, 64 on CQ). At enrollment primiparae and secundingparae were significantly malaria - infected than multiparae. CQ - women experienced 130 malaria breakthrough episodes compared to 63 for PROG. group. Similarly, 95% of CQ episodes ended into clinical attacks compared to 78% of PROG-episodes. Haemoglobin levels was raised among the primiparae of both groups, unchanged in secundiparae and fell down among the multiparae.

Reduction of malaria infection and morbidity plus prevention of the consequent anaemia is one of the major objectives for prophylaxis. Comparatively proguanil has met this objective. it is therefore concluded that daily proguanil is better malaria prophylactic than once weekly chloroquine.

ARE LACTATING WOMEN A RISK GROUP FOR IRON DEFICIENCY

By: Dr. J. Greiner
International Child Health Unit
Uppsala University,
Sweden

Abstract

Iron deficiency anaemia is commonly stated to be a disease of pregnant and lactating women.

The statement, hence implies that pregnancy and lactation are part of cause of anaemia. While this is clearly true for pregnancy, especially when birth spacing is short, the opposite is true for lactation.

The paper dwells on the reasons as to why a lactating women should not become anaemic.

COMPLIANCE TO IRON SUPPLEMENTATION DURING PREGNANTY

RESEARCH PROPOSAL

MS. L. EKSTROM WHO/TFNC, DAR ES SALAAM

Abstract

Iron supplementation during pregnancy is a recognized efficietn means to reduce anaemia prevalence. However, in supplementation programmes it is often suggested that due to gastro-intestinal side-effects, compliance to the prescribed regimen is low resulting in a less than optimal biologic impact. Information on magnitude of side-effects and patterns of compliance is limited.

Objectives

- Study patterns of compliance and its determinants.
- Test if use of a iron preparation with less side-effects improve compliance and hematologic outcome

Study design

The study is proposed for Ilula Villages in Iringa district. Two-hundre-fifty pregnant women registering at the MCH clinic before 28 weeks of gestation will be enrolled.

Two types of iron preparation a) conventional ferrous sulphate and b) ferrous sulphate in a gastric delivery system, will randomly be assigned to the subjects. Data will be collected at five occassion during a twelve week period.

- Base-line information: household socio-economic and demographic information, reproductive history,
- Morbidity: recall of mordbity, including symptoms of side-effects will be done repeatedly

APPENDIX 4

Control of Nutritional Anaemia in Tanzania - Program Proposal

July 1991

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Control of Nutritional Anaemia in
Tanzania - Programme Proposal

Introduction

ANAEMIA

The word "anaemia" has its original meaning of "no blood"! Anaemia is a condition characterized by a REDUCTION IN THE CONCENTRATION OF HAEMOGLOBIN in the blood below the normal range. In most cases the reduction in haemoglobin is accompanied by reduction in the number of red blood cells (RBC), with blood volume being maintained at a nearly constant level. The reductions develop when the rate of normal RBC production by the bone marrow fails to keep pace with their destruction or with any losses from haemorrhage.

However Haemoglobin concentrations in healthy persons vary widely. World Health Organisation (WHO) has set up standards below which anaemia is likely to be present.

Table 1: Haemoglobin Concentrations below which anaemia is likely to present at sea level

Age/Sex group	Hb level in g/dl
1. Children 6 months - 5 months	11.0
2. Children 6-14 years	12.0
3. Adult males	13.0
4. Adult females (non-pregnant)	12.0
5. Adult females (pregnant)	11.0

Source: World Health Organisation: Nutritional anaemias.(1) 1972.

Anaemia is diagnosed as MILD when the haemoglobin concentration is above 10.0 g/dl but below the cut off level shown above; MODERATE when the concentration is between 7.0 and 10.0 g/dl: and SEVERE when it is below 7.0 g/dl. (1).

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NUTRITIONAL ANAEMIA

Nutritional anaemias are dyschaemopoietic anaemias in which bone marrow activity is limited by deficiency of erythrocyte and haemoglobin building blocks. The most important nutrients are iron and folic acid.

In Tanzania the most prevalent nutritional anaemias are:

i) IRON DEFICIENCY ANAEMIA and
ii) FOLIC ACID DEFICIENCY ANAEMIA

The two are also known as MICROCYTINC and MEGALOBLASTIC anaemias respectively.

EXTENT OF THE PROBLEM

Nutritional anaemia is a widespread health problem, worldwide. It is estimated that 1 billion people are affected by iron deficiency. Women and children constitute the most vulnerable group

According to WHO, in 1979 in the developing countries, it was estimated that at least half of the non-pregnant women and nearly two thirds of pregnant women had haemoglobin levels below those established by the WHO as indicative of anaemia. The overall proportion of anaemic women was highest in Asia and Oceania (58%), followed by Africa (40%) and Latin America (17%). Accurate information on the prevalence of anaemia in preschool children is not available but figures of 20-50% have frequently been mentioned. The total figure of anaemic individuals, according to the WHO estimates, may be 500 million and perhaps more.

National figures for nutritional anaemia in Tanzania are yet to be worked out. As a nutrition disorder, nutritional anaemia ranks second among the four most serious nutritional problems in Tanzania, being outranked only by Protein-Energy-Malnutrition). Surveys so far done in various parts of the country reveal significantly high prevalence rates. For example, anaemia survey carried out in the Zanzibar Islands in January/February 1990 which covered 24 Party Branches (a Party Branch comprises of several villages) among pregnant and lactating women and underfives, revealed, in accordance to the WHO cut offs, anaemia prevalence rates of: (2)

- 31.6% in pregnant women
- 42.5% in lactating women
- 56.4% in underfives.

But it is also noted that nutritional anaemia does take the first place as a nutrition disorder depending on the population considered and geographical location.

Geographical location is an important factor in the prevalences of anaemia. It is observed that anaemia is highest along the coastal belt and low lands, where malaria and the blood depleting intestinal parasites are most prevalent.

EFFECTS OF THE PROBLEM

Anaemia is an important cause of morbidity and, when severe, mortality. Anaemia reduces work capacity with adverse effects on productivity and earnings. In Pregnant women it can lead to abortions, intrauterine growth retardation, low birth weights and increased perinatal mortality. Maternal mortality rate is increased by anaemia.

Childhood anaemia is associated with apathy, inactivity and significant loss of cognitive abilities. Studies on anaemic children have shown them to be more prone to infections.

CAUSES OF NUTRITIONAL ANAEMIA

As already mentioned iron deficiency and folic acid deficiency or combination of both are the most common types of nutritional anaemia. The causes are as follows:-

1) Low dietary intake of the nutrients

Good sources of iron include green leafy vegetables, cereals, fish, meat liver heart and kidney. Dietary intake of iron may not be a problem in Tanzania. Most of the local foods consumed in the country have sufficient quantity of iron.

However foods mostly consumed are of plant origin, whose iron is the non-haem type and therefore not easily absorbable. The problem is much less with communities whose foods are of animal origin. Foods of animal origin contain haem iron, the easily absorbable type.

As for folic acid, deficiency arises as a result of non-consumption of foods rich in the nutrient.

2. Reduced food intake

This is likely to be observed in conditions in which there is lowered appetite. Chronic infections eg malaria may be a cause of long standing anorexia, resulting in a significant reduction in food intake.

But inadequate food availability and inappropriate child feeding practices may contribute more than the actual loss of appetite. Reduced food intake is the major cause of varying levels of undernutrition: iron and folic acid deficiencies are often detected among the Protein-Energy-Malnutrition cases.

3. Iron Bioavailability

The role of gastrointestinal diseases (eg diarrhoea) as a cause of malabsorption of iron is an established fact. But of much significance as a cause of anaemia is the non-consumption of vitamin C (the enhancer of iron absorption). Another, is the presence in the diet of iron absorption inhibitors such as tannins, found in tea, coffee and phytates found in pulses and cereals.

Folic acid Availability

A condition known as "congenital malabsorption of Folate"; an inherited defect in the intestinal mucosa transport system for folic acid contributes to malabsorption of folic acid.

Drugs such as phenytoin, oral contraceptives and co-trimoxazole may cause folic acid deficiency.

4. Increased requirement for the nutrients

Increased iron requirements are marked in actively growing young children and women of child bearing age, especially the pregnant and lactating ones. Hence women and children are at highest risk of developing nutritional anaemia.

Folic acid requirements increase during pregnancy and lactation, in leukemia and other diseases in which there is an increase in haemopoietic activity (in haemolytic states like malaria and neoplastic conditions).

5. Blood losses

Acute haemorrhages (bleedings) cause rapid loss of blood for example during complications in pregnancy, deliveries and traumas. Large amounts of iron can be lost during chronic blood losses, and this is not easily noticed. Also intestinal parsites such as hookworm and schistosoma can cause iron losses leading to. Chronic iron deficiency states.

CLINICAL FEATURES OF ANAEMIA

Haemoglobin, the red pigment of blood, has a function of transporting oxygen from the lungs to the tissues. Clinical features of anaemia, therefore, are due to the reduced capacity of Haemoglobin to transport sufficient oxygen to different parts of the body.

Symptoms include:

1. Fatigability, the tendency to tire easily. An anaemic person can't sustain activities for long, be it physical or mental. This is because the muscles and the brain are not getting sufficient oxygen needed for the metabolic activities.

2. Breathlessness. The subject experiences laboured breathing even on mild physical activity.

3. Palpitation. Decreased haemoglobin concentration means decreased capacity of the blood to transport oxygen and therefore resulting into tissue hypoxia. This leads to increased cardiac output, hence palpitation.

4. Pallor. This is detected on mucous membranes (sclera, gum, tongue) and nail beds.

5. Pica. Anaemic subjects are observed to be craving for unnatural articles of food, like dirt, soil, chalk. This may be due to inadequate iron in the body.

Other symptoms include dizziness, faintness, headache and blurring of vision. Splenomegaly (and even hepatomegaly) may develop.

2. CONTROL OF NUTRITIONAL ANAEMIA

Interventions for the control of nutritional anaemia can be short or long term. Short term intervention may be for example. provision of iron and folic acid supplements to pregnant women. Long term measures, may be:

food fortification of suitable food with a suitable iron compound. The implementation of this control measure has been observed to be successful in developed countries where most foods are industrially processed.

Another long term intervention may be improvement of iron bioavailability from foods consumed. This can be achieved by increasing the intake of haem iron obtained from foods of animal origin. Intake of vitamin C leads to increase in iron absorption. Therefore consumption of foods rich in Vit. C should be promoted. Measures musut be taken to minimise the effect of iron absorption inhibitors such as tannins found in tea, phytates found in cereals and oxalates found in green leafy vegetables.

Intestinal parasites and malaria control are other interventions of highest priority. It has been observed that malaria is the number one cause of anaemia in Tanzania. Hookworm and shistosoma also contribute significantly to iron deficiency states. Control measures are improving sanitation, deworming, and health education.

Nutrition education should be a necessary component of all long term interventions. There are situations where appropriate foods are readily available, but not consumed regularly; adequate food intake are often counter acted by heavy worm loads; iron and folic acid supplements may be adequately supplied to pregnant women, but not taken by them due to poor compliance! These problems can be greatly reduced through creating awareness of the target populations.

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BACKGROUND TO THE DRAFT PROPOSAL FOR A 5 YEAR PROGRAMME FOR THE CONTROL OF NUTRITIONAL ANAEMIA IN TANZANIA

Nutritional Anaemia as a TFNC. Project started way back in the year 1978/79. A number of activities both aimed at research and intervention were initiated and implemented. (3, 4, 5, 6, 7, 8.).

These activities however lacked a long term intervention approach. Therefore, in 1989 with the loan from World Bank to the Government of Tanzania, it was decided to formulate a 5 year national programme for the control of Nutritional Anaemia.

The programme was to have 4 lines of action:- (9)

- training of MAs and RMAs in the recognition of clinical iron deficiency and in the effective administration of iron supplements to vulnerable groups.

- promotion of the production and consumption of iron-rich foods (such as green leafy vegetables) and particularly vitamin C rich fruits which assist the body in the uptake of iron from the food.

- development and utilization of appropriate IEC materials, targeted particularly pregnant women.

- a programme of research of morbidity, mortality risk related to iron-deficiency, the socio-cultural factors contributing to the inadequate intake of iron-rich foods and a strong programme of operational research on compliance with iron supplementation tablets and the trials of alternative pharmaceutical compounds.

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PROGRAMME OBJECTIVES

Broad Objective

To reduce the problem of Nutritional Anaemia in Tanzania by 1/3 by the year 2000.

Specific Objectives

- To create and increase awareness on the problem of nutritional anaemia and ways for its control.
- To generate more knowledge on the status and underlying causes of nutritional anaemia in the country
- To monitor and ensure availability and utilization of iron and folic acid supplements in the MCH clinics for women during pregnancy and early lactation.
- To promote adequate and regular dietary intake of the micronutrients essential for the control of nutritional aneamia
- To facilitate appropriate intervention measures for the control of blood depleting parasites
- To establish mechanisms for food fortification with iron in Tanzania.
- To establish monitoring and evaluation system for the programme.

Strategies

1. Nutritional Anaemia should be an essential component of nutrition education programmes in the country.
2. Nutrition anaemia should feature in the syllabuses of all Health and related training institutions.
3. Reorientation of extension workers in nutritional anaemia through seminars, workshops, short courses.
4. Preparation of relevant and appropriate educational materials for the field workers and the community.

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5. Effective dissemination of knowledge and information on nutritional anaemia through appropriate mass communication channels to the target population.
6. Carrying out of Situation Analysis on nutritional anaemia in Tanzania in order to have all available information reviewed and compiled.
7. Carrying out of applied and operational research on nutritional anaemia
8. Provision of screening facilities for anaemia in all MCH clinics in the country.
9. Conducting regular needs assessment of haematinics (iron and folic acid tablets) in all MCH clinics.
10. Promotion of adequate production, consumption, preservation and appropriate preparation methods of foods rich in iron, folic and ascorbic acids.
11. Promotion of consumption of foods with readily available iron, and improving bioavailability of iron from the commonly consumed foods.
12. Integration of nutritional anaemia component into already on-going parasitic control and environmental sanitation programmes.
13. Carrying out of feasibility studies on food fortification with iron
14. Initiation of pilot food fortification programmes
15. Strengthening of the current MCH data reporting system which includes nutritional anaemia
16. Making use of the MCH data on nutritional anaemia to assess the overall impact of the programme.

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Activities undertaken by TFNC since January 1991 are:

1. Vitamin C supplementation study carried out in the Mandaka area, Moshi.
2. Study on sociological factors contributing to non compliance to iron and folic acid tablets by pregnant women
3. Carrying out of situation analysis on nutritional anaemia in Tanzania
4. Ordering of laboratory items for the various investigations as would be demanded by the programme.
5. Training of one laboratory technician on haematological investigations, and
6. The preparation of the national workshop on nutritional anaemia

The proposed programme is still a draft, and subject to alteration and modification as per discussions and recommendations by the workshop participants. The programme will therefore finally formulated by this workshop.

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APPENDIX 5

Proposal for a research study on

"Compliance to iron supplementation during pregnancy"

To be discussed at

The First National Nutritional Anemia Workshop

Dar es Salaam, July 1-5, 1991

Eva-Charlotte Ekström

Nutrition officer
WHO

Tanzania Food and Nutrition Center
PO Box 977, Dar es Salaam
Tanazania

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I. Background

Iron deficiency anemia among pregnant women has for a long time been recognized as a major public health problem. This is particularly true for developing countries where anemia makes a large contribution to pregnancy complications and maternal mortality (WHO 1979, Fleming et al 1986).

It is quite clear from a number of studies that supplementation of elemental iron during pregnancy is a possible means to prevent a fall in hemoglobin levels and decrease the prevalence of anemia (Simmons 1990, Charoenlarp et al 1988, Fleming et al 1986) So far, most efforts to solve the anemia problem has focused on identifying appropriate dose, iron compound and formulation to ensure a positive change in the blood parameters. However, when attempts have been made to apply these clinical findings in public health programmes results have been less successful. A major reason for this is suggested to be non-compliance to the iron regimen on part of the pregnant woman (WHO 1990).

One of the most commonly proposed reason for non-compliance is the gastro-intestinal side-effects which iron supplementation often causes (DeMayer 1989, Simmons 1990, Fochi et al 1985). Some other suggested factors are related to health beliefs, (WHO 1990), patient-health personnel interaction and social support.

Although compliance is a prerequisite for a successful iron supplementation programme limited research has been done on the determinants and constraining factors for compliance. Therefore the major focus of this study will be on compliance to iron supplementation. However, measurements of hematologic outcome will also be included to assess if a variation in compliance is of importance for anemia prevalence.

In this study a new type of iron formulation with reported less side-effect will be tested. It contains ordinary ferrous sulphate but combined in a matrix of hydrocolloid which in contact with gastric juices dissolve and form a mix which will float on top of the stomach content. This gastric delivery system (GDS) has the advantages of having less side-effects but also better absorption than both the conventional iron preparations and the so called slow-release types.

The importance of using a theoretical models in studying health behavior, like compliance, has been stressed (Christensen 1978). Application of a theoretical model assists the researcher in identifying the determinants for the particular behavior which is studied and for examination of their interrelationships. Use of a model also enables comparisons to be made between studies in different settings.

In studies of compliance to medication the most frequently used model is the "health belief model". It was originally developed by Rosenstock 1966 and further developed by Becker and Maiman 1975 (Christensen 1978). The theory argues that whether a person will comply to recommended health behavior depends on the individual's: 1) perceived susceptibility of the disease or condition, 2) the perceived severity of the condition if contracted, 3) perceived efficacy of the treatment recommended and 4) perceived difficulties or barriers to the recommended behavior. In this model side-effects sort under barriers for compliance. The health belief model will be tested as a part of the sociological component in this study.

II. Objectives

The overall objectives for the study are:

- Study pattern of compliance to iron supplementation and factors that determine it, focusing on importance of side-effects.

- Make a cost-effectiveness analysis of the use of GDS iron supplement as a means to increase compliance and improve hematologic outcome.

III. Relevance of study.

In the overall efforts to reduce maternal mortality one of the recognized strategies is to prevent iron deficiency anemia. It is established that iron supplementation during pregnancy has a positive effect, - if the supplement is taken as prescribed.

However, although there is little known why, there is evidence that compliance to supplementation is low. In the literature addressing the issue there is no consensus on the causes. For example, side-effects being a central issue, have in some sources been referred to as a major cause while others state that the importance is limited. In discussions with practicing health personnel side-effects is frequently regarded as a plausible reason for non-compliance.

In order to develop appropriate strategies for improving compliance it is regarded as useful to investigate if reducing side-effects of supplementation is a useful approach.

In the study the newly developed GDS iron preparation is going to be used. Compared to other iron formulations that has been developed to have less side-effects, the so called "slow release" preparations, the GDS preparation has a good iron absorption ratio. The iron absorption from GDS supplement is even three to four-fold higher than conventional formulations. Theoretically, if the dose and compliance level is the same as a conventional preparation

the GDS tablets would result in a better hematologic outcome. The cost of the GDS supplement is competitive to conventional iron preparations and the cost-effectiveness analysis will provide basis for decisions whether to make recommendations to substitute conventional iron preparations with GDS ones.

The study will also include a sociologic descriptive part which will address health beliefs, social support, and patient (client) - health personnel interaction. These factors have all been mentioned as playing a role for compliance. In the current study they will be assessed at the same time enabling their relative importance to observed. As the information to be collected is detailed it will show potentially effective strategies for interventions to improve compliance. For example it is expected that educational or information gaps regarding anemia and iron supplementation will be identified which will enable improvement in future communication of the importance of use of iron supplements. At the same time the knowledge gathered about beliefs and practices during pregnancy can be used in developing a more culturally acceptable message.

IV. Study area and magnitude of problem

A. Nutritional anemia during pregnancy in Tanzania

Anemia during pregnancy is a major health problem in Tanzania. It is one of the most common reason for antenatal hospital admission in Dar es Salaam region and it is also a leading factors for maternal mortality (Kavishe 1982). A study at Muhimbili Medical Centre showed that in early 1988, 26 of 71 (37%) maternal deaths could be attributed to anemia (Kaisi 1988). A large study of anemia during pregnancy was conducted in 1977. A total of 1317 women were evaluated when they came for their first antenatal clinic visit. The mean hemoglobin level was 9.3 gm% and none of the women had a hemoglobin level above the recommended cut-off of 11 gm% (Mwanukuzi E and Nhonoli AM 1972).

The major causes of anemia during pregnancy are malaria and iron deficiency. However there is limited information on their relative contribution to anemia. It is likely that is depending on region and prevalence of malaria, as well as of season. A recent study in Zanzibar (TFNC 1991) showed a prevalence of 41.2 of anemia in women using WHO cut-off of <12 g/dl for non-pregnant and <11g/dl for pregnant women. The same study demonstrated that 29.2 of the women were iron deficient as defined by a free erythrocyte protoporphyrin level above 70 microgram/dl of red blood cells. Anemia and iron deficiency showed a statistical significant correlation while malaria (prevalence 8.1 %) did not in this particular study. Although there is a need of more comprehensive information on anemia in Tanzania it is beyond all doubts that it is a major problem that need to be tended to.

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B. Study site.

The study is proposed for Ilula villages in Iringa region about 45 km from Iringa town. The villages lie on the main road Dar es Salaam to the Zambian boarder. The census for the villages in 1978 was 5657. Health care is provided by the units, one Roman Catholic dispensary and a Lutheran dispensary and this study will have the Lutheran dispensary as a basis. From previous studies in the area it is reported that antenatal coverage is high but with a big variation in gestational age at register (mean 23, range 9-35 weeks) and number of visits to the clinic (mean 6.3 and range 0-1[illegible]). Ilula villages are comparable with other rural villages in Tanzania although they probably are somewhat more developed due to the closeness of the road. There is neither electricity nor telephone.

Although no quantitative information on anemia is available from Ilula anemia is perceived by the medical assistant at the MCH to be very common (Ballart 1991). In the previous study in the area anemia was noted as the most common pregnancy complication (Kavishe et al 1987).

V. Study design

A. Overall design.

The study is divided into three phases. The first phase includes an assessment of the coverage and attendance of pregnant mothers at Ilula MCH clinic. It is a retrospective study where all women who has given birth the last three months will be visited and interviewed. Included in this phase is also collection of descriptive information regarding food and agricultural system of the community, general and specific health beliefs, and identifying factors which can be used for differentiating socio-economic status. This information will be used in development of the questionnaires to be used in the main part of the study.

The second phase is a pretest of the developed questionnaire as well as a test of the intervention components and the methodology for their assessment, in particular the measurement of compliance. The last phase is the actual study where all the proposed factors affecting compliance will be measured as well as compliance itself and hematologic outcome.

B. Phase I. Assessment of MCH coverage and development of questionnaires.

1. specific objectives

The specific objectives for phase 1 are:

- To assess coverage of pregnant women by the antenatal

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clinic.
- To describe common health beliefs related to pregnancy.
- To develop draft questionnaires.

2. Coverage of pregnant women by the MCH clinic.

In the villages to be included in the main study all household where a child has been born the last three months will be visited. The household to be visited will be identified by ten-cell leaders. At the visits information on mother's attendance at MCH clinic, such as number of visits, gestational age at registration and reason for participation or non-participation will be collected. The aim is to find out how large proportion of the pregnancies in the area are covered by the MCH clinic.

3. Development of questionnaires.

At the visits to the household for assessment of coverage of the MCH service material will be collected for preparations of the questionnaires to be used in the main study. Information on; food and agricultural system; socio-economic differentiating variables; beliefs around pregnancy and anemia; as well as common symptoms and their treatment during pregnancy will be gathered. The latter will be used to create a list of conditions common during pregnancy which also will include the anticipated side-effects of the iron supplement. The list will be part of the "morbidity" questionnaire.

C. Phase II. Pilot test of questionnaires and outcome measures.

1. Specific objectives.

The specific objectives of phase 2 are;

- Pre-test of questionnaires.
- Pre-test of methodologies and procedures for assessing outcome variables.

2. Methodology.

Phase 2 involves a testing of all variables to be measured in the main study. For a period of two months data will be collected according to the schedule set for the main study. This involves a pretest of the questionnaires developed in phase 1 for the assessment of a) household characteristics; b) sociologic aspects of compliance; and c) morbidity.

Measurement of the outcome variables of compliance by microprocessor, stool or urine test will be tested as well as the measures of hematology and parasites.

Apart from testing the actual assessment instrument for the various variables this phase is a practical preparation for management of data collection in the main study.

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D. Phase III. Intervention study

1. Specific objectives

The specific objectives for the intervention part of the study are the following;

- To assess level of initial compliance to iron supplements.
- Study pattern of continuous compliance and its determinants.
- Test if use of gastric delivery system (GDS) iron preparation with reported lower frequency of side-effects has an improved effect on continuous compliance compared to conventional preparations.
- Compare cost-effectiveness of conventional and GDS iron preparation on continuous compliance.
- Compare effectiveness in improving hematological parameters related to anemia (in particular hemoglobin) between conventional and GDS iron preparations.
- Compare cost-effectiveness of conventional and GDS iron preparations on hemoglobin level.
- Test the explanatory capacity of the components in the health belief model on variation in compliance.

2. Study population and sample size

Of all women registering for MCH clinic at Ilula dispensary women with a gestational age less than 27 weeks will be offered to participate in the study and their consent to do so will be sought. Women with Hb less than 7 g/dl (cut-off for severe anemia) will be excluded from the study and referred to Iringa hospital as the routine is. Women with, or developing pregnancy complications such as: requiring blood transfusion; emergency obstetric intervention; eclampsia; or chronic diseases will also be excluded from the study.

The following calculations were made to estimate the number of women it may be possible to enroll per month. Based on information from the health center approximately 50 children are born at the clinic per month. If 80 percent of these have participated in MCH clinic before delivery there will be 40 children per month. Of these, assume 75 percent have participated before week 27 in pregnancy which lead to 30 pregnant women per month who can be offered to participate. If 80 percent accept to participate about 25 women per month can be entered in the study.

The proposed sample size is 250 women which will require a data collection of 13 months. A sample size of this magnitude will enable a difference in compliance proportion between the two iron preparation of 20 percent to be detected and a difference in Hb level of 0.35 g/dl to be identified. A difference of less than 0.5 g/dl is probably of little biologic importance (INACG 1984).

3. Treatment groups

Initially all women that agree to participate in the study will be treated for hookworm infestation with a single dose of Albendazole 400 mg.

The women will randomly be assigned to receive either conventional or GDS iron preparation. The dose suggested by WHO 1989 is 120 mg Fe per day when ferrous sulphate is used. The iron absorption from GDS iron supplement is three to four-fold as high as the conventional preparation and it is thus suggested that the dose should be lower. In this study it is proposed that it will be 60 mg.

The dose of conventional ferrous sulphate can be taken once a day or as two administrations of 60 mg each. To take the tablets twice a day is more difficult to remember and for comparative reasons because the GDS supplement is to be taken once a day also the conventional iron preparation will be scheduled for administration once a day. To reduce the side-effects both preparations will be recommended to be taken in the evening after the main meal.

This study does not include a formal placebo group. If a placebo group was to be included it would, due to ethical reasons, be necessary to exclude all subjects with a low Hb and provide them with iron supplement. Since the group with low Hb level is really the group of major interest for improving compliance this would be a serious draw-back for the study. It may also be that there is a differential compliance due to initial Hb and it is already known that the hematologic response is larger in a deficient population.

However, designing a study without a placebo group also create some problems. One of these is difficulties in evaluating the side-effects as there are a number of placebo side-effects. Furthermore, an assessment of the absolute effect of iron supplementation is not possible to achieve.

However if, which is likely, there will be a non-compliant group in the study it may to certain extent be possible to use this group as a quasi-experimental control group. In interpretation of the results considerations have of course to be made to the fact that the group is a self selected one and not randomly assigned. Still the use of this group as a control group may be acceptable, in particular if it is possible to statistically show that it, regarding essential variables, is comparable with the rest of the study population. Suggestions when non-compliers are to be used as control group is outlined in the analysis section.

4. Data collection frame

For each of the recruited subjects data will be collected at registration (week 0 of supplementation) and at week 2, 4, 8, and 12 of supplementation. The information that will be collected at these occasions are:

Registration:

- base-line information
- morbidity
- sociologic aspects of compliance
- hematology (Hb, FEP, sickle-cell, reticulocyte count),
- parasites; malaria

Week 2 of supplementation:

- morbidity, 2 weeks recall
- compliance by microprocessor

Week 4 of supplementation:

- morbidity, two weeks recall
- compliance by microprocessor and urine or stool sample
- sociologic follow-up

Week 8 of supplementation:

- morbidity, four weeks recall
- compliance by urine or stool sample

Week 12 of supplementation:

- morbidity, four weeks recall
- compliance by urine or stool sample
- hematologic data
- sociologic follow-up
- parasites; malaria

Provision of iron supplements will be made until end of pregnancy.

Methodology

a.Base-line information

The base-line information include household composition and characteristics such as major means of living, and socio-economic variables; reproductive history of the pregnant woman and obstetric including gestational age.

A previous study in the area showed that using last menstrual period gave a valid assessment of duration of pregnancy (Möller 1989). Even so fundus height will also be measured as a complementary method.

The base-line questionnaire will be developed and pretested in phase 1 and 2 of the study.

b.Hematology

Because of difficulties to handle venous blood sample under the existing field condition the hematologic measurements selected have been limited to those which can be done by use of capillary blood and do not need to be centrifuged or stored in a freezer. Capillary blood will be collected by finger prick and all samples will be taken by the same

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person. The measurement are: hemoglobin, free erythrocyte protoporphyrin (FEP), reticulocyte count and sickle cell test.

Hemoglobin level will be assessed by Hemocue system which use disposable microcuvettes. The technique has been compared with standard laboratory Hb methods and good reliability and accuracy has been found (Bridges et al 1987). The method particular convenient for field conditions as it does not require any sample or reagent preparation and can be run by use of a battery.

FEP will be assessed by a microflourometric method (Orfanos et al 1977). The method needs a small amount of blood 2-3 drops which can be spotted on a filter paper and stored protected from sunlight for analysis later in the laboratory.

Sickle cell,

Reticulocyte count

Parasites

Malaria will be assessed by preparing blood slides for microscopic examination.

d.Morbidity questionnaire

The aim with this section is to probe about symptoms and illnesses which are common during pregnancy and among these include the expected manifestations of side-effects of the iron supplement. A list of symptoms will be developed which include symptoms described by medical personnel as well as symptoms or conditions described by the women in the community. For each of the symptoms in the list there will be a complementary question on the cause of the it. The list of symptoms is to be developed through individual and group discussion in phase 1 and pretested in phase 2 of the study.

Apart from this list of symptoms there will be a number of more general question addressing the woman's assessment of her health status.

Compliance

In general it is difficult to assess compliance without affecting the event of interest which will bias the study results.

This study intend to use a new equipment for measuring compliance to medical treatment "Medication Event Monitor System" MEMO (Cramer et al 1989). It is a small microprocessor imbedded in a pill bottle cap. The microprocessor register each time and date the pill bottle is opened. Information can be collected for weeks and then be recovered by processing it by the computer software developed with the microprocessor. This equipment allow

detailed information about compliance to be collected without the knowledge from the pregnant women which decrease the risk of altering the compliance behavior due to the study intervention. The assumption is that every time the pill bottle is opened the tablet is actually taken.

The microprocessor method of measuring compliance will be used during the first month of supplementation where there is a need of accurate information on a daily basis. As the possibility exist that the woman will open the pill bottle regularly and through away the tablet this method will be validated by either testing for iron in stool samples (Afifi et al 1966) or by a riboflavin marker in the iron supplement which can be detected in urine samples.

For the follow-up of compliance assessment (week 4, 8 and 12 weeks) the methods using either stool or urine samples will be used.

f.Sociologic aspect on compliance

In the sociologic section the major area is the health beliefs which are relevant during pregnancy. Apart from the general descriptive information collected in phase 1 of the study the health belief model is going to be applied as a framework for gathering more detailed information of the factors that affect compliance behavior.

Health belief model:

The components in the health belief model which the questionnaire is going to address are;

1.The perceived susceptibility to anemia as conceptualized and explained for the women at registration. e.g Do you there is a risk that you will get "anemia"

2. Perceived severity of the illness; If you get "anemia" how dangerous is it?

3.Perceived efficacy of iron supplement in preventing the condition; e.g. Do you think taken the tablets will prevent you from getting "anemia"?

4. Barriers or expected difficulties or constraint in taking the supplement, for example side-effects. e.g. Do you think taking this tablets will give you any problems?

In the health belief model social support has a function of giving cues for action. Questions related to social support will be included to assess this aspect.

Another important factor for compliance is the interaction between the patient (client) and the health care personnel. Also this aspects will be addressed in the questionnaire.

g. Cost-effectiveness part

To calculate cost the following information will be collected:

- cost of the two different iron supplement per pregnancy.
- cost for health personnel
- cost for transport
- cost for facilities

The first cost, the iron supplement, will differ between the two types of formulation. The cost difference will be used for calculation of marginal cost of using the GDS supplement instead of the conventional preparation.

The cost for the next three ingredients in the supplementation programme will not differ between the iron supplements. For later calculations of total programme cost the assumption will be that a basic health delivery system already exists and that the iron supplement will be one of several drugs delivered to the center. The cost factors for the mentioned items will thus be calculated as shares of the total budget for the health centers cost for the mentioned ingredients. The share which will be attributed to the iron supplementation will be based on an assessment of how large proportion of the health center's time is used for information about, and distribution of iron supplement.

The effectiveness part of the calculation will have two variables. The first is a measure of the process of the supplementation programme, an output measure, which in this case will be number of compliers. The second variable is an outcome measure, per definition a biologic variable, in this case it will be hematologic outcome.

VI. Analysis

A. Hypothesis testing;

The intervention part of the study is the use of GDS iron preparation. A number of hypotheses regarding characteristics of the GDS supplement will be tested

Hypothesis 1. GDS iron preparation produce less side-effects compared to conventional iron preparation.

Definition of side-effects as dependent variable;

i) Number of different types of iron treatment related side-effects
ii) total reported number of episodes of a specific side-effect
iii) prevalence of a specific side-effect at a given time
iv) cumulative prevalence of a specific side-effect

Comparisons groups:

a) GDS iron preparation

b) Conventional iron preparation

Hypothesis 2. Use of GDS iron preparation will lead to better compliance to regimen.

Definition of compliance as dependent variable;

i) proportion of prescribed dose taken the first two weeks (by microprocessor)
ii) proportion of the prescribed dose taken the first four weeks (by microprocessor)
iii) positive or negative stool or urine sample at week 4, 8 and 12.

Comparisons groups:

a) GDS iron supplement
b) Conventional iron supplement

Hypothesis 3. Use of GDS iron preparation will maintain or improve hematologic status better than conventional iron supplement.

Definition of hematologic outcome as dependent variable:

i) mean value of HB at week 12 of supplementation
ii) prevalence of anemia (Hb<11 g/dl) at week 12 of supplementation
iii) mean change in Hb between registration and week 12 of supplementation
iv) mean value of FEP at week 12 of supplementation
v) prevalence of iron deficiency (FEP >80 ug) at week 12 of supplementation
vi) mean change in FEP between registration and week 12 of supplementation.

Comparisons groups:

a) GDS iron supplement
b) Conventional iron supplement
c) Non-compliers; less then 10 percent compliance during the first month and negative test week 4, 8 and 12.

The last comparison group is a self-selected group and precaution has to be taken in interpretation of the results. Before trying to use this group in analysis tests will be made if the group is comparable to the others regarding factors that may be correlated with hematologic outcome such as for example, parity, age, socio-economic status, education etc.

Hypothesis 4. Side-effects of iron supplementation have a negative effect on compliance.

Tests will be done for each of week 2, 4, 8 and 12's recall of side-effects and compliance plus the cumulative effect of the variables.

finition of compliance as dependent variable:

The same definitions as in hypothesis 2 will be used.

Definition of side-effects as independent variable:

i) Number of different types of iron treatment related side-effects
ii) prevalence of a specific side-effect
iv) cumulative prevalence of a specific side-effect

Hypothesis 5. A better compliance will result in better hematologic outcome.

Definition of hematologic outcome as dependent variables;

The same variables as in hypothesis number 3. will be used.

Definition of compliance as independent variable:

A compliance variable for the 12 weeks of supplementation will be constructed classifying compliance into three groups, non-compliers, low compliers and high compliers. The classification criteria for these groups will be developed after information on range and patterns of compliance have been collected.

Hypothesis 6. GDS iron supplement is more cost-effective in terms of compliance than conventional iron supplement.

Cost effectiveness in terms of process output, cost per complier.

a) marginal cost: the additional cost for using GDS supplement will be calculated by assessing the cost of supplement per complier and compare the two drugs.

b) total cost: the total cost of using the two different drugs will be calculated per complier. This calculation will enable an assessment of how the proportional extra cost for using the GDS supplement. (Assuming it is more expensive).

Hypothesis 7. GDS iron supplement is more cost-effective in terms of hematologic outcome.

a) marginal cost: additional cost per difference in average Hb (FEP) level between the two supplement or per anemia case averted.

b) total cost of using the drugs will be compared with average Hb (FEP) and per case anemia averted.

B. Analysis of the sociologic part.

The health belief model:
The components in the health belief model will be assessed on their respective importance for the variation observed in compliance. This will be done by pathway analysis.

The health beliefs contribution to compliance patterns will be investigated by assessing the overall explanatory capacity of the model.

The general questions will be compiled into a descriptive section.

VII. Human subjects considerations

The subjects in the proposed research project are pregnant women attending an antenatal clinic Ilula village, Iringa district in Tanzania.

The subjects will be recruited at the MCH center when they register for MCH service. At the first visit, before they are requested to take part in the study, they will be informed about the research project, its aims and what a participation would involve on their part. Since the population to large extent is illiterate written consent is not feasible and oral consent has to be taken. The subjects will be informed that if they choice to participate and later change their mind they can withdraw any time from the study without any penalties or consequences for their continuous participation and use of the clinic's service.

If the ongoing research for some reasons has to terminate and debriefing can't be done within the project's time frame the village leaders will be asked to inform the subjects.

The intervention part of the study is the test of two iron supplement which are randomly assigned to the women. Both preparations have the same active compound, ferrous sulphate. The dose of the conventional formulation is the one recommended by WHO. The GDS iron preparation has a lower amount of elemental iron but its absorption is much higher making dose comparable with the conventional iron preparation. Thus, the efficacy of the treatments offered are as good or better as the one recommended by WHO (DeMaeyer 1989).

The research involve taking two finger prick blood samples. Finger prick sample is routinely taken in the clinic at registration but an additional sample is needed after 12 weeks of iron supplementation. Finger prick blood samples as such does not involve any risks for the patient. However, HIV virus is prevalent in Tanzania and strict precautions has to be taken to avoid transmission of the virus. The steps that will be taken are the following; only one person will take and handle all the blood samples in the study. The

existing lab technician at the clinic will be requested to take the study's samples. This person will, even if previously trained, receive further training in safety measures regarding handling blood samples possibly contaminated with HIV virus. If the person in the clinic does not agree to take the blood samples for the study someone will be hired and trained specifically for this purpose. Training will be done by a medical doctor at the laboratory department of TFNC who has previously trained persons in blood sampling techniques.

Protective gears for the person taking the blood samples will be made available as well as equipment for handling contaminated supplies and for its destruction. Finger prick will be done by use of disposable supplies.

The data collection and interviews will be carried out by field workers living in the area who are familiar with local customs and fluent in kiswahili.

The data collected will be confidential. The subjects privacy will be ensured by limiting the number of persons who have access to the raw data. In analysis and presentation of the results all subjects will be codified and no names will be revealed.

Due to ethical reason the study does not include a placebo group which does not receive any elemental iron.

The research proposal will be submitted for ethical considerations to the Committee for Research quality and human subject considerations.

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APPENDIX 6

THE IMPACT OF COFFEE INTAKE ON IRON STATUS AND BEHAVIOR OF INFANTS IN GUATEMALA

Kathryn G. Dewey, PhD
Department of Nutrition
University of California, Davis

Noel Solomons, M.D.
Jesus Bulux, M.D.
Julieta Quan, M.S.
CeSSIAM, Guatemala

Patrice Engle, PhD
Psychology and Human Development Department
California Polytechnic State University, San Luis Obispo

Background and Rationale

Coffee is a widely consumed beverage throughout the world. Caffeine, a natural constituent of coffee, has been the subject of numerous studies due to its pharmacological and possibly teratogenic effects (1-3). However, caffeine is not the only metabolically active substance in coffee; there are other methylxanthines, aromatic volatile and nonvolatile acids, piperidines, chlorogenic acid, tannins, trigonelline and ketones which may have physiological effects as well (4). Several indices of nutritional importance have been shown to be affected by coffee and/or caffeine intake, including low birth weight (5), calcium metabolism (6), thiamin status (7-9) and zinc and iron absorption (10-11). In our own research, we found that mothers in Costa Rica who drank 3 or more cups of coffee per day during pregnancy and lactation were more likely to be anemic and had infants with lower hemoglobin and hematocrit levels (at one month of age) than mothers who did not drink coffee, even though both groups were very similar in socioeconomic status, maternal anthropometric indices, use of prenatal iron supplements, and dietary intake (12). Similarly, in studies with rats we found alterations in trace-mineral metabolism in offspring of dams who consumed coffee during pregnancy and lactation (13). Hemoglobin and hematocrit values of pups of coffee-exposed rats were significantly lower whereas liver iron, zinc and copper concentrations were significantly <u>higher</u> than in pups of control rats. Because the rats were fed a diet with an adequate level of iron, these findings suggested that coffee may interfere with the mobilization of iron from the liver to sites of hematopoesis. Furthermore, we determined that the causative factor was not caffeine, but some other compound(s) (as yet unidentified) in coffee.

In many parts of Latin America, coffee is a common beverage not only among adults but among children as well. In Guatemala, for example, coffee is given as early as 2 months of age, either with other foods (e.g., bread soaked in coffee) or by bottle or cup. As described above, our previous studies examined the effects of <u>maternal</u> coffee intake on the offspring, but not the effects of coffee consumed <u>directly</u> by the infant. We are not aware of any published reports on the nutritional effects of coffee in children, but an analysis of data collected in Nicaragua indicates that the frequency of coffee consumption among preschoolers is inversely associated with weight-for-age and

height-for-age, even when controlling for socioeconomic status, maternal education, child age and frequency of consumption of animal foods or milk (LaMontagne, Zeitlin and Engle, personal communication). No data are available regarding iron status. The potential effect of coffee on iron status is of considerable importance, given the high prevalence of iron deficiency anemia among young children worldwide and its association with functional outcomes such as immunological competence and behavioral development.

Preliminary Studies

To determine the extent of coffee intake and the doses consumed by infants in Guatemala, we recently completed an informal infant feeding survey of 100 mother-infant pairs recruited through out-patient clinics at 3 hospitals serving low-income families in Guatemala City. Mothers of infants ranging in age from 5 to 36 mo were interviewed regarding the types and amounts of foods and fluids given and the age of introduction of each. If coffee was given, the dose was calculated based on dry weight and adjusted for the weight of the child. In our previous study in Costa Rica, the selection criterion for the coffee-consuming group was 3 cups per day, which is equivalent to about 0.18 g/kg based on dry weight. Of the 100 Guatemalan infants, 28 were not given any coffee, 41 were given doses less than 0.18 g/kg, and 31 were given doses greater than or equal to 0.18 g/kg. The age at which coffee was introduced ranged from 2 to 24 mo: 78% of those given coffee began to consume it by 10 mo of age. These findings suggest that coffee consumption is widespread among infants in this population and thus it would not be difficult to find sufficient subjects for the proposed study.

We have begun a second study to determine whether there is any observable relationship between coffee intake and indices of iron status in Guatemalan infants. In this study, we plan to survey approximately 200 mothers of infants between the ages of 6 and 24 months, using the same questionnaire as in the first study. Blood samples are collected by venipuncture from the infants for assessment of hematocrit, zinc protoporphyrin/heme (ZPP/H) ratio (14) and plama ferritin. Additional information on infant feeding practices, recent infections, family socioeconomic and demographic variables, and the mother's reproductive history and coffee consumption are collected in order to interpret the results. At the present time we have completed collection of blood samples from 43 subjects. Preliminary analyses suggest an association between coffee intake and ZPP/H levels indicative of iron deficiency.

In this survey, we expect that there will be significant confounding variables that may exaggerate the relationship between coffee intake and iron status. For example, mothers with less education are more likely to give coffee to their infants, and those infants may also be at higher risk of anemia for other reasons. We will adjust for these confounding variables in our statistical analysis, but we recognize that a purely observational

study of this nature is not the ideal way to determine if there is a causal association between coffee and iron status. Therefore, the next phase of the research, described below, will be designed as a randomized intervention trial. The data collected from the preliminary observational study will be used to determine the selection criteria for the intervention trial.

Specific Aims

The purpose of the proposed research is to determine whether discontinuation of coffee intake among infants 12-18 months of age who have received coffee for at least two months prior to the intervention will have an impact on trace element status, prevalence of anemia, growth rate or behavioral indices.

Hypotheses

1) Infants 12-18 mo of age whose mothers discontinue giving coffee for 4 months will have better iron status (as reflected by hemoglobin, hematocrit, ZPP/H, plasma ferritin and transferrin receptor) and better zinc and copper status (as reflected by plasma Zn, Cu and ceruloplasmin activity and by erythrocyte copper-zinc superoxide dismutase [SOD] and metallothionein) than infants who continue to receive coffee.

2) Infants whose mothers discontinue giving coffee for 4 months will have more rapid growth in weight and length than infants who continue to receive coffee.

3) Infants whose mothers discontinue giving coffee for 4 months will be less irritable, will have a more regularized sleep pattern, and will show a more rapid behavioral development than infants who continue to receive coffee.

Procedures

The study will be designed as a randomized intervention trial with infants 12-18 mo of age who have been receiving coffee (without milk added) for at least 2 months. Sujects will be recruited from one or more of the peripheral outpatient "well-baby" clinics associated with the Pediatric Hospital of the Guatemalan Institute of Social Security. These clinics are located in several zones of Guatemala City and serve primarily low-income families. Infants with chronic medical conditions will be excluded. Subjects will be randomly assigned to one of two groups:

1) Control group - mothers will continue feeding their infants the usual foods and fluids, including coffee, with no instructions to alter their practices.

2) Intervention group - mothers will be asked to discontinue giving coffee for 4 months, and will be provided with a substitute (an "instant" coffee-colored, fruit-flavored powder containing the same amount of sugar as their usual coffee preparation, to be mixed

with hot water) to give instead.

Results of the ongoing observational study will be used to determine the minimum dose of coffee for selection into the intervention study, and whether selection criteria regarding maternal coffee consumption during pregnancy or lactation should be imposed. With regard to the latter issue, 93% of mothers in the first pilot study consumed coffee (even during pregnancy), although the dose varied. The majority (63%) of infants 12-18 months of age were still being breastfed. Thus, it is likely that most infants in this age group would still be getting some of the compounds in coffee through breast milk even if they were not given any coffee directly. We will examine data from the observational study to determine if maternal coffee intake is associated with infant iron status among breastfed infants not given coffee directly. If so, we may need to restrict the intervention study to non-breastfed infants, or to those who nurse less than 3 times per day. Results from the ongoing observational study will also be used to determine whether stratification by infant level of coffee intake or baseline hematocrit would be desirable.

The intervention depends entirely on the willingness of mothers to make this change in feeding practices. Initial discussions with 20 mothers indicated that if it was felt that this might be beneficial to their infants, they would be very willing to discontinue coffee. We have included a question in the ongoing observational study regarding the reasons for giving coffee. In almost all cases the reasons are because it is a custom or because it is a beverage consumed by other members of the family and is available. In nearly all cases (97%), coffee is given without milk. Thus, the change requested is not a major one and in fact, the mothers who do not give coffee to their infants often use a beverage ("fresco") similar to the substitute we will provide, except that it is consumed cold rather than hot. The substitute chosen is intended to minimize disruptions in the child's dietary pattern; other substitute fluids (e.g. milk, juice) have nutrients that might cause a change in the outcome variables independently of the discontinuation of coffee. Because carbohydrate (e.g. sugar) may facilitate iron absorption, the same amount of sugar will be used in the substitute as in the coffee preparation.

Random assignment will be done as follows: the first time an eligible subject is encountered, a card will be drawn from an envelope containing an equal number of cards for control and intervention groups. A card will be drawn for each subsequent subject from the same envelope, without replacement. Control subjects will not be informed of their "assignment" so as not to bias their subsequent feeding practices. Intervention subjects will be asked if they are willing to discontinue coffee. Background information will be requested from all eligible subjects. If they are not willing to participate (either intervention or control subjects), the card drawn will be returned to the envelope.

Four months is considered to be an adequate time period for the potential effects of coffee to disappear, based on studies of iron supplementation among children of the same age. In Costa Rica, 3 months of iron therapy among children 12-23 mo of age resulted in normal hematological indices among those who had previously been anemic or iron deficient (14).

The necessary sample size is estimated as 30 per group, based on an expected difference of 2 in hematocrit (%) and a standard deviation of 3, using a one-sided test with a=0.05 and b=0.20. In the Costa Rican study (14), the standard deviation in hematocrit was 2-3 and the improvement after 3 months of iron therapy ranged from 7 in anemic subjects to 1 in non-anemic, iron-sufficient subjects. The other outcome variables for iron status (hemoglobin, ferritin, ZPP/H, transferrin receptor) have a higher ratio of expected change to standard deviation, so the sample size for detecting differences in hematocrit should be more than sufficient for those variables. No data are available for this age group to estimate variation in the other indices reflecting trace element status. The sample size of 30 per group will be sufficient for detecting a difference of 25% between groups in weight and length gain, assuming a standard deviation of about 100 g/mo in weight gain and 0.35 cm/mo in length gain. (add sample size calc. for behavioral indices). To allow for attrition we plan to recruit 40 subjects per group.

Baseline measurements will be made immediately after recruitment, unless the child has a current infection, in which case they will be rescheduled after recovery. Measurements will include collection of a venous blood sample, measurement of weight and length, behavioral assessment (a modified temperament questionnaire, an interview regarding sleep patterns, and the Bayley test of motor development), dietary history (timing of introduction of all foods and fluids, breastfeeding patterns, food frequency questionnaire for current intake), morbidity questionnaire (history since birth, plus illness currently and in the previous week), and collection of other socioeconomic and demographic data, including maternal reproductive history and consumption of coffee during pregnancy and lactation. The anthropometric and dietary measures will be repeated at a home visit two months after recruitment, and the entire battery of tests will be repeated at a visit conducted at the end of the study (four months after recruitment). In addition, subjects will be visited in the home every two weeks to collect morbidity data and to investigate any reported difficulties in compliance in the intervention group. Once a month, unannounced spot visits to the homes of intervention subjects will be made to collect infant urine samples, which will be analyzed for caffeine to monitor compliance.

Analysis of variance will be used to determine the effect of the discontinuation of coffee on trace element status, prevalence of anemia, growth rates and behavioral indices. Other factors such as infant sex, initial weight, birth order, duration of breastfeeding, frequency of consumption of iron-rich foods, dietary

scores reflecting estimated bioavailability of trace elements (including vitamin C sources, phytate, dietary fiber, etc.), maternal coffee consumption during pregnancy and lactation and socioeconomic status will be included in the statistical analyses as potential confounding variables. Analyses will also be conducted separately for infants found to be anemic (based on hematocrit), iron-deficient (based on ferritin, transferrin receptor and ZPP/H), or iron-replete at baseline.

Significance

The results of this study will indicate whether coffee intake among young children in Latin America has adverse nutritional consequences. If coffee consumption is a major etiological factor for iron-deficiency anemia, it represents a readily altered behavior that could make a potentially large difference in this widespread problem at minimal cost. In the study among pregnant women in Costa Rica, all of whom took prenatal iron supplements, 23% of coffee-consuming mothers were anemic (hemoglobin < 11 g/dl) whereas none of the non-coffee-consuming mothers were. We don't know if the same magnitude of difference would exist among infants, but given the likelihood that the half-life of many of the constituents of coffee would be longer in infants than in adults, it is possible that the effect could be even greater.

Although the hypothesized effects of coffee on other trace elements such as zinc are more speculative, they also represent potentially important public health concerns. Moderate zinc deficiency among preschool children is now thought to be relatively common and is likely an important cause of growth faltering during the critical period from 6 to 24 months. If coffee consumption interferes with zinc metabolism, as suggested by our study with rats, elimination of coffee from the diet of young children might improve zinc status and consequently have a salutary effect on child appetite and rate of growth.

Time Schedule

Jan-Feb, 1992:	Recruitment of personnel Training and standardization Logistical arrangements
Mar, 1992,- Feb, 1993:	Subject recruitment (2-3/wk) Data collection and lab analysis
Mar-Dec, 1993:	Follow-up of remaining subjects to 4 mo Data analysis and write-up

Collaborative Arrangements

Laboratory analysis of blood samples will be conducted in collaboration with Dr. Robert Labbe, University of Washington (ZPP/H), Dr. James Cook, Kansas University Medical Center (transferrin receptor) and Drs. Bo Lonnerdal and Carl Keen, University of California, Davis (plasma ferritin and indices of trace element status).