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ICCNA PROGRESS REPORT AND WORK PLAN

IRON DEFICIENCY SUPPORT PROGRAM

AID COOPERATIVE AGREEMENT DAN-5115-A-00-7908-00
7098

1989 - 1990

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ICCNA PROGRESS REPORT

IRON DEFICIENCY SUPPORT PROGRAM

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

September 1989 - August 1990

The following summarizes activities from September 1, 1989 to August 31, 1990 performed under the AID Cooperative Agreement. The overall objective of this program is to support field activities designed to assess and combat iron deficiency in developing countries and to conduct a variety of intramural activities at the International Center for Control of Nutritional Anemia (ICCNA) for the purpose of developing newer techniques to assess iron status and newer methods of intervention.

I. PUBLICATIONS

1. **Flowers CH, Skikne BS, Covell AM, Cook JD. The clinical measurement of serum transferrin receptor. J Lab Clin Med 1989;114:368-377.**

This report culminates several years of effort to develop a more sensitive method for detecting mild iron deficiency. The report describes the assay of serum transferrin receptor using monoclonal antibodies developed in our laboratory against transferrin receptor purified from human placenta. The

assay technique is virtually identical to that used for serum ferritin, a sensitive enzyme-linked immunoassay. The average level of circulating receptor in 82 normal subjects was 5.63 mg/L. In patients with complete marrow ablation, the level fell to 2.6 mg/L, indicating that non-erythroid tissues contribute significantly to the circulating protein. The most significant finding was a sharp elevation to an average of 18 mg/L in patients with iron deficiency anemia. The assay for serum transferrin receptor has a between assay variability of <3% when examined over a 12 month period and is completely free of matrix effects of whole serum that has plagued prior assays of this type. We also observed that the serum receptor level is sensitive to expansion of erythroid marrow and is therefore sharply elevated in patients with hemolytic anemia. In the present report, patients with sickle cell anemia had a mean increased to 33 mg/L. It is not believed that hemolysis will interfere significantly with the use of serum transferrin receptor for prevalence studies because inherited abnormalities in erythropoiesis are readily identified by other techniques. An accompanying editorial to this report attests to the value of this new measurement and indicates that it will play a key future role in the assessment of iron status and in the evaluation of the anemic patient.

2. **Cook JD, Skikne BS. Iron deficiency: Definition and diagnosis. J Int Med 1989;226:349-355.**

This review article summarizes the present state of the art with respect to laboratory measurements of iron deficiency. The article points out that the optimal techniques differ for clinical and epidemiological purposes. The major task in a clinical setting is to distinguish iron deficiency from other disorders which frequently masquerade as iron deficiency such as the anemia of chronic disease. On the other hand, the optimal techniques epidemiologically are those that provide quantitative estimates of body iron. In both situations, the serum ferritin is the key laboratory measurement because it provides a quantitative measure of body iron stores in epidemiologic surveys and reliably identifies causes of anemia that are not due to iron deficiency. The evolution of serum transferrin receptor measurements is described in this report and predictions are made about their potential utility in epidemiologic studies of prevalence.

3. **Cook JD. Adaptation in iron metabolism. Am J Clin Nutr 1990;51:301-308.**

This extended review of iron balance and the factors influencing it was presented as part of a FASEB symposium on Adaptation and Nutrition.

Variables in iron nutriture are described in relationship to the adaptation that can be achieved in response to differences in dietary intake and iron bioavailability. Successful adaptation in iron metabolism is defined as a state in which the iron needs of all body tissues are satisfied. From a laboratory perspective, this is defined as a serum ferritin level >12 μg/L since lower levels indicate storage depletion and the onset of a deficiency in functional iron. The determinants of iron balance are described under the headings of Physiological Iron Demands, Dietary Iron Supply, and Adaptation. With respect to Dietary Iron Supply, the key variables are total iron intake, heme iron content, and nonheme iron bioavailability. Radioiron absorption studies in human subjects have demonstrated a 15-fold difference in bioavailability at the extremes of meals containing inhibitors or enhancers of absorption. Data from human absorption studies is presented to indicate the relative contribution of heme and nonheme dietary iron in individuals with normal, reduced, or deficient iron reserves. This monograph provides an extended review of the extent to which individuals adapt to variations in iron supply and reviews current information on the mechanisms by which this adaptation is achieved.

4. **Skikne BS, Flowers CH, Cook JD. Serum transferrin receptor: A quantitative measure of tissue iron deficiency. Blood 1990;75:1870-1876.**

This report describes the first detailed evaluation of serum transferrin receptor for the assessment of iron status. Normal volunteer subjects were serially phlebotomized for periods ranging up to 6 months to achieve a wide range of iron status. At each phlebotomy, a battery of iron measurements were performed including serum iron, total iron binding capacity, mean cell volume, free erythrocyte protoporphyrin, red cell mean index, serum ferritin, and importantly, serum transferrin receptor levels. The purpose of this study was to define the point at which serum receptor levels became abnormal and to determine their relative sensitivity in the detection of iron deficiency. The expected sharp decline in serum ferritin levels during the period of storage iron depletion was observed. Serum receptor levels remained essentially normal throughout the period of storage depletion, but a progressive rise occurred subsequently that continued with an advancing deficit in functional iron. The serum receptor level proved to be a more sensitive and reliable guide to the degree of functional iron deficiency than either the free erythrocyte protoporphyrin or mean cell volume which have been used extensively in prevalence studies. The serum receptor was of

particular value in identifying the development of mild iron deficiency before the occurrence of frank anemia. This study provides the framework for the future assessment of iron status in population studies. It appears that the quantitative assessment of body iron can be made with only three parameters: serum ferritin as an index of iron stores, serum receptor as a quantitative measure of tissue iron deficiency, and the hemoglobin concentration as a measure of more advanced iron deficiency. By calculating the ratio of serum ferritin:serum receptor with varying degrees of iron depletion, a single parameter covering the entire range of iron status can be obtained.

5. **Cook JD, Carriaga M, Kahn SG, Schalch W, Skikne BS. Gastric delivery system for iron supplementation. Lancet 1990;335:1136-1139.**

This publication describes the most important advance in iron supplementation for developing countries in the past several decades. The results of more than 10 years of studies with a new gastric delivery system for iron supplementation are described. The report includes radioisotopic studies performed several years ago in normal volunteers demonstrating that absorption from a gastric delivery system containing ferrous sulfate provides three times the amount of absorbed iron as conventional ferrous sulfate. More importantly, a double-blind placebo-controlled trial in 200

normal women showed that gastrointestinal side-effects associated with the administration of 50 mg of iron daily were completely eliminated when ferrous sulfate was given as a gastric delivery system rather than as a conventional tablet. Our studies therefore indicate that a single GDS tablet daily provides the same amount of absorbed iron as conventional ferrous sulfate given three times daily and importantly, is not associated with gastrointestinal side-effects. Problems in compliance continue to be cited as the major obstacle to the development of effective supplementation programs for pregnant women. To the extent that these are explained by gastrointestinal side effects, this new iron preparation should improve compliance. This article has received wide spread attention both in the US and overseas, and has prompted further assessment by Hoffman-La Roche of the potential value of this pharmaceutical preparation.

6. **Klemow D, Einsphar D, Brown TA, Flowers CH, Skikne BS. Serum transferrin receptor measurements in hematologic malignancies. Am J Hematol 1990;34:193-198.**

In using serum transferrin receptor measurements to assess iron status in population surveys, it is important to identify other conditions that might be associated with an elevated level. In this report, a wide range of hematologic malignancies were assessed to determine those associated

with significant elevations in serum receptor levels. Surprisingly, the majority of hematologic malignancies were not associated with an elevation. Consistent elevations occurred only in chronic lymphocytic leukemia. Because of the limited number of hematologic disorders that are associated with an elevation of serum transferrin receptor, this measurement can be equated with iron status in an otherwise normal population.

7. **Cook JD, Dassenko S, Skikne BS. Serum transferrin receptor as an index of iron absorption. Br J Haematol 1990;75:603-609.**

This report describes the potential value of serum receptor levels in predicting iron absorption. The relationship between serum receptor and iron absorption is of key interest because the signal provided by tissue to the intestinal tract for iron absorption has been elusive. In this report, the absorption of either inorganic iron or dietary nonheme iron was measured in 234 normal subjects. The results were correlated with serum receptor and serum ferritin levels. As in prior studies, there was a close correlation between serum ferritin and iron absorption. A much lower correlation was seen between serum receptor and iron absorption, and this disappeared completely when subjects with mild iron deficiency were excluded. This study provides additional evidence that the serum receptor is a sensitive index of mild iron deficiency. It also reaffirms the importance of iron stores

as measured by serum ferritin in regulating the amount of iron absorbed from the gastrointestinal tract.

8. **Baynes RD, Bothwell TH. Iron deficiency. Annu Rev Nutr 1990;10:133-148.**

This report was co-authored by Dr. Roy Baynes who joined the Division of Hematology at Kansas University Medical Center in 1989 and plays a key role in our intramural program of iron metabolism. This review is a comprehensive update of methods to assess iron status, factors affecting food iron absorption, current estimates of the prevalence of iron deficiency, biological consequences of iron lack, and current techniques to prevent nutritional iron deficiency. The review is particularly comprehensive in regard to factors affecting the absorption of dietary iron and includes an extended review of the efficacy of fortifying curry powder with sodium iron EDTA in a population of Indian women living in South Africa. This report provides an excellent summary on a broad range of topics relating to nutritional iron deficiency and provides a comprehensive rationale for developing methods to combat it.

II. PRESENTATIONS

The following presentations were made to increase awareness of iron deficiency as an important nutritional problem and to outline recent advances at ICCNA in developing newer intervention strategies and evaluating iron status in prevalence surveys.

1. Drs. James Cook, Manju Reddy, Judy Shih, Roy Baynes, and Richard Hurrell attended the FASEB Meetings held in Washington DC, April 1-5, 1990. A variety of papers were presented relating to recent studies performed at ICCNA. The following presentations were made:

   A. Reddy MB, Cook JD. Assessment of a rat model for estimating nonheme iron availability.

   This paper describes the use and limitations of rat measurements in predicting human iron bioavailability.

B. Shih YJ, Baynes RD, Hudson BG, Flowers CH, Skikne BS, Cook JD. Characterization of human serum transferrin receptor.

This paper describes biochemical studies identifying the nature of the circulating immunoreactive material measured in assays for serum receptor.

C. Hurrell RF, Juillerat M, Reddy M, Dassenko S, Lynch SR, Cook JD. Iron absorption from soy protein isolate in man: Effect of phytate removal and protein hydrolysis.

This paper describes the interaction between soy protein and phytate and their inhibiting role in food iron absorption.

D. Baynes RD, Shih J, Skikne BS, Flowers CH, Cook JD. An in vitro model of the circulating transferrin receptor.

This describes specialized studies of transferrin receptor fragment generated in a leukemic cell line.

2. Drs. James Cook and Barry Skikne attended the American Society of Clinical Nutrition Meetings in Washington DC, May 25, 1990. The following papers were presented:

   A. Cook JD, Carriaga M, Kahn SG, Schalch W, Skikne BS. Gastric delivery system for iron supplementation.

   This describes the gastric delivery system for iron supplementation.

   B. Skikne BS, Flowers CH, Cook JD. Serum transferrin receptor: A quantitative measure of tissue iron deficiency.

   This describes the use of serum transferrin measurements in the assessment of iron status.

3. Dr. James Cook attended a meeting sponsored by the International Atomic Energy Agency (IAEA) in Galveston, Texas, on April 23-25, 1990. This IAEA consultants meeting was held to provide advice on the establishment of a global coordinated research program on isotope-aided studies of bioavailability of iron and zinc from human diets. It is planned to involve 12-15 participating institutes, mainly from developing countries. IAEA will provide modest funding of approximately $20,000 (US) in each developing

country program spread over 4-5 years. A major focus of the proposed coordinated research program (CRP) as evolved from discussions at this meeting will be on iron nutrition. High priority will be given to studies in developing countries related to the planning and/or support of appropriate intervention programs for iron deficiency. These programs will include: 1. food fortification including type and level of fortification required and monitoring of effectiveness, 2. dietary modification including selection of appropriate local food-stuffs containing bioavailable iron, and reduction in substances inhibiting absorption of iron. Although the funding for this program is relatively modest, it is believed that it will greatly stimulate interest in the field of iron nutrition, particularly in relation to those aspects that can be addressed by the use of stable or radioactive isotopes. Regular meetings will be held in the future to evaluate submitted protocols and to advance the cause of this important new research program.

4. Dr. James Cook attended a meeting held in Dublin, Ireland, June 6-8, 1990. The purpose of this meeting was to develop a report on iron deficiency control for the Administrative Committee on Coordination, Subcommittee on Nutrition (ACC/SCN) of the United Nations. This meeting was jointly sponsored by World Bank, UNICEF, and WHO. The prime focus of this conference was to examine the reasons why iron supplementation has diminished effectiveness in developing countries as a means of combatting

iron deficiency during pregnancy. A variety of developing countries were surveyed prior to this workshop to identify the major causes of failed supplementation programs. Extensive discussions were held during this workshop to develop methods to circumvent problems in iron supplementation programs. Various aspects of ICCNA activities, such as serum receptor measurements and particularly the gastric delivery system for iron, were presented at this meeting. An important by-product of this meeting was a commitment of several major donor agencies to petition Hoffman-La Roche to expand the availability of GDS for distribution to developing countries.

5. Drs. Manju Reddy and James Cook attended a workshop at Nestlé in Vevey, Switzerland, August 23-24, 1990. The purpose of this was to review ongoing collaborative studies of food iron bioavailability and to prepare detailed plans for collaborative studies in this area over the next 12 months.

III. FIELD PROGRAMS

1. GDS Evaluation, Kingston, Jamaica

This activity involves a trial of the gastric delivery system (GDS) for iron supplementation in pregnant women. The objective is 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.

A detailed review of this project was made by Dr. Cook during a visit to Kingston, Jamaica, January 9-12, 1990. A summary of that review is included in Appendix B. At that time, 357 women had entered the trial and approximately 200 had completed three months of iron administration. The study, including most biochemical determinations, was completed in June 1990. The final group included almost 300 women as originally planned. During June, July, and August, Marjorie Thomas performed measurements of serum transferrin receptor at ICCNA in Kansas City. These measurements were completed in mid-August.

In general, the study demonstrated a highly significant hematological response with both conventional iron and GDS as compared to a placebo. No major differences were seen between the response obtained with two tablets of ferrous sulfate daily as compared to a single GDS capsule. Because of the intensive interim evaluation during the study, a reliable assessment of compliance was difficult to obtain. Preliminary evaluation of

the data indicates that an adequate hematological response can be obtained with a single GDS tablet. By reducing administration to a single tablet a day and eliminating gastrointestinal side-effects, it is anticipated that GDS will improve compliance significantly in iron supplementation programs.

This field study is presently undergoing detailed statistical analysis. This is being performed jointly by Keith Bingham at the University of the West Indies, Kingston, Jamaica, and personnel at ICCNA. One current problem is a significant difference in the hematologic and iron status data among the three study groups at their initial visit, despite careful attention to a randomized selection procedure. A detailed discussion of the limitations of this study as assessed during its conduct is outlined in Appendix B. Quality control data indicate that the biochemistry and hematology were done with a high degree of precision, accuracy, and reproducibility. Although extensive and detailed analysis will be required before this work is ready for publication, it is believed that it will be of major value in documenting the potential benefits of GDS.

2. Grenada Fortification Project

A second major activity during the past year was the development of a project to assess the efficacy of fortifying wheat flour in Grenada, West Indies. A site visit was conducted by Drs. James Cook and William Simmons, CFNI, May 13-18, 1990, for the purpose of developing a suitable protocol. A detailed description of that site visit is included in Appendix B. This study will be administered by PAHO and supported by AID funds. It is an outgrowth of a prevalence survey conducted in nearly 1000 subjects living in Grenada in 1986 which showed a high prevalence of iron deficiency. At that time it was found that approximately 60% of the wheat flour used in the country was not fortified with iron. Because of the extensive use of this so-called counter flour by the lower socioeconomic segments of the population, it was believed that its fortification would reduce the prevalence of nutritional anemia significantly.

The protocol is in the final draft stages at the time of this report. In general, it will involve, during the first 6 months, an island-wide survey of wheat intake to be performed on a household basis. The purpose is to estimate the per capita intake of flour and the proportion of unfortified flour used within each household. Following this survey, it is planned to conduct a

baseline household survey at the start of the fortification project and again 18 and 36 months later. A total of 600 households at each sampling time is planned. Capillary blood samples will be obtained at these visits for measurements of hemoglobin, serum ferritin, and serum transferrin receptor. It is planned that approximately 2000 subjects will be sampled on each occasion. Funding for this project has not yet been identified although it is anticipated that funds will become available within the next few months. The study provides an ideal opportunity to assess the impact of iron fortification of wheat flour because, although the strategy has been used for several decades in industrialized countries, its nutritional benefit has never been documented. The study will be simplified because of the ability to fully control the point of fortification within the country. However, there are several details and obstacles before this study can be implemented.

IV. INTRAMURAL PROGRAM

1. Assessment of Iron Status

Emphasis continues to be placed on better defining the role of transferrin receptor measurements in the assessment of iron status. One of the key studies that has just been completed is the evaluation of serum receptor in third trimester pregnancy. A total of 176 women attending a hospital outpatient maternity clinic at Kansas University Medical Center had blood

samples drawn for several iron-related measurements including serum iron, total iron binding capacity, red cell mean index, mean corpuscular volume, erythrocyte protoporphyrin, and serum receptor. Although the utility of serum receptor for detecting iron deficiency has been clearly documented in nonpregnant subjects, its use in pregnancy was clouded by a recent report from Japan indicating that a progressive rise in serum receptor levels occurs in pregnancy. These workers attributed the rise to a placental origin of the serum receptor rather than the development of progressive iron deficiency.

In our present study in third trimester pregnancy, the frequency distribution of serum transferrin receptor levels was virtually identical to the frequency distribution in 175 nonpregnant control subjects. More importantly, elevation in serum receptor above the upper cut-off level of 8.5 μg/L invariably occurred in women with a serum ferritin level <12 μg/L. This indicates that an elevated receptor level occurs only in women with depleted iron stores indicating that the measurement can be used to assess iron status during pregnancy. The majority of our pregnant women were taking iron supplements regularly and the prevalence of iron deficiency was therefore low. However, in 13 subjects that were identified as having iron deficiency anemia based on several laboratory measurements, the receptor level alone was able to identify iron deficiency in 11 out of 13. This study represents an

important intermediate step in the application of serum transferrin receptor levels for the assessment of hematological response in the Jamaican GDS study. This work is now being prepared for publication and hopefully will be submitted within the next few months.

Another important aspect of serum transferrin receptor measurements is to determine whether they are capable of distinguishing iron deficiency anemia from the anemia of chronic disease. This distinction is one of the most vexing problems in the assessment of epidemiological data because most of the measurements used to assess iron nutriture are effected similarly by these two disorders (iron deficiency and chronic infection). We undertook an extensive clinical trial to examine serum receptor levels in patients with varying degrees of acute and chronic inflammation. Patients with acute and chronic liver disease were included because these disorders are known to affect the serum ferritin levels and diminish the value of serum ferritin for detecting iron deficiency. More than 200 clinical patients were examined in this trial. In patients with acute infection such as pneumonia or bacterial sepsis, there was no elevation in serum receptor. More importantly, in a large group of patients with the anemia of chronic disease, receptor elevations were detected in less than 10%, whereas in a parallel group of patients with iron deficiency anemia, more than 90% of the values were elevated. Similarly, acute or chronic liver disease had no influence on serum

receptor levels. These results indicate that receptor levels will be of value in distinguishing the anemia of chronic disease from true iron deficiency and will therefore be valuable in developing countries where infection is often highly prevalent.

2. Iron Fortification Strategies

Most of this effort during the past year has been directed to collaborative studies with Richard Hurrell at Nestlé, Vevey, Switzerland. One of the most important areas of completed work was the **evaluation of ferrous fumarate** as an iron fortificant for a chocolate milk powder targeted to children and adolescents. In human studies performed at ICCNA, iron absorption from ferrous fumarate (3.3%) was similar to that for ferrous sulphate (2.8%) and ferric pyrophosphate (2.1%) when these labelled iron compounds were added to the drink immediately prior to consumption. On the other hand, when the iron compounds were processed during the manufacture of the chocolate drink, the absorption of ferrous fumarate was 5.3% and remained significantly higher than either ferrous sulphate (2.6%) or ferric pyrophosphate (0.6%). Interestingly, ascorbic acid had little or no effect on the absorption of ferrous fumarate. Organoleptically, ferrous fumarate was acceptable when the chocolate drink powder was

reconstituted in milk or water that was heated to <80°C. Unfortunately, unacceptable color changes occurred when boiling milk or water was used. The study demonstrated that ferrous fumarate is a useful compound for fortification of chocolate milk powders unless constituted with boiling milk or water and has a particular advantage in products undergoing extensive heat processing following the addition of the fortification iron compound. This work is completed and is being prepared for publication.

There are several areas of collaboration with Nestlé in which work is in progress. One of the most important is the production of **phytate-free cereals**. It has been possible to show, in studies during the past year, that if phytate is completely removed from a cereal by enzymatic hydrolysis, absorption increases dramatically. However, the extraction of phytate must be virtually complete. Most of this work has been performed with isolated soy protein because of the strong inhibitory effect of soy on iron absorption. The method for extracting phytate and the absorption data demonstrating the increase with complete removal, is currently being prepared for publication.

There are several other ongoing projects with the Nestlé collaboration. One of these is the **evaluation of bush teas** and comparison of their inhibiting effect on iron absorption with commercial tea. Although tea is known to

have a dramatic inhibiting effect on iron absorption, it is not known whether the large variety of bush teas used in several developing countries have a similar adverse effect. Several teas have been evaluated at ICCNA with respect to their inhibiting properties. There is a precise correlation between the degree of inhibition in iron absorption and total polyphenol content. A monograph describing the inhibiting effect of bush teas is in preparation.

Another activity during the past year is to better define **the role of an inhibiting or enhancing diet in iron balance** in subjects consuming a varied diet. A method was developed at ICCNA for measuring iron absorption from a complete diet by giving a labelled wheat roll with 28 self-selected meals over a 14 day period. The results were compared with absorption from representative single meals fed in a laboratory setting. When the subjects were allowed to consume their own diet, absorption from the single meal and from the complete diet was similar. However, when subjects were instructed to maximize their diet by taking meat and ascorbic acid with each meal, a much more dramatic increase in iron absorption from the single meal occurred (13.5%) than from the complete diet (8.0%). Thus, the use of a single meal to measure iron absorption in fasting subjects exaggerated the importance of bioavailability by nearly 50%. Similarly, when subjects were instructed to consume a diet that

maximally inhibits iron absorption, a much greater decrease in absorption from a single meal occurred as compared with a complete diet. The exaggeration of the effect of the inhibitor was also about 50%. This study indicates that in subjects consuming a varied diet, conventional studies in which extrinsically labelled meals are fed to fasting subjects may exaggerate the importance of dietary bioavailability. We also demonstrated that a typical American diet is nearly maximal with respect to iron availability. It is planned in future years to extend this work to developing countries, where the diet is more monotonous. This refinement for assessing bioavailability from a complete diet will be important for determining the role of the diet in iron nutriture.

ICCNA WORK PLAN

IRON DEFICIENCY SUPPORT PROGRAM

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

SEPTEMBER 1990 - AUGUST 1991

The following is a 12-month work plan proposed for the Iron Deficiency Support Program beginning in September 1990. Much of the background for the proposed studies is outlined in the preceding progress report. The following work plan is divided into two major sections: Extramural Programs or field studies, and Intramural Programs to develop new interventions and methods to assess iron status.

I. EXTRAMURAL PROGRAMS (Field Studies)

It should be noted in regard to overseas programs that funds to conduct the following projects have not yet been identified. The ability to proceed with these programs is entirely dependent on funding outside of this contract and none of the work can proceed without support. The only exceptions are the GDS studies in Jamaica and studies in Ethiopia where funds are available through UNICEF to perform the field work.

1. GDS Evaluation - Kingston, Jamaica

This trial is now finished as discussed in the progress report. However, a major task remains in the next 12 months of preparing the work for publication. One of the immediate questions is the randomization of the three study groups. Sometime within the next few months, Dr. Cook will travel to Kingston, Jamaica to discuss this problem in detail with the statistician at CFNI (Keith Bingham) and the principle investigator, Dr. William Simmons. The approach taken in preparing this work for publication will depend on the reasons initial iron status measurements differed among the 3 study groups.

2. The Evaluation of Wheat Fortification with Iron, Grenada, WI

This project has also been outlined in the progress report and in further detail in Appendix B. The initial phase of this study will involve an island-wide assessment of wheat intake on a household basis to estimate the per capita intake of wheat flour and the proportion of unfortified (counter) flour within each household. Following this, it is planned to conduct baseline household surveys at the start of fortification and again at 18 and 36 months following the institution of fortification. ICCNA will provide most of the laboratory backup for this program especially measurements of ferritin

and transferrin receptor. Although there is apparently a high prospect of funding for this study by AID Washington, a final determination has not yet been made.

3. Work Productivity, Kingston, Jamaica

A very attractive research protocol has been developed by Dr. Patterson, Director of CFNI, in Kingston, Jamaica. The purpose is to examine the impact of iron supplementation on the productivity of women working on garment manufacturing in an industrial estate. A total of 14 companies make up an industrial estate within the Free Zone, each company employing between 30 and 3000 workers. Workers are paid on an incentive basis and the salary is computed on the basis of work output. This provides a precise quantitative measure of work output by which to gauge the possible benefit of improving iron nutriture. Virtually all of the workers are females between the age of 18 and 30 years. It is planned to select 2-3 companies for this project, which seeks to define the relationship of iron deficiency anemia to work output with tasks that involve dexterity, attention span, and learning ability. A randomized double-blind controlled clinical trial is planned in two groups, one given a placebo, and the other given 60 mg of iron supplement daily over a 12 week period. Blood chemistry will be examined 3 times during the intervention and some, or all, of these

measurements (serum ferritin, serum receptor) will be performed at ICCNA. The projected length of the study is approximately 4 months at a total cost of $30,000-40,000 (US). Further details about the proposed study are given in Appendix B. Funding for this project has not yet been identified.

4. Prevalence and Etiology of Anemia in Ethiopia

During September 1990, Dr. Hana Neka Tebeb visited ICCNA for the purpose of laboratory training and discussing collaborative studies between the Ethiopian Nutrition Institute, Addis Ababa, Ethiopia, and ICCNA. A protocol for proposed studies, drafted by Dr. Tebeb in April of 1990, is included in Appendix B. Anemia is known to be highly prevalent in Ethiopia, but the cause is uncertain. Some of the highest daily intakes of iron in the world have been reported in this country, largely due to the contamination of grains during processing. However, anemia remains highly prevalent in the country and under support by UNICEF, the Ethiopian Nutrition Institute desires to obtain more information on the prevalence and cause of this anemia. A variety of study plans were discussed, but it was decided to limit the prevalence sample initially to segments of the population more susceptible to iron deficiency, i.e. women in the childbearing age and school children. Dr. Tebeb plans to draft a revised protocol on her return to Ethiopia, but our preliminary plan involves sampling children at the time of

their first entry to school. The purpose is to link prevalence data with a potential for intervention because these children are readily accessible for iron supplementation. Blood samples will be drawn when first entering school. Household dietary information will be obtained in parallel by interviewing mothers in the homes of these children. It is tentatively planned to perform the majority of the immunoassays (serum ferritin, transferrin receptor) at ICCNA.

II. INTRAMURAL PROGRAMS

1. Assessment of Iron Status

Emphasis will continue to be placed, in the coming year, on the use of serum receptor levels to assess iron nutriture. One segment of the population that has not yet been studied with this new measurement is **preschool and school aged children**. In view of this, a large study is tentatively planned with Dr. Susan Fairweather-Tait at the Norwich Agricultural Laboratory, UK. It is anticipated that approximately 2000 samples will be obtained on newborns with repeated sampling at 2-3 month intervals during the first 2 years of life. The extensive database generated should clearly define the role of serum receptor measurements in identifying iron deficiency during infancy.

Experience with transferrin receptor measurements in epidemiological surveys is still limited. In collaboration with Dr. Ray Yip at CDC, it is planned to undertake serum receptor measurements in samples drawn in **HANES III**. Several thousand samples have already been obtained in this survey and there is residual serum from many of these samples. It is planned to perform between 500 and 1000 analyses on selected segments in the HANES III sample with emphasis on women during the childbearing age and preschool and school-aged children. Because of the extensive iron data and nutritional parameters collected as part of HANES III, we believe that this pilot study will be of key importance in defining the sensitivity of serum receptor measurements for the detection of mild iron deficiency.

2. Development of Fortification Programs

Several collaborative studies are now underway with a view to developing more effective means of supplying dietary iron. One of these projects is in support of the **rice fortification project** at Rutgers University. A variety of approaches to fortifying rice using a synthetic rice granule is being examined in this project. It is planned that studies of iron availability of the fortified product will be conducted in human volunteers at ICCNA.

Collaborative studies will continue with Richard Hurrell at Nestlé. There are several proposed studies, some of which are a continuation of ongoing work. Further effort will be made to obtain **phytate-free infant cereals** using a variety of cereal products to determine the benefit of fully extracting phytate from weaning foods. A new batch of radioiron compounds will be prepared to extend studies of **fortification compounds** that might be used to fortify a chocolate drink powder. Because ferrous fumarate was not fully satisfactory, we plan to evaluate ferrous succinate and ferrous saccharate.

It is also planned to perform further studies with **NaFe EDTA** for iron fortification. An intramural study at Nestlé is currently examining the influence in traditional metabolic balance studies of the effect of NaFe EDTA on calcium and zinc balance. However, the influence of NaFe EDTA on dietary iron absorption was not examined in this project. ICCNA will prepare some radiolabelled sodium iron EDTA and use it to fortify the same wheat rolls that are being used in that study. Additional studies on the efficacy of NaFe EDTA for fortifying weaning food will be undertaken if time permits.

A new form of fortification iron will be evaluated. This compound, referred to as **biglycino iron**, is presently marketed by Albion Laboratories. This product employs USP grade amino acids as the ligand in a bidentate

chelate with iron. Glycine has been used traditionally for this purpose because of its low molecular weight. This preparation is said to be low in cost, good tasting, and safe. There are several reports of using such chelates with other minerals. Dr. Heney has shown superior absorption of a calcium chelate in human subjects and another report by Dr. Sacks had demonstrated the efficacy of a zinc histidine chelate over zinc sulfate in human subjects. Human studies with the biglycino iron have not yet been conducted, but information with other metals suggests that it may be useful for iron fortification. Representatives from Albion Laboratories will travel to ICCNA where the radiolabelled complex will be prepared. It is planned to examine its efficacy as a means of fortifying wheat flour, but additional studies will be performed with infant cereals.

The precise experimental design in these studies depends on the nature of the investigation. Typically, we perform approximately 20 studies per year in groups of 6-10 subjects to give a total of approximately 200 human subjects annually, or 800 separate measurements of iron absorption (four measurements are usually performed in each subject using dual radioiron tags). Partial support for this investigation is obtained from Nestlé.

APPENDIX A

THE CLINICAL MEASUREMENT OF SERUM TRANSFERRIN RECEPTOR

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Reprinted from
THE JOURNAL OF LABORATORY AND CLINICAL MEDICINE,
St. Louis

Vol. 114, No. 4, pp. 368-377, Oct., 1989
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(Printed in the U.S.A.)

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The clinical measurement of serum transferrin receptor

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Monoclonal antibody reagents were used to develop a sensitive enzyme-linked immunoassay for clinical measurement of circulating transferrin receptor. By using transferrin-bound receptor for the preparation of the immunologic reagents, we developed an assay that gives an identical dose-response curve with either free or transferrin-bound receptor. The mean concentration of circulating receptor in 82 normal male and female volunteers was 5.63 ± 1.42 mg/L. The level was reduced significantly in patients with primary aplastic anemia and post-transplant aplasia (2.58 ± 1.07 mg/L and 2.32 ± 0.48 mg/L, respectively) and was sharply elevated in patients with hemolytic anemia and iron deficiency anemia (33.1 ± 17 and 18.0 ± 11.4 mg/L, respectively). Our assay values are approximately 20-fold higher than results published previously in a study that used an immunoradiometric assay. The disparity apparently relates to a difference in sensitivity of the latter assay for free and transferrin-bound receptor. Measurements of serum transferrin receptor provide a useful clinical index of either total or iron-deficiency erythropoiesis. (J LAB CLIN MED 1989;114:368-77)

Abbreviations: BSA = bovine serum albumin; ELISA = enzyme-linked immunosorbent assay; HEPES = *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; HRP = horseradish peroxidase; IRMA = immunoradiometric assay; SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PBST = phosphate-buffered saline solution containing 0.05% Tween 20; TfR = transferrin receptor; Tf-TfR = transferrin–transferrin receptor complex

There is now abundant evidence that the number of TfRs on the surface of a cell controls its rate of iron uptake from circulating diferric transferrin.[1-5] The TfR number on the cell surface reflects the iron requirement of the cell and, accordingly, is increased sharply in rapidly growing tissues and in hemoglobin-synthesizing cells. Two general approaches have been used to measure the density of surface TfR on intact cells: mesurement of the uptake of radiolabeled transferrin[6,7] and immunofluorescent techniques with monoclonal antibodies used against surface TfR.[8] Immunoassays have also been developed for soluble TfR extracted from tissues or cellular extracts.[9-11] Application of these methods has provided invaluable information regarding the mechanisms by which cells satisfy their iron requirements.

A series of recent reports indicates that soluble TfR can also be detected in human serum or plasma.[12-15] Elevated levels occur in patients with iron deficiency anemia, autoimmune hemolytic anemia, and polycythemia, and lower values are seen in patients with aplas-

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Supported by National Institutes of Health Grant DK39246 and AID Cooperative Agreement DAN-515-A-00-7908-00.

Submitted for publication March 23, 1989; revision submitted May 16, 1989; accepted May 17, 1989.

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An editorial relevant to this article appears on p. 336 of this issue of the JOURNAL.

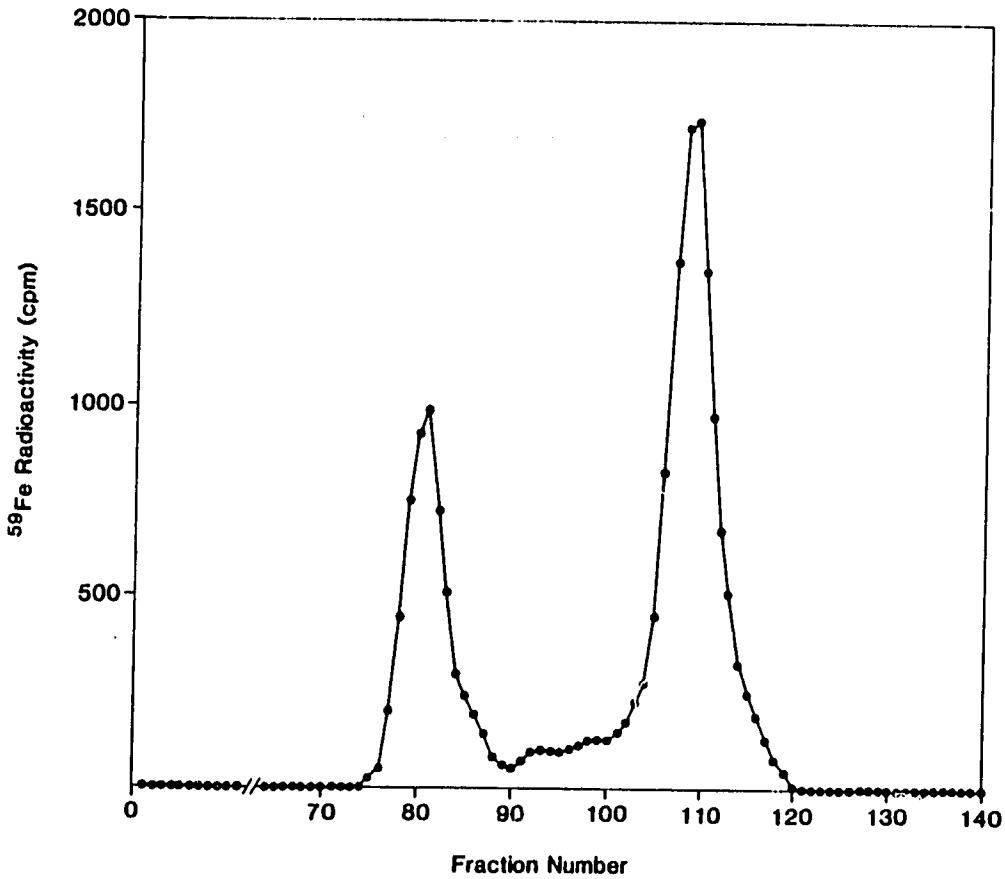

Fig. 1. Fractionation of human placental receptor on AcA34 Ultrogel column. First, higher molecular weight peak contains Tf-TfR. Second radioactivity peak contains free transferrin.

tic anemia. The monoclonal antibodies OKT9 and B3/25 that were used for the IRMA in these studies were developed against intact cells[16,17] and presumably react only with the extracellular portion of the TfR molecule. The objective of the present study was to develop an enzyme immunoassay for soluble or serum TfR that is equally sensitive to free or transferrin-bound receptor. To accomplish this, our monoclonal reagents were prepared against soluble Tf-TfR rather than surface TfR. Our serum TfR values are much higher than those reported previously, a difference that may be related to the nature of the material used as standard in the assay.

METHODS

Clinical samples. Blood was obtained from 45 female and 37 male healthy volunteer subjects, 18 to 45 years of age, who were hematologically normal and had serum ferritin values between 12 and 160 μg/L. Serum TfR level was also measured in four patients with acquired aplastic anemia, eight patients from whom samples were taken 10 days after bone marrow transplantation and prior conditioning with cyclophosphamide (Cytoxan; Bristol-Myers Oncology Division, Evansville, Ind.) and total body irradiation, 19 patients with iron deficiency anemia as determined by a serum ferritin concentration <12 μg/L and hematocrit <36%, and 11 patients with sickle cell anemia. Serum was separated from whole blood within 24 hours and stored at −30° C until assayed. All specimens were obtained according to procedures approved by the Human Subjects Committee of the Kansas University Medical Center.

Preparation of monoclonal antibodies. TfR was isolated from fresh human placental tissue. After removing the cord and membranes, we cut the placenta into pieces weighing 10 to 12 gm. The tissue was washed extensively with ice-cold saline solution and frozen at −70° C within 1 hour of delivery. The initial homogenization procedure was based on previously described methods.[10,18] All procedures were carried out at 4° C unless otherwise stated. A 150 gm sample of placental tissue was partially thawed and then homogenized in 300 ml 0.01 mol/L phosphate buffer, pH 7.5, first in a blender (Waring Products, New Hartford, Conn.) for 5 minutes, and then with a tissue homogenizer (Polytron; Brinkmann Instruments Inc., Westbury, N.Y.) for 5 minutes. The homogenate was filtered through several layers of gauze and centrifuged at 10,000 g for 45 minutes. The pellet was resuspended in fresh phosphate buffer and recentrifuged. This procedure was repeated three times, until the supernatant was

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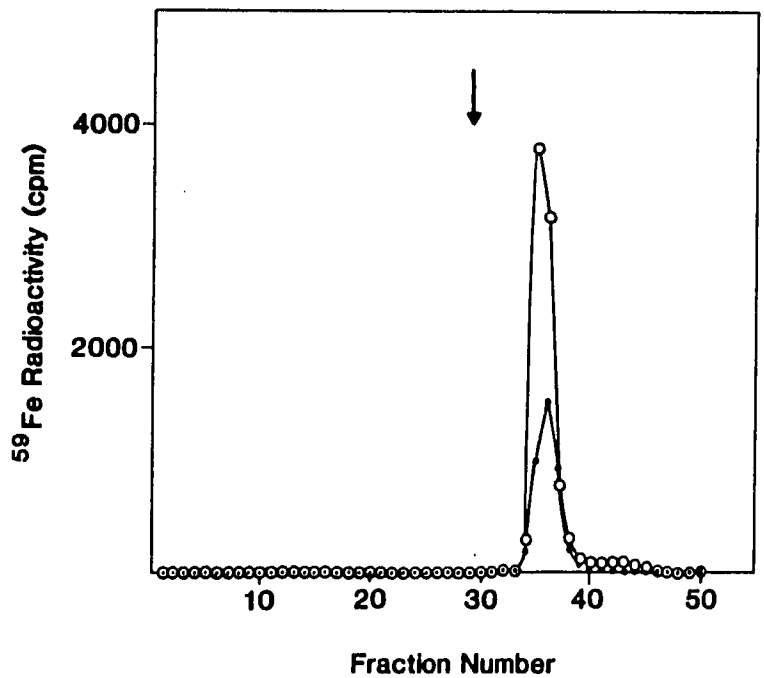

Fig. 2. Ion-exchange chromatography of human placental receptor. Pooled fractions from AcA34 were applied to DEAE Sephacel column. *Arrow* indicates start of gradient elution.

clear of contaminating blood. The final pellet was resuspended in 0.02 mol/L Tris-HCl, pH 8.3, containing 2% nonionic detergent (Teric 12A9; Sigma Chemical Co., St. Louis) used for solubilizing membrane components[10,19] and was stirred for 16 hours. The solution was centrifuged at 10,000 *g* for 45 minutes. Solid ammonium sulfate was added to the supernatant (31.5 gm/100 ml), and the solution was allowed to stand overnight. After centrifugation at 10,000 *g* for 60 minutes, the supernatant was gently suctioned from underneath, the floating pellet. The latter was resuspended in 0.02 mol/L Tris-HCl buffer, pH 8.3, stirred for 3 hours, and dialyzed overnight against the same Tris buffer.

The supernatant was then trace labeled with diferric iron 59-transferrin and applied to a diethylaminoethyl Sephacel column. Free transferrin and Tf-TfR were eluted as a single peak with a 0.05 mol/L to 0.3 mol/L Tris-HCl, 0.6 mol/L NaCl gradient. The peak radioactive fractions were then pooled and applied to a gel filtration column (Ultrogel AcA 34; LKB Instruments Inc., Gaithersburg, MD.),[19] which yielded two separate radioactive peaks, Tf-TfR complex and free transferrin (Fig. 1). The higher molecular weight peak fractions were pooled, dialyzed against 0.02 mol/L Tris-HCl, pH 8.3, applied to a second diethylaminoethyl Sephacel column for concentration, and eluted as before (Fig. 2). The peak fractions, identified by both radioactivity and protein (A_{280}), were pooled. Purity of the Tf-TfR was confirmed by demonstration of a single protein band after PAGE.

Six-week-old male Balb/C mice were injected subcutaneously with 40 μg Tf-TfR in complete Freunds' adjuvant. Two additional injections were given in incomplete Freunds' adjuvant at 2-week intervals. A final injection of 40 μg Tf-TfR was given 72 hours before fusion. Spleen cells from the immunized mice were fused with mouse myeloma line P3/X63-AG8 6.5.3 as previously described.[20] Hybridomas were selected in hypoxanthine aminopterin thymidine medium and screened for antibody production by adding 100 μl of culture supernatant to microtiter plates coated with 2 μg/ml Tf-TfR in carbonate buffer. Antireceptor antibody was then detected by using HRP-conjugated anti-mouse immunoglobulin G. Cultures positive for antireceptor were also screened for antitransferrin by incubating culture supernatants in plates coated with 5 μg/ml transferrin (Sigma Chemical Co., St. Louis). Cultures from wells that showed no reaction with transferrin were then cloned three times by limiting dilution. Expanded cultures from four hybridomas, A4A6, D3A12, E2H10, and A10A9, were injected into pristane-primed mice, and ascites fluid was collected 10 to 21 days later.

These antibodies were partially purified by sodium sulfate precipitation.[21] All four were of the immunoglobulin G_1 subclass as determined by immunoelectrophoresis. The antibodies were conjugated to HRP as previously described.[22] Combinations of all four monoclonals were evaluated for their performance in an ELISA, E2H10 and A4A6 were selected for use as the coating and indicator antibody, respectively, on the basis of their high titers and dose response and lack of interference by transferrin in the ELISA assay.

ELISA protocol. The technique is similar to that described previously for the measurement of serum ferritin.[22] Flat-bottomed 96-well microtiter plates (NUNC-Intermed; NUNC Inc., Naperville, Ill.) were coated with 2 μg/ml monoclonal antibody E2H10 diluted in 0.05 mol/L carbonate buffer, pH 9.6, and incubated overnight. Unreacted sites were blocked

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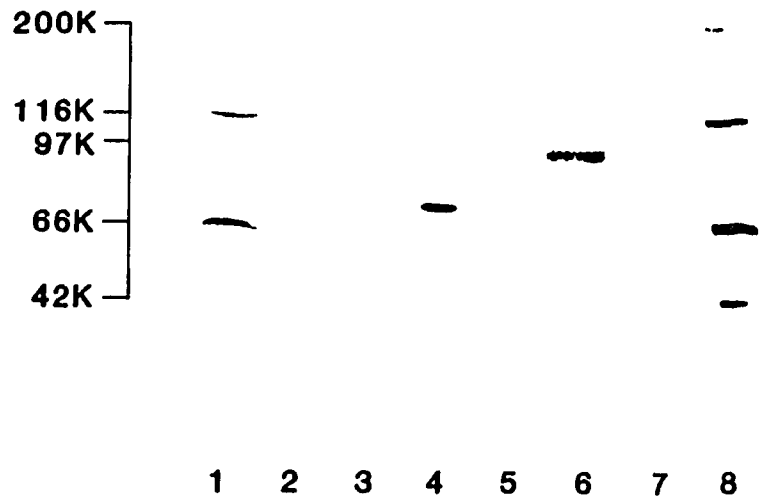

Fig. 3. SDS-PAGE of placental receptor. *Lane 1*, Molecular weight standards; *Lane 2*, buffer, *Lane 3*, placental receptor, nonreduced; *Lane 4*, transferrin, nonreduced; *Lane 5*, buffer; *Lane 6*, placental receptor, reduced; *Lane 7*, transferrin, reduced; *Lane 8*, molecular weight standards.

with 0.5% BSA in carbonate buffer for 30 minutes at room temperature. The plates were then washed three times with PBST.

Standards or serum samples were added in a volume of 200 μl to the appropriate wells. Purified TfR was diluted in PBST containing 0.5% BSA. Serum was diluted 1:500 in PBST. Standards containing 0.5 to 50 ng/ml and serum unknowns were run in triplicate. The plate was covered and incubated for 2 hours at room temperature. After being washed three times with PBST, 200 μl of HRP-conjugated A4A6 diluted in PBST containing 1% BSA was added to all wells, and the plate was incubated for an additional 2 hours at room temperature. The plate was again washed three times, and 200 μl of the substrate (34 mg of *o*-phenylenediamine in 0.15 mol/L citrate-phosphate buffer, pH 5.0, containing 0.01% H_2O_2) was added. After incubation for 30 minutes at room temperature in the dark, the reaction was stopped by adding 50 μl of 25% sulfuric acid. The optical density was read at 492 nm on a microplate reader (Titertek Multiskan; Flow Laboratories, McLean, Va.). The dose-response curve was linear when log net absorbance was plotted against log receptor concentration.

Standardization. Because the long-term stability of TfR may be enhanced when it is saturated with diferric transferrin,[23] the assay was standardized with Tf-TfR prepared as described for the preparation of monoclonal antibody. Tf-TfR was diluted to a concentration of 1 μg/ml TfR protein in 0.02 mol/L Tris buffer, pH 8.3, containing 1% Teric, was stored at 4° C, and was used to prepare working standards each week.

To determine the TfR protein concentration of the Tf-TfR standard, we isolated free TfR from human placenta tissue by using the affinity purification protocol described by Turkewitz et al.[24] After iron was released from the transferrin affinity column with deferoxamine, the column was washed rapidly with HEPES buffer, and TfR was eluted with sodium thiocyanate buffer. The eluate was immediately desalted through gel filtration columns (PD10; Pharmacia Inc., Piscataway, N.J.), and the fractions were pooled and analyzed for protein content by the method of Lowry et al.[25] and the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.). The purity of the isolated TfR was confirmed by electrophoresis on SDS-PAGE with a 4% to 20% gradient gel (Fig. 3). The purified complex gave a single protein band at 190,000 daltons without reduction and 95,000 daltons after reduction with 2-mercaptoethanol. On the basis of comparison of protein concentrations and dose response in the ELISA, the Tf-TfR standard was found to contain 54% TfR protein, consistent with evidence that one molecule of TfR binds two transferrin molecules.[4,12,16,26] When adjusted for differences in protein concentration, the standard curve with free TfR was identical to that obtained with Tf-TfR (see later).

IRMA. An IRMA with OKT9 and B3/25 monoclonal antibodies was used previously for measurement of serum TfR.[13] To compare this assay with our ELISA, B3/25 and A4A6 monoclonal antibodies were iodinated in chloramine-T.[27] Iodinated antibody was separated from free iodine on a Sepharose G25 (Pharmacia) column (1 × 10 cm) equilibrated with 50 mmol/L barbital buffer, pH 8.0. Pooled antibody fractions were diluted with an equal volume of barbital buffer containing 0.1% BSA. The IRMA was performed in 12 × 75 mm tubes (Maxisorp; NUNC-Intermed, NUNC Inc.). The tubes were coated with OKT9 antibody (5 μg/ml) in 0.1 mol/L carbonate buffer, pH 9.6, 0.1 ml per tube, and incubated for 48 hours. The coating buffer was then removed and 0.5% BSA in carbonate buffer was added to block un-

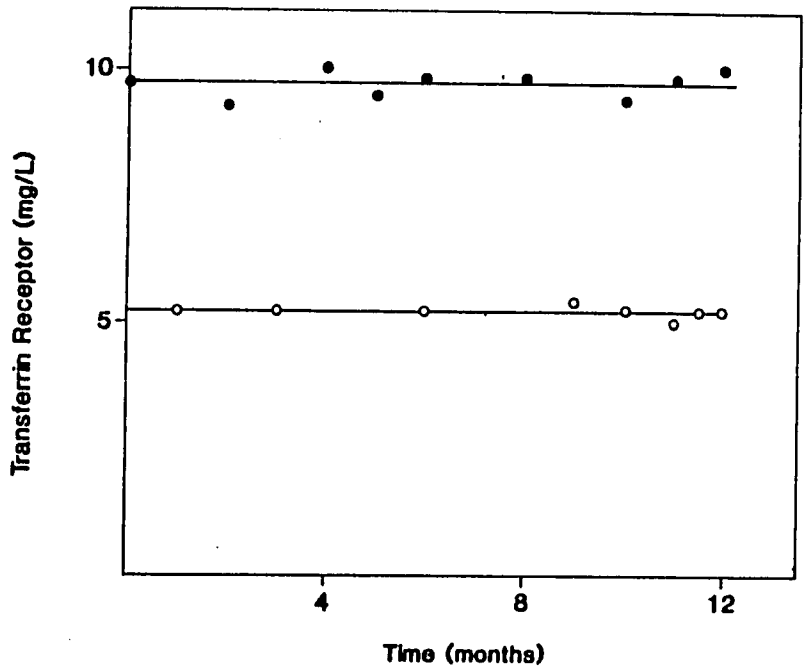

Fig. 4. Between-assay variability and stability of transferrin receptor measurements on quality control serum samples measured over 12 months.

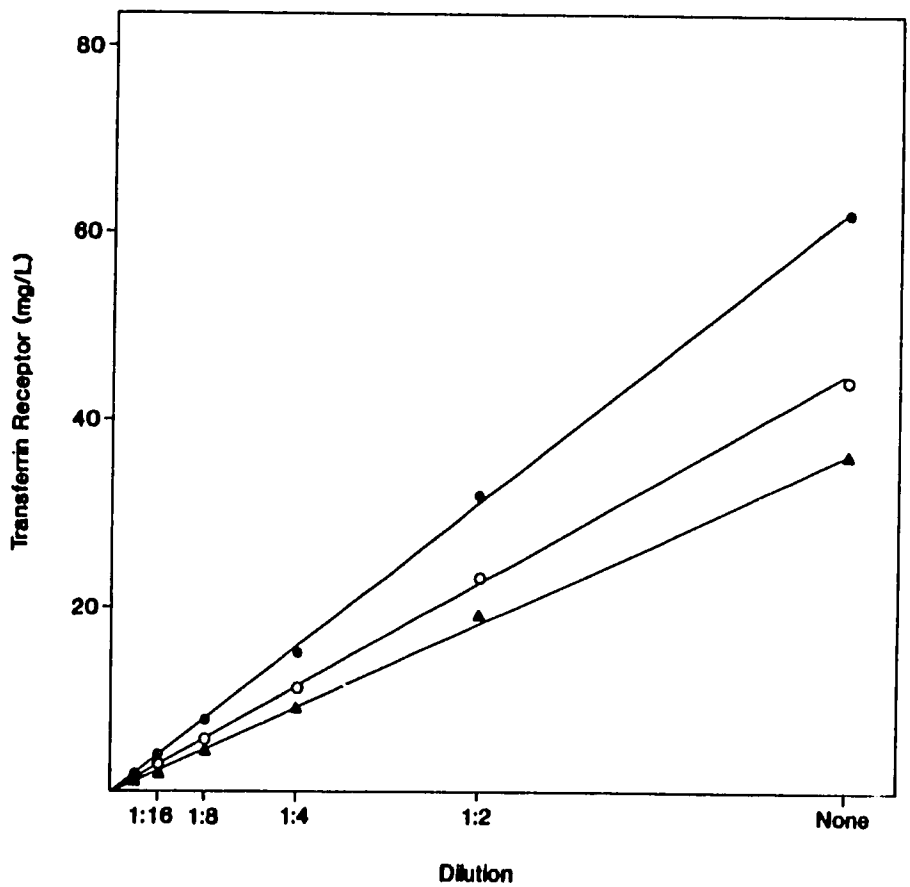

Fig. 5. Serial dilutions of three serum samples with initial transferrin receptor levels of 62 mg/L (●), 44 mg/L (○), and 36 mg/L (▲).

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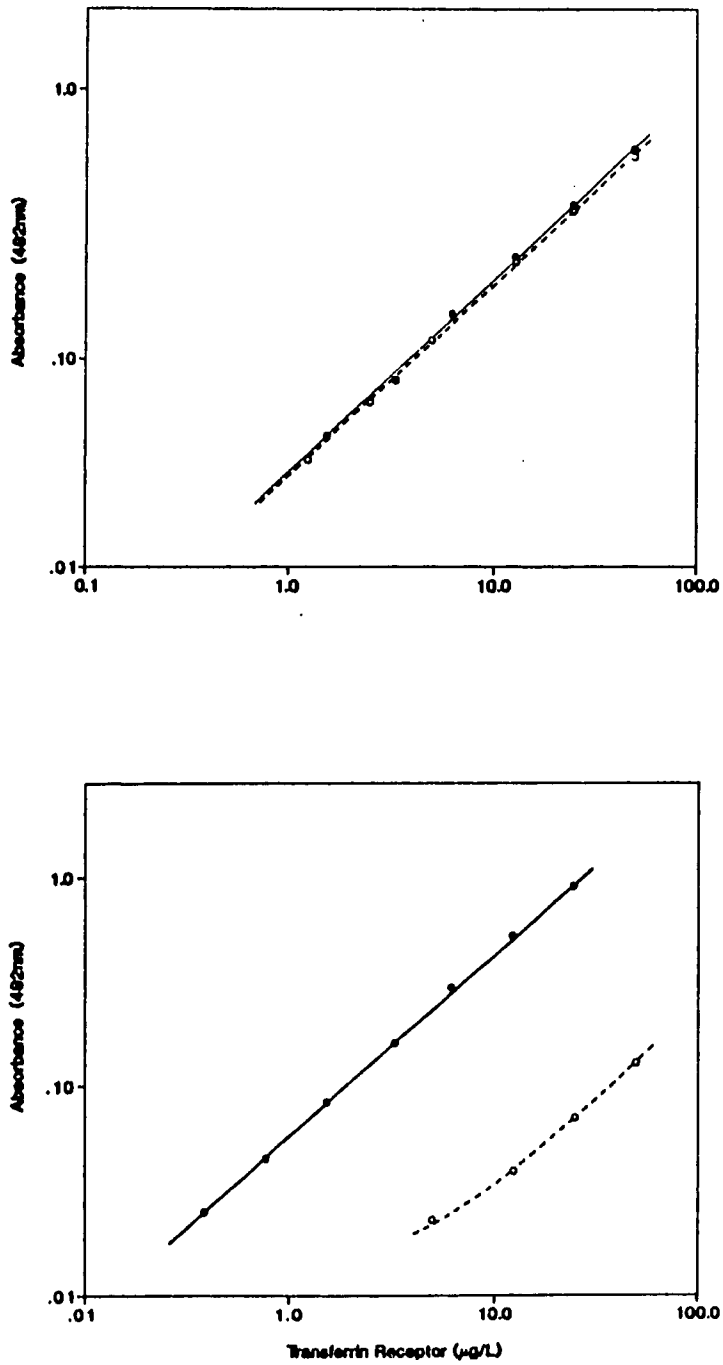

Fig. 6. ELISA standard curves with E2H10 and A4A6 monoclonal antibodies *(upper panel)* or OKT9 and B3/25 monoclonal antibodies *(lower panel)*. ●, Free transferrin receptor used as standard. ○, Transferrin-transferrin receptor used as standard.

reacted sites. The tubes were washed three times with PBST and 50 μl of standard containing 10 to 1000 μg/L or serum unknown diluted 1:200 in PBST was added. After the tubes were incubated for 18 hours and washed, 50 μl iodine 125-labeled monoclonal anti' 'dy diluted in 1% BSA in PBST to a final concentration of .),000 cpm/ml was added and incubated overnight. The tubes were washed three times with PBST and counted in a gamma counter (Searle Inc., Chicago, Ill.).

RESULTS

One important advantage of using monoclonal antibodies for enzyme immunoassays is low background

Table I. ELISA standardization with TfR and Tf-TfR when using different monoclonal systems

	Circulating transferrin receptor (mg/L)			
	Bound TfR		Free TfR	
Sample	E2H10 A4A6	OKT9 B3/25	E2H10 A4A6	OKT9 B3/25
1	4.0	8.6	3.8	0.42
2	4.9	5.6	4.7	0.28
3	10.5	10.5	10.0	0.54
4	5.2	7.2	4.9	0.35
5	9.8	14.2	9.3	0.75
6	8.0	5.4	7.7	0.27
7	6.0	5.6	5.8	0.31
8	8.2	7.8	7.9	0.40
Mean	7.1	8.1	6.8	0.42

absorbance.[22] In the present assay, background absorbance values typically range between 0.01 and 0.02. The sensitivity of the assay as defined by the lowest measured receptor concentration that differs significantly from zero ($p < 0.001$) is 0.5 μg/L. The within-assay variability, calculated on the basis of 10 replicate measurements on serum samples containing 3.5 to 10.5 mg/L TfR, ranged between 3% and 5%. The between-assay variability, as assessed by performing repeated measurements over a 12-month period in samples containing 5.2 and 9.7 mg/L TfR, was 1.9% and 2.5%, respectively (Fig. 4). No matrix effect of whole serum could be demonstrated by recovery studies in which free TfR and Tf-TfR complex were added to whole serum; recovery was 100%. Serial dilutions of three serum samples containing 36, 44, and 62 mg/L TfR, were linear (Fig. 5).

Initial assays of normal serum samples gave values for circulating TfR that were roughly 20 times higher than those reported previously.[13] A series of studies was undertaken to explain this disparity. Our initial approach involved substituting monoclonal antibodies used previously in our ELISA. Thus, OKT9 replaced the E2H10 monoclonal antibody used for the solid phase, and B3/25 was conjugated with HRP and substituted for the A4A6 as the indicator antibody. Substitution of OKT9 in our assay had no effect on the standard curve when either free TfR or Tf-TfR was used as the standard. When both OKT9 and B3/25 were substituted, the dose-response curve with free TfR was also similar to that obtained in our standard assay system. However, when Tf-TfR was used as standard, there was a dramatic inhibition of the dose-response curve (Fig. 6).

Additional studies with an IRMA demonstrated that the difference in sensitivity of the B3/25 monoclonal antibody to Tf-TfR was not related to conjugation with HRP. When the IRMA was established with OKT9 as the coating antibody and ^{125}I-labeled B3/25 as the indicator antibody, there was a similar dramatic inhibition of the dose-response curve when Tf-TfR was substituted for TfR as the standard. Thus, the findings with the ELISA and IRMA were similar.

A comparison of serum TfR values with free TfR and Tf-TfR used as standards with each monoclonal tandem in the ELISA assay is shown in Table I. With our E2H10 and A4A6 monoclonals antibodies, the TfR for eight normal serum samples was 7.1 mg/L when Tf-TfR was used as standard and virtually the same (6.8 mg/L) when the assay was calibrated with free TfR. With the monoclonal antibodies OKT9 and B3/25, the serum TfR averaged 0.42 mg/L when free TfR was used as standard; however, this increased to 8.1 mg/L when the assay was calibrated with Tf-TfR.

Serum TfR averaged 5.7 ± 1.4 mg/L in 37 healthy men and 5.56 ± 1.44 mg/L in 45 healthy women, a difference that was not significant ($t = 0.43$, $p = 0.67$). No effect of age could be demonstrated in either group. In patients with aplastic anemia, the mean TfR fell approximately 50%, to 2.58 ± 1.07 mg/L. Similar values were obtained in eight patients with aplastic anemia after transplant, with average levels of 2.32 ± 0.48 mg/L. Both of these levels were significantly lower than those in the normal controls ($t = 4.23$ and 6.51, respectively, $p < 0.001$). In 19 patients with iron-deficiency anemia, the mean serum TfR rose sharply, to 18 ± 11.4 mg/L, a highly significant increase ($t = 9.65$, $p < 0.001$). The level was also sharply higher in 11 patients with sickle cell anemia 33.1 ± 17 mg/L ($t = 14.77$, $p < 0.001$) (Fig. 7).

DISCUSSION

Human TfR is a transmembrane glycoprotein comprised of two identical subunits each weighing 95,000

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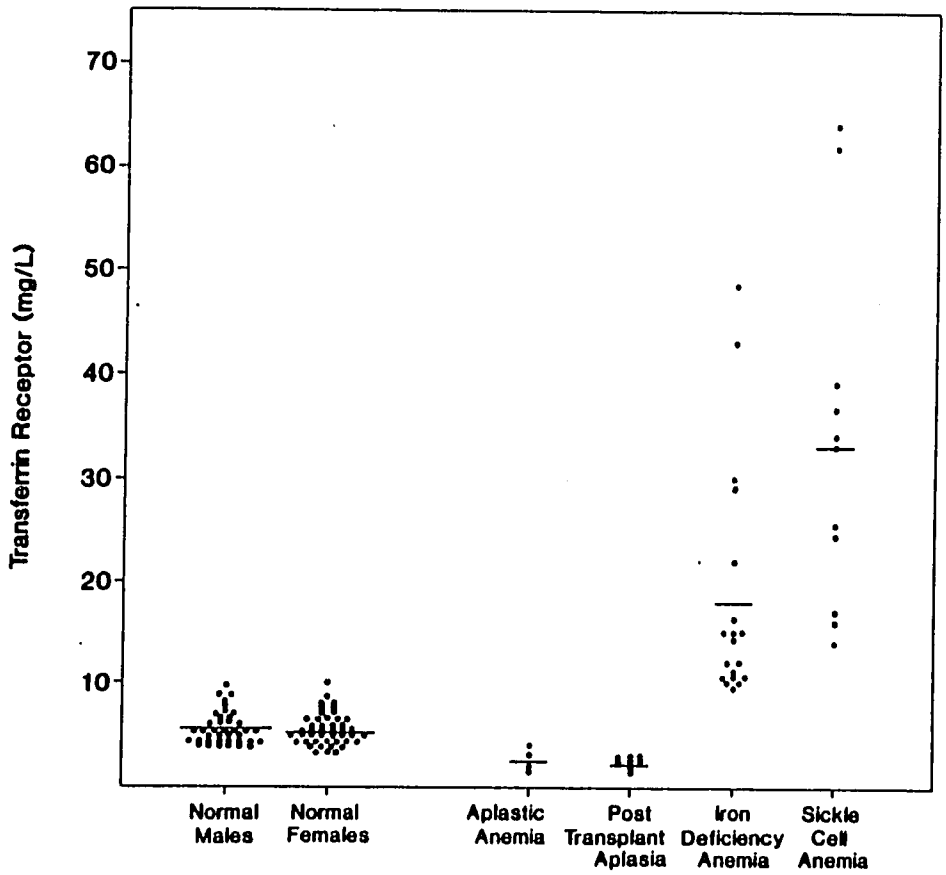

Fig. 7. Transferrin receptor levels in normal subjects and patients with various hematologic disorders.

daltons and linked by disulfide bridges. Each subunit is believed to combine with one molecule of transferrin, giving a molecular weight of approximately 350,000 daltons for the Tf-TfR complex. TfR consists of a small internal cytoplasmic domain, a larger hydrophobic region normally positioned within the cell membrane, and a large C-terminus extracellular polypeptide chain. Treatment with trypsin results in the formation of two fragments of 70,000 daltons each, which retain their transferrin binding capability.[16,24,28] TfRs isolated from human placenta tissue and reticulocytes are apparently identical on the basis of proteolytic patterns, molecular size, and immunoreactivity.[29] The affinity of TfR is known to be much higher for diferric transferrin than for either monoferric or apotransferrin.

The exact biochemical nature of TfR, or its immunoreactive fragments in human serum, has not been fully defined. When human serum was electrophoresed on PAGE, transblotted to nitrocellulose, and reacted with four different monoclonal antibodies, a single band between 80,000 and 100,000 daltons was observed.[13] This contrasted with an estimated molecular weight of 180,000 daltons when serum was subjected to gel filtation on Sephadex G200. The authors concluded that circulating TfR is in monomeric form bound to one molecule of transferrin and that transferrin is dissociated during electrophoresis. In a more recent study with SDS-PAGE, a single 110,000 dalton band in normal serum was observed on the basis of both immunologic and transferrin binding properties. Under reducing conditions, bands of 46,000 and 23,000 daltons were observed in normal subjects and patients with iron deficiency anemia, autoimmune hemolytic anemia, and polycythemia vera.[15] It was postulated that TfR immunoreactivity in human serum is caused by nicked dimers with a molecular weight of 110,000 daltons, which are capable of binding two molecules of diferric transferrin. These observations differ from those in studies of circulating TfR in rat plasma in which the immunoreactive band after PAGE migrated in the same position as intact placental receptor.[23] It was also noted that an excess antitransferrin added to rat serum precipitated 92% of the immunoreactivity, indicating that nearly all circulating TfR is bound to transferrin.

The objective of the present investigation was to establish an enzyme immunoassay for circulating TfR by using principles similar to those in the sandwich radioimmunoassay used previously.[13] To detect all circulat-

ing immunoreactive material, we elected to prepare our monoclonal antibodies against soluble TfR rather than against whole cells, as used in the preparation of OKT9 and B3/25. We also used Tf-TfR complex so that the assay would be unaffected by the presence or absence of bound transferrin. Similar results can also be obtained by using polyclonal antiserum prepared in rabbits immunized with Tf-TfR and subsequently adsorbed with a transferrin affinity column to remove antitransferrin antibody (Finch, personal communication). Hybridoma cultures, however, offer significant advantages over polyclonal antisera because they provide an unlimited supply of defined immunologic reagent that facilitates long-term standardization and eliminates the need for repeated antigen purification and antibody production. Monoclonal antibodies also permit the detection of lower levels of protein because of reduced background.[22]

One of the surprising findings in the early phase of our investigation was the marked difference in values in normal subjects as compared with those reported previously.[13] Our studies suggest that this disparity relates to the reagents used for standardization. When we used Tf-TfR as the standard with OKT9 and B3/25, the results agreed with our ELISA. Because substitution of Tf-TfR for free TfR inhibits the dose-response curve, higher values are obtained for serum unknowns. This difference was not anticipated, because neither OKT9 or B3/25 inhibit transferrin binding to cells. Other monoclonals, such as 42/6, that have been developed against intact cells do inhibit transferrin binding and impair the growth of tumor cells in vitro.[30] An assay for soluble TfR that does not require assumptions about its binding status with transferrin may offer advantages in the assay of TfR in culture systems or in body fluids that may contain both free and transferrin-bound TfR.

Despite differences in the level of serum TfR from results reported by Kohgo et al.,[15] the relative decrease or increase in circulating TfR in various clinical disorders is remarkably similar. For example, a mean serum TfR of 730 μg/L was observed previously in 41 patients with untreated iron deficiency, as compared with a normal value of 253 μg/L, giving a ratio of 2.88. In the present report, we observed a mean TfR level of 18 mg/L in patients with iron deficiency as compared with a mean of 5.6 in normal subjects, a ratio of 2.98. The relative decrease in patients with aplastic anemia and the elevation in patients with chronic hemolytic anemia were also similar. The major difference between the assays appears, therefore, to relate to standardization.

Our clinical experience with measurements of serum TfR to date suggests that the assay will assume major importance in the assessment of hematologic disorders. A close correlation was observed previously between the reticulocyte count and serum TfR in compensated autoimmune hemolytic anemia.[14] Because the TfR number on reticulocytes is much lower than that of earlier erythroid precursors, it must be assumed that circulating TfR is derived primarily from normoblasts. In support of this, we have observed marked TfR elevations in thalassemia major despite a normal reticulocyte count. Measurements of serum TfR may therefore provide a reliable measurement of total erythropoiesis.

At the present time, the effectiveness of red cell production can be determined quantitatively with ferrokinetic measurements or qualitatively by comparing the degree of erythroid cellularity on bone marrow examination with the reticulocyte count. Ferrokinetic measurements are rarely justified for clinical purposes because of cost. If a reliable quantiative relationship can be established between serum TfR and total erythroid mass, the effectiveness of erythropoiesis can be determined by comparing serum TfR levels with a corrected reticulocyte count. This TfR-to-reticulocyte ratio would be useful in the diagnosis of disorders in which erythropoiesis is characteristically ineffective, but variable over time, such as the myelodysplastic syndromes. Serum TfR measurements will be less reliable in detecting reduced erythropoiesis. In the present study, the serum TfR was decreased by about 50% in patients with complete marrow aplasia, suggesting that a portion of circulating TfR is also derived from nonerythroid tissues. This is analogous to ferrokinetic measurements where a variety of corrections must be applied before the plasma iron turnover can be used to assess diminished erythropoiesis.[5]

Another important application of TfR measurements is for the detection of iron deficiency. A high correlation has been observed between serum TfR and other iron parameters, such as serum iron, total iron binding capacity, and serum ferritin level, in patients with iron deficiency anemia but not in patients with hemolytic anemia.[15] Presumably, the sharp elevation in serum TfR in patients with iron deficiency is related to an increased transferrin receptor number on individual normoblasts rather than to an increase in erythroid cellularity.

Because the serum TfR may reflect either iron-deficient erythropoiesis or increased erythroid mass, elevated levels will require additional laboratory determinations to distinguish these. Nevertheless, in patients in whom ineffective erythropoiesis and hemolysis can be excluded, the serum TfR should be a reliable guide to the severity of iron deficiency. Serial phlebotomy studies in normal subjects indicate that the serum TfR is a highly sensitive index of iron-deficiency erythropoiesis (Skikne et al., unpublished observations).

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Journal of Internal Medicine 1989, 226: 349–55

Iron deficiency: definition and diagnosis

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Abstract. Cook JD, Skikne BS (Division of Hematology, Department of Medicine, Kansas University Medical Center, Kansas City, KS 66103, USA). Iron deficiency: definition and diagnosis. *Journal of Internal Medicine* 1989; 226: 349–55.

There has been a continuous refinement over the past several decades of methods to detect iron deficiency and assess its magnitude. The optimal combination of measurements differs for clinical and epidemiological assessment. Clinically, the major problem is to distinguish true iron deficiency from other causes of iron-deficient erythropoiesis, such as the anaemia of chronic disease. Epidemiologically, techniques that provide quantified estimates of body iron are preferable. For both purposes, the serum ferritin is the focal point of the laboratory detection of iron deficiency. Serum ferritin measurements provide a reliable index of body iron stores in healthy individuals, a cost-effective method of screening for iron deficiency, and a useful alternative to bone marrow examinations in the evaluation of anaemic patients. Preliminary studies indicate that measurement of the serum transferrin receptor may be the most reliable way to assess deficits in tissue iron supply.

Keywords: iron deficiency, serum ferritin, serum transferrin receptor.

Introduction

There have been major advances over the past several decades in our ability to detect iron deficiency and to estimate its magnitude. Old methods have been improved, new methods have been introduced and our ability to interpret both has increased. These refinements have decreased the cost of laboratory screening and diminished the need for invasive procedures such as bone marrow examination. Because of the large array of available methods, it is possible to tailor the diagnostic approach to specific situations. Refinements in the measurement of iron status are continuing, as evidenced by the introduction of the serum transferrin receptor assay, an important new tool for assessing iron status.

It is useful to distinguish between two broad applications of iron-related measurements: the clinical detection of iron deficiency, and the assessment of iron status in the population. Although several tests are used for both purposes, the optimal combination differs for clinical and epidemiological evaluations. Cost is of less concern in clinical diagnosis, but is often a major limitation in epidemiological surveys. Diseases that masquerade as iron deficiency complicate clinical diagnosis, but these do not occur frequently enough in population surveys to influence the interpretation if their presence is known in advance. Another difference between clinical and epidemiological studies is the usefulness of estimating body iron quantitatively. For clinical purposes, the detection of iron deficiency is more important than gauging its severity, whereas in population studies estimates of body iron are of considerable value in the assessment of the impact of interventions and the detection of long-term trends in iron status.

Definition of iron deficiency

Epidemiological studies indicate that body iron stores in a given population sector form a single distribution curve [1]. It is therefore as difficult to select a cut-off point for iron deficiency as it is to define abnormal body weight or height. Experts agree that iron deficiency exists when body iron has declined to the extent that overt anaemia occurs. Difficulties arise in defining milder iron deficiency.

Iron status is best characterized in relation to the

amount of iron contained in the storage and functional compartment [2]. Storage iron is located primarily in the parenchymal cells of the liver and the reticuloendothelial cells of the bone marrow, liver, and spleen. These stores constitute about one-third of body iron in healthy adult men, but are smaller, more variable, and frequently absent in children and menstruating women. Storage iron functions as an iron reserve to replenish deficits in functional iron. Iron stores that are reduced but still present are not associated with any known liabilities and should not be taken to represent iron deficiency.

Functional iron exists mainly as haemoglobin in circulating red cells, and in lesser amounts in myoglobin and various tissue enzymes. Because all body tissues depend on circulating transferrin for their iron supply, any sustained reduction in plasma iron should be regarded as iron deficiency. In serial phlebotomy studies, a fall in transferrin saturation below 16% and a decrease in serum ferritin below 12 $\mu g\ l^{-1}$ occur at approximately the same time, indicating that both can be used as evidence of depleted iron stores (B. S. Skikne *et al.*, unpublished observations). Because the major outflow of plasma iron is directed to the erythroid marrow, functional iron deficiency is recognized within weeks of onset by signs of impaired haemoglobin synthesis in circulating red cells, such as an elevated erythrocyte protoporphyrin and microcytosis.

In defining iron deficiency as the point at which iron stores are completely exhausted, we imply that even small functional iron deficits are associated with certain liabilities. The consequences of mild iron deficiency have been the subject of extensive study in recent years and are reviewed elsewhere in this minisymposium (see Dallman, page 367). There is evidence that iron deficiency is associated with a variety of non-haematological symptoms such as impaired attention span and learning ability [3–6], impaired growth and enhanced morbidity in children [7], defects in cell-mediated immunity and bactericidal function of leucocytes [8], reduced work capacity of the endurance type [9,10], impaired athletic performance [11], increased frequency of premature uterine contractions during pregnancy [12], and possibly an increased rate of premature births [13]. Any reduction in cellular iron supply should be regarded as iron deficiency.

Laboratory evaluation of iron status

It is helpful to classify iron measurements as those that indicate either residual iron in the storage compartment or depleted iron in the functional compartment (Table 1). Estimates of storage iron are important because the presence of any store excludes the diagnosis of iron deficiency. Bone marrow examination to estimate stainable iron in reticuloendothelial cells is still regarded by many as the gold standard for storage iron assessment, especially in hospitalized anaemic patients [14]. Because serum transferrin or total iron-binding capacity (TIBC) is inversely related to serum ferritin in otherwise normal subjects [15], it also provides an indirect measure of storage iron. However, transferrin measurements are relatively insensitive, as the degree of change with variation in iron stores is small relative to assay variability. Serum ferritin measurement is a key indicator of iron stores and deserves detailed discussion.

There are many methods of identifying functional iron deficits. Plasma iron levels or transferrin saturation are useful for screening purposes, but their value is lessened by the large number of clinical disorders which induce secondary changes in plasma iron transport. Highly automated chemical techniques and disposable supplies have largely eliminated the iron contamination that plagued earlier manual measurements of plasma iron. The diagnostic specificity of a low transferrin saturation is enhanced if associated with a TIBC above 400 $\mu g\ l^{-1}$ but, in general, plasma iron transport measurements provide only circumstantial evidence of iron deficiency.

The most important indices of iron-deficient erythropoiesis are the erythrocyte protoporphyrin and

Table 1. Laboratory measurements of iron deficiency

	Diagnostic range
a) Storage iron	
Bone marrow examination	Absent
Total iron-binding capacity	> 400 $\mu g\ dl^{-1}$
Serum ferritin	< 12 $\mu g\ l^{-1}$
b) Functional iron	
Transferrin saturation	< 16%
Erythrocyte protoporphyrin	> 70 $\mu g\ dl^{-1}$ RBC
Mean cell volume	< 80 fl
Serum transferrin receptor	> 9 mg l^{-1}
Anaemia	< 130 g l^{-1} (men)
	< 120 g l^{-1} (women)

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mean cell volume (MCV), measured electronically. Each has its advantages and drawbacks [14]. Both are more stable than transferrin saturation because changes in circulating red cells only occur after several weeks of iron deficiency, and disappear slowly with iron replacement. Red cell protoporphyrin measurements have been facilitated by the development of haematofluorometers which require only a couple of drops of blood and minimal technical experience. Elevations also occur with lead poisoning and acquired defects in haemoglobin synthesis. Because of the simplicity of the procedure, red cell protoporphyrin has been widely used in population surveys, though only infrequently by clinicians.

A reduction in MCV below 80 fl has approximately the same diagnostic specificity as elevated erythrocyte protoporphyrin levels. Slight microcytosis commonly occurs in the anaemia of chronic disease, but more pronounced microcytosis below 70 fl greatly increases the probability of true iron deficiency, especially if thalassaemia can be excluded. The MCV is of particular value in screening hospitalized patients because it is often measured routinely on admission. The main disadvantage of all available measurements of functional iron is that they do not distinguish true iron deficiency from secondary iron-deficient erythropoiesis due to inflammatory or neoplastic disease. An important new laboratory parameter, the serum transferrin receptor, may circumvent some of these problems.

Serum ferritin

Laboratory measurement of serum ferritin has emerged during the past two decades as the single most important indicator of iron deficiency. It is the focal point of any iron assessment, whether performed to screen healthy individuals for latent iron deficiency, to diagnose iron deficiency in overtly anaemic patients, or to assess iron stores in population surveys. However, the serum ferritin is not an infallible guide to iron status and its usefulness can be enhanced by a thorough knowledge of factors that influence it in both healthy and diseased individuals. There are several reviews available on the subject [16–18].

In healthy individuals, the level of serum ferritin provides a reliable measure of storage iron [15, 19, 20]. Quantified phlebotomy studies have demonstrated that within the range of 20–150 $\mu g\ l^{-1}$, 1 $\mu g\ l^{-1}$ serum ferritin is equivalent to 8–10 mg of iron in an average-sized adult [14]. A more reliable correlation between storage iron and serum ferritin can be obtained using a logarithmic transformation [1]. There is a three-fold difference in mean serum ferritin levels between adult men and women, which reflects the difference in their levels of storage iron. Following menopause, serum ferritin increases abruptly as the iron balance improves; as a result, by 60–70 years of age, the difference in serum ferritin levels between the sexes has largely disappeared. Serum ferritin has been reported to increase with age [21], but the cause of this is not adequately defined. It may represent a further increase in body iron stores, a higher prevalence of inflammatory disorders in the elderly, or a gradual redistribution of iron from the functional to the storage compartment.

Serum ferritin measurements are most informative when assessing iron-replete sectors of the population, when the normal level is well above the 'cut-off' point for iron deficiency of 12 $\mu g\ l^{-1}$. Serum ferritin is an excellent indicator of iron status in adult males because of their larger iron reserves. In a recent survey in the USA, only 20% of men between 18 and 65 years had serum ferritin levels of less than 50 $\mu g\ l^{-1}$, and only 5% of men had levels of less than 20 $\mu g\ l^{-1}$ [1]. A reduction in serum ferritin in an otherwise healthy male to below 50 $\mu g\ l^{-1}$ has as much clinical relevance as an iron-deficient value below 12 $\mu g\ l^{-1}$ in a menstruating woman.

By the same token, the clinical utility of serum ferritin is much less in sectors of the population in which iron deficiency is common. Because iron reserves are marginal throughout most of infancy and early childhood, serum ferritin is of limited value in screening for iron deficiency in this paediatric age group. For similar reasons, obstetricians find serum ferritin to be of only limited use in assessing iron status during pregnancy. Serum ferritin levels fall dramatically to below 20 $\mu g\ l^{-1}$ during the second and third trimester, even in women taking iron supplements [22]. Whereas the serum ferritin level during the first trimester may predict the risk of development of iron deficiency later in pregnancy, measurements are of limited value during the second and third trimester.

Serum ferritin measurements are important in the clinical assessment of anaemic patients. In all forms of anaemia except iron deficiency, a decrease in circulating haemoglobin is accompanied by a reciprocal increase in storage iron and a proportional rise in serum ferritin levels. If iron deficiency is the

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sole cause of the anaemia, the serum ferritin level is almost invariably below 12 $\mu g\ l^{-1}$. In early clinical reports of over 150 cases of iron deficiency anaemia defined by the absence of stainable marrow iron, only 3% of patients had serum ferritin levels above 12 $\mu g\ l^{-1}$ [14]. The overriding clinical advantage of serum ferritin measurement is that a value below 12 $\mu g\ l^{-1}$ in an anaemic patient is diagnostic of iron deficiency.

A serum ferritin level within the normal range in an anaemic patient is less useful diagnostically because many clinical disorders cause an increase in serum ferritin that is independent of iron status. Chronic infection or inflammation, liver disease, and malignancy increase serum ferritin at any given level of storage iron [20]. The rise is about threefold in patients with infection or inflammation, and nearly fivefold in patients with liver disease. Because of the higher prevalence of disorders which independently increase the serum ferritin levels of patients seen in the hospital or out-patient clinic, it is desirable to use less rigid diagnostic criteria in these anaemic patients. Values below 20 $\mu g\ l^{-1}$ can be considered diagnostic of iron deficiency (Table 2). Patients with serum ferritin levels between 20 and 100 $\mu g\ l^{-1}$ may have iron deficiency as defined by absent marrow iron stores or a positive response to oral iron, but the probability of iron deficiency declines sharply at levels above 60 $\mu g\ l^{-1}$. Iron deficiency is usually excluded at values above 100 $\mu g\ l^{-1}$, although it is interesting that in patients with renal failure that has been treated with recombinant erythropoietin, signs of iron-deficient erythropoiesis may develop despite relatively high serum ferritin levels [23].

One final point concerning serum ferritin measurements deserves emphasis. Although a low serum ferritin defines the onset of iron deficiency, values in the iron-deficient range provide no guide as to its severity. This is partly explained by the higher assay variability at low levels which often obscures any diagnostically useful information. Using a highly sensitive immunoassay developed with monoclonal antibodies, we have observed a significant negative correlation between the deficit in functional iron and serum ferritin levels below 12 $\mu g\ l^{-1}$.

Table 2. Diagnosis of iron deficiency in anaemic patients by use of serum ferritin levels

Serum ferritin ($\mu g\ l^{-1}$)	Diagnosis of iron deficiency	Oral iron therapy
< 20	Unequivocal	Will respond
20–60	Possible	May respond
60–100	Rare	Rarely responds
> 100	Excluded	No response

Serum transferrin receptor

Knowledge of the process by which iron is transferred from its plasma transferrin carrier to an intracellular compartment has expanded greatly during the past decade [24–26]. Although a non-endocytotic mechanism has not been completely excluded, the major influx of iron occurs by a receptor-mediated mechanism. Circulating diferric transferrin binds to its specific receptor on the cell membrane, and this is followed by endocytosis of the ligand receptor complex. Iron is then released within the cell as the pH of the vesicle decreases. On return of the vesicle to the cell surface and restoration of the pH to 7.4, apotransferrin is promptly released into the circulation. This is an ingenious mechanism by which the iron requirements of a cell are directly linked to the means of obtaining it. When the iron supply is inadequate, there is an up-regulation of transferrin receptors to enable the cell to compete more effectively for iron. Consequently, the density of transferrin receptors is much higher in rapidly growing tissues, in haemoglobin-synthesizing red cell precursors, and in the placenta, which must acquire iron for the foetus. The density of surface receptors on intact cells can be measured either by direct uptake of radiolabelled transferrin [27,28] or by immunofluorescent techniques using monoclonal antibodies directed against the surface receptors [29].

Tissue measurements of transferrin receptor have few clinical applications because of their invasive nature. However, it has recently been reported that soluble transferrin receptor can be detected in sera by sensitive immunological techniques [30–32]. These findings were prompted by *in vitro* studies which showed that receptors on reticulocytes and K562 cells are shed into the medium during incubation. In the original reports, an increase in serum receptor levels was seen primarily in patients with enhanced erythropoiesis, but also in those with iron deficiency anaemia. In both iron deficiency and chronic haemolysis, a correlation was observed between the reticulocyte count and the serum receptor level. In iron-deficient patients, significant correlations were also observed between serum receptor and TIBC, serum iron, and serum ferritin. In patients with aplastic anaemia, the receptor level declined to

approximately 50% of normal, implying that the erythroid marrow accounts for only about one-half of the circulating protein [32].

We have recently established an enzyme-linked immunoassay for the measurement of serum transferrin receptor using monoclonal antibodies [33]. The mean level in 82 normal male and female subjects was 5.6 mg l^{-1} (normal range 3–9 mg l^{-1}), about 20-fold higher than the levels reported previously. The disparity appears to be related to a difference in the sensitivity of the original assay for free and transferrin-bound receptor. Our clinical findings are similar. We observed a significant rise in iron deficiency anaemia (mean 18 mg l^{-1}) and chronic haemolytic anaemia (mean 33 mg l^{-1}), and a significant reduction in patients with aplastic anaemia (mean 2.5 mg l^{-1}). We did not observe a correlation between serum receptor and reticulocyte counts in patients with iron deficiency anaemia, indicating that enhanced erythropoiesis and iron deficiency increase the serum receptor levels independently.

Further studies are required to define the precise role of serum receptor measurements in the diagnosis of iron deficiency, but our initial results are encouraging. In controlled phlebotomy studies, the receptor does not increase until iron stores are completely exhausted (B. S. Skikne *et al.* unpublished observations). Beyond this point, the increase in serum receptor levels is roughly proportional to the deficit in functional iron. It may be possible to substitute serum receptor measurements for the battery of standard laboratory indices of iron-deficient erythropoiesis, especially if enhanced red cell production can be excluded. The serum receptor level provides a more stable measurement than transferrin saturation, and is affected earlier in the development of functional iron deficiency than the erythrocyte protoporphyrin or MCV. Our preliminary results obtained with this new measurement suggest that it will assume a major role in the evaluation of iron status.

Clinical diagnosis

There are two main clinical situations in which iron status measurements are required: screening for iron deficiency in otherwise healthy individuals, and diagnosis of iron deficiency in patients with overt anaemia. A cost-effective screening method is to use the serum ferritin and haemoglobin levels in tandem (Table 3). If both values are within the normal range, iron deficiency can be excluded. Low serum ferritin and normal haemoglobin levels indicate storage depletion, and additional measurements such as erythrocyte protoporphyrin, MCV, or serum receptor are required in order to determine the severity of the iron shortage. If both serum ferritin and haemoglobin are low, iron replacement should be initiated. If anaemia is detected together with normal or elevated serum ferritin levels, a haematological search for other causes of anaemia is required.

Table 3. Screening for iron deficiency

Serum ferritin	Haemoglobin	Diagnosis
Normal	Normal	Iron deficiency excluded
Low	Normal	Storage depletion
Low	Low	Iron deficiency anaemia
Normal or elevated	Low	Other cause of anaemia

A major difficulty that arises when evaluating anaemic patients involves distinguishing iron deficiency from the anaemia of chronic disease which often mimics it. If the serum ferritin level falls within the diagnostic 'grey zone' between 20 and 100 μg l^{-1}, details of history and physical examination will often provide clues that aid interpretation. Because of the high cost of hospital care, it may be preferable to examine the bone marrow rather than to wait for the results of a serum ferritin assay. In an out-patient clinic, a therapeutic iron trial may be an acceptable approach when the serum ferritin is within the low normal range and iron deficiency seems likely. Follow-up of these patients is essential because of the risk of overlooking a more serious underlying disease. Because non-compliance with chronic iron therapy is common, a baseline serum ferritin should always be obtained to assist in the later evaluation of patients who do not respond to iron. The measurement of serum ferritin is an excellent means of monitoring the restoration of iron deficiency and determining the precise end-point of iron therapy.

Epidemiological assessment

Nutritional surveys of iron status are important for estimation of the prevalence of iron deficiency, measurement of the efficacy of iron supplementation or fortification, and assessment of long-term changes in iron status. Such studies have benefited from increased laboratory sophistication in recent years.

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Earlier population surveys relied solely on haemoglobin or haematocrit determinations: iron status was defined by the proportion of individuals with values below an arbitrary cut-off point. Studies which rely solely on anaemia measurements provide little useful information because of the extensive overlap in levels between normal and iron-deficient populations [21, 34, 35]. Moreover, while haemoglobin measurements give an approximate indication of the severity of iron shortage they provide no information about the iron-replete sector of the population.

More recent population survyes of iron nutrition have employed a battery of iron measurements. Rather than using each measurement in isolation to determine iron status, combinations of measurements have been attempted. For example, the specificity of a low haemoglobin level is greatly enhanced by requiring the association of more specific iron measurements such as a low transferrin saturation, low ferritin, or elevated erythrocyte protoporphyrin [21]. In an extensive survey of iron status in the USA, impaired iron status was defined with models based on either the transferrin saturation, serum ferritin, and erythrocyte protoporphyrin, or MCV, transferrin saturation, and protoporphyrin. Iron deficiency anaemia was diagnosed when these measurements were also associated with a low haemoglobin level [36].

In a recent study we attempted to assess iron status in the population by estimating body iron in each individual sampled [1]. In individuals with a serum ferritin level above 12 $\mu g\ l^{-1}$, the ferritin value was used to estimate body iron. At the other end of the spectrum of iron status, a deficit in functional relative to storage iron was estimated from the haemoglobin level. Quantitative estimates are more difficult in cases of mild iron deficiency without anaemia. Empirical values based on serum ferritin, transferrin saturation, and erythrocyte protoporphyrin were used to estimate deficits in functional iron between 0 and 300 mg. Estimation of iron stores proved to be more reliable than the use of criteria based on multiple laboratory cut-off levels, and both approaches were superior to estimates based on haemoglobin alone. This model has been used recently to assess the outcome of a sugar fortification trial in Guatemala [37], and to measure the impact of fortified curry powder in South Africa [38]. There are several advantages of estimating iron status individually. By increasing the sensitivity of the evaluation, relatively small changes in iron status can be detected with a reduction in the time and cost required to conduct field trials. This technique can be used in surveys to examine the effects of factors such as diet or socio-economic status. Future refinements using this approach will increase its applicability.

Acknowledgements

This work was supported by NIH grant DK39246 and AID Cooperative Agreement DAN-515-A-00-7908-00.

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Received 16 June 1989, accepted 20 June 1989.

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Adaptation in iron metabolism[1-3]

James D Cook

ABSTRACT Humans can adapt successfully to a wide range of iron requirements and intakes. The lower limit of the adapted state is best defined by serum ferritin ≥ 12 μg/L, because lower values indicate that iron stores are fully depleted. Successful adaptation is achieved by modifying the rate of gastrointestinal absorption according to body iron needs. Several dietary variables, including total intake, content of heme, and bioavailability of nonheme iron, determine the ceiling of the adaptive response when iron demands are high. With lesser demands the nature of the diet has a limited influence on body iron reserves, although there is some evidence that the adaptive response to variations in heme iron intake is less complete than the response to differences in nonheme bioavailability. How the intestinal mucosal cell achieves adaptation is one of the most important unsolved questions in iron metabolism. *Am J Clin Nutr* 1990;51:301–8.

KEY WORDS Adaptation, iron absorption, dietary iron

Introduction

The term adaptation is not used very often in iron literature but the phenomenon is well known. One of the most characteristic features of iron metabolism in humans is their ability to maintain adequate body iron concentrations despite wide differences in physiological requirements and dietary iron supply. The highly refined adaptive mechanisms for iron reflect not only the critical importance of the metal in oxygen transport but also the potential toxicity of iron when the body concentration exceeds the capacity of proteins designed for its transport and storage. Although the major focus of this review is on adaptive mechanisms to prevent iron deficiency, adaptation to prevent a harmful accumulation in body iron in the face of excessive iron supply is no less important.

Definitions of adaptation in iron metabolism

There is still no complete agreement as to what constitutes success or failure in adaptation for a given nutrient, although several excellent reviews on the subject were published (1–3). An enormous range in body iron is compatible with prolonged survival in human subjects. The consequences of a marked deficit or excess in body iron are well defined in the clinical literature. Advanced iron deficiency is associated with severe anemia, which not only impairs the quality of life but eventually leads to failure of the cardiac and respiratory systems. Chronic iron overload, on the other hand, produces fibrotic scarring and eventual failure of several organs, including the liver, pancreas, heart, and endocrine system. These extremes in iron balance are obviously beyond the limits of successful adaptation. On the contrary, it is preferable to define adaptation in iron metabolism as a state that is free of any known or suspected liabilities. A precise definition requires a knowledge of body iron distribution.

Iron status is defined conveniently in relationship to the amount of iron contained in two main body compartments: functional and storage iron (4). The functional compartment consists mainly of hemoglobin in circulating red cells but includes smaller amounts in myoglobin and various tissue enzymes. Storage iron is a smaller and more variable compartment that serves as an emergency reserve of iron to replenish sudden deficits in functional iron. Storage iron is located primarily in reticuloendothelial cells located in bone marrow, liver, and spleen.

Nutritional iron deficiency is associated with periods of heightened physiological requirements and usually develops slowly over months or even years. The earliest phase is accompanied by a gradual depletion of storage iron. Iron stores can be measured quantitatively by performing repeated phlebotomies to induce iron deficiency anemia and then calculating iron stores as the difference between the amount of iron removed and the induced deficit in circulating hemoglobin iron (5, 6). For clinical purposes iron stores are usually assessed from the amount of stainable iron in reticuloendothelial cells seen on bone marrow examination. In recent years serum ferritin measurements have provided a simpler, more precise, measure of storage iron (6–9). Within the range of 20–150 μg/L, 1 μg serum ferritin/L corresponds to 120 μg storage iron/kg body wt, or 8–10 mg iron stores in an average sized adult (6, 10, 11). False increases in serum ferritin are seen clinically in patients with chronic inflammatory, neoplastic, or hepatic disease (7, 8). In otherwise-healthy individuals a serum ferritin < 10–12 μg/L is a reliable guide to the point at which iron stores are fully exhausted (12).

Once iron stores are depleted, any further decrease in body

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

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Received August 9, 1989.
Accepted for publication August 16, 1989.

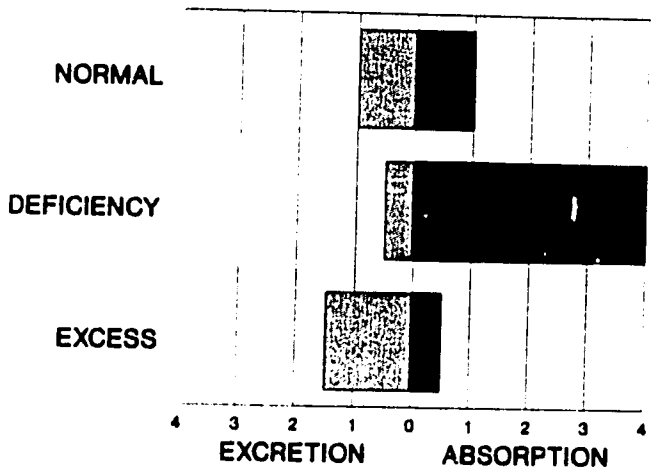

FIG 1. Limits of adaptation in iron excretion and absorption at extremes of body iron status.

iron is associated with a curtailment in iron supply to body tissues. Because red cell precursors in the bone marrow have a much higher iron requirement than other tissues, a deficit in functional iron is associated with the development of iron-deficient erythropoiesis. This state is recognized in the laboratory by a decrease in serum iron, an increase in total iron-binding capacity (TIBC), or a reduction in transferrin saturation (serum iron × 100/TIBC) (4, 12). Iron-deficient erythropoiesis is also recognized within a few weeks of onset by an increase in free erythrocyte protoporphyrin in circulating red cells (13). As long as there is a sufficient supply of iron to immature red cells, protoporphyrin is fully utilized by combining with iron to form heme, but when iron supply to the erythron is compromised, an unbound excess of protoporphyrin remains in circulating red cells. Eventually, iron-deficient erythropoiesis produces a smaller red cell containing a reduced quantity of hemoglobin, changes referred to as microcytosis and hypochromia. As the deficit in functional iron increases, an obvious decline in hemoglobin concentration occurs, which defines the onset of iron deficiency anemia.

There is now considerable evidence that mild iron deficiency even without anemia is associated with significant health consequences and should be considered failed adaptation. The greatest impact of iron deficiency is in growing children, who develop defects in attention span leading to learning and problem-solving difficulties (14–17). This learning deficit may have lasting consequences by limiting an individual's subsequent achievement. Iron supplementation has been shown to increase growth velocity and level of morbidity in children (18). Several studies showed that iron supplementation of workers in regions of the world where iron deficiency is highly prevalent improves work productivity of the endurance type even in the absence of anemia (19, 20). Mild iron deficiency produces abnormalities in cell-mediated immunity and the ability of neutrophils to kill several types of bacteria, although it has not been proved conclusively that these abnormalities produce an increased incidence or duration of infection (21). Iron deficiency may have its greatest impact on perinatal morbidity and mortality. In a recent study a close inverse relationship was observed between the rate of prematurity and the maternal hematocrit (22). A woman with a hematocrit of 0.37 had twice the risk of having a premature birth as a woman with a hematocrit between 0.41 and 0.44. A decrease in hematocrit by even a single point was associated with a 24% increase in the risk of prematurity. Although the nature of the anemia was not defined in this study, iron deficiency is known to be the main factor in pregnant women. Even mild iron deficiency during pregnancy may be important. In a recent study only 11% of pregnant women with serum ferritin values > 20 μg/L had premature labor contractions as compared with 48% with serum ferritin values < 10 μg/L (23).

Because even small deficits in functional iron are associated with health liabilities, adaptation is best defined as a state in which the iron needs of all body tissues are satisfied. Because a decrease in serum ferritin to < 12 μg/L marks the onset of a deficiency in functional iron, only individuals with a serum ferritin > 12 μg/L should be considered adapted.

It has been known for over half a century that the ability of normal humans to maintain constant body iron concentrations resides with the gastrointestinal mucosa. In the classical monograph by McCance and Widdowson (24), it was concluded that once iron is absorbed there is little or no ability to excrete the metal and that the intestine must somehow regulate iron absorption. After the introduction of radioactive iron in 1938, Hahn et al (25) were quick to conclude from their studies in dogs that the need of the body for iron in some manner dictates the absorption of the metal. These two basic principles of iron metabolism, that body iron is regulated by the gastrointestinal mucosa and that the gut performs according to body iron needs, are the foundations on which any discussion of adaptation must be based.

Extensive application of radioisotopic measurements in humans over the next several decades substantiated these early conclusions (**Fig 1**). Long-term isotopic studies in human subjects have shown that daily iron losses can be reduced to ~0.5 mg in advanced iron deficiency and increased to 1.5 mg/d in subjects with iron overload (4). These losses are of a passive nature and apparently reflect differences in the rate of transferrin iron supply to tissues. Far more dramatic responses occur in iron absorption. Basal absorption of 1 mg/d decreases by ~50% in iron overload but increases dramatically to between 3 and 5 mg/d in iron deficiency.

Determinants of iron balance

There are three major determinants of body iron in healthy individuals: physiological iron demands, dietary supply of available iron, and adaptation (**Table 1**). Note that two of these

TABLE 1
Determinants of iron status

Physiological iron demands
Basal
Growth
Menstruation
Pregnancy
Blood loss
Dietary iron supply
Total iron intake
Heme iron content
Nonheme bioavailability
Adaptation

determinants, dietary supply and adaptation, are concerned with the process of iron assimilation. Factors relating to the diet are commonly termed the *luminal* phase of iron absorption and include both the amount ingested and the proportion available for uptake by the mucosal cell. Factors relating to adaptation are concerned with the *mucosal* phase and include the amount of luminal iron taken into the mucosal cell and the fraction of this iron subsequently retained in the body.

Physiological iron demand

The physiological demand for iron is unquestionably the most important determinant of body iron status. There is a basal loss from the gastrointestinal tract, urinary tract, and skin amounting to 12–14 $\mu g \cdot kg^{-1} \cdot d^{-1}$ or ~1 mg in a normal adult male. About two-thirds of this loss is gastrointestinal and is from bleeding and exfoliation of epithelial cells. Isotopic measurements of iron loss have only been performed in adult men, but it is assumed that the basal loss is similar in both sexes and at all ages when expressed by body weight.

The high prevalence of iron deficiency in infants, children, and adolescents reflect the growth demands for expanding body tissues and the red cell mass in particular. These relative growth demands gradually decline during childhood, but iron deficiency may again arise during the adolescent growth spurt. Menstrual blood loss averages 25–30 mL/mo, or 0.5 mg iron/d, when calculated over the entire menstrual cycle. Menstrual losses in the population are highly skewed and are more than three times this amount in ~5% of otherwise healthy women (26). The iron requirement of a normal pregnancy is roughly 500 mg, which is distributed to the fetus, placenta, and expanded maternal red cell mass. Depletion of iron stores will occur in the majority of pregnant women who do not take iron supplements during pregnancy. Finally, there are several types of blood loss that are not pathological and that increase requirements in certain individuals. For example, regular aspirin usage (27) and long-distance running (28) increases gastrointestinal blood loss. Regular blood donations obviously increase iron requirements although in most regular donors these needs are offset readily by enhanced dietary absorption.

Dietary iron supply

Dietary iron supply encompasses both the total amount of ingested iron and its bioavailability. Before 1950, nutritionists emphasized only total iron intake as a measure of dietary adequacy. Wider application of isotopic techniques during the 1950s and 1960s led to the realization that the bioavailability of ingested iron may be more important than total intake. There are two separate pathways of iron entry into the mucosal cell. The largest fraction of dietary iron is in the form of inorganic or nonheme iron, the absorption of which is determined largely by the nature of the meal. Nonheme-iron absorption occurs mainly from the duodenum because of the greater solubility of luminal iron in the proximal, more acid, region of the small intestine. Isotopic studies with extrinsic labeling demonstrated that all dietary forms of nonheme iron ingested in the same meal form a common pool within the intestinal lumen. Absorption from this pool is determined not by the type of the iron ingested but by enhancers, which promote absorption by maintaining iron in a reduced soluble form, and inhibitors, which bind iron or make iron insoluble and prevent its uptake

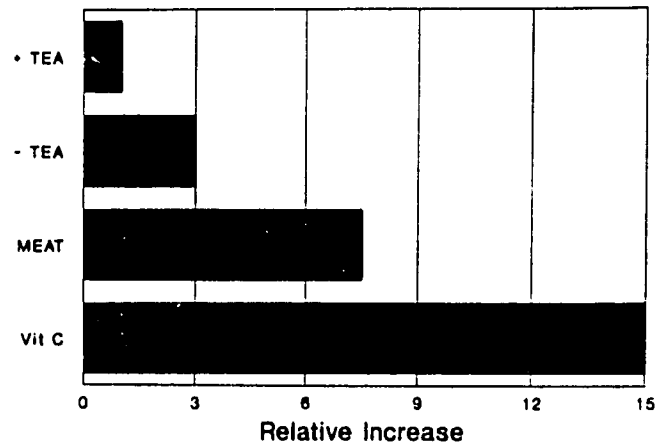

FIG 2. Range in bioavailability of nonheme iron. The most inhibitory meal, which would be cereal-based and consumed with tea, is assigned a relative absorption value of 1.

by the brush border (29–32). The bioavailability of nonheme iron is enhanced by ascorbic acid and various tissue foods, such as meat, fish, and poultry, but not dairy products (33). A large number of dietary constituents impair iron absorption and these factors have been the major focus of absorption studies during the past decade. The most important inhibitors include tea, coffee, bran, calcium phosphate, egg yolk, polyphenols, and certain forms of dietary fiber. The extremes in bioavailability of nonheme iron as measured from isotopically labeled single meals served in a laboratory setting is nearly 15-fold (Fig 2). If tea is eliminated, absorption will increase about threefold. If meat is added, absorption will again increase 2–3 times. Maximal enhancement absorption occurs when a large quantity of ascorbic acid (eg, 2 g) is taken with the meal.

The second dietary iron fraction is heme, which is absorbed into the intestinal cell as an intact porphyrin complex. Specific receptors for heme iron have been identified in laboratory animals (34) but not in humans. After heme iron enters the cell it is rapidly degraded by heme oxygenase (35), and the released iron then enters the common intracellular iron pool. Subsequent mucosal handling of this iron appears to be identical to that of inorganic iron. Because heme iron remains protected within the porphyrin complex before its uptake by the mucosa, it does not interact with dietary ligands and is therefore unaffected by the nature of the meal. Percentage absorption of heme iron is 5–10-fold higher than from nonheme iron. Although heme represents only 10–15% of dietary iron in meat-eating populations, it may account for nearly one-third of absorbed iron (36). Because absorption of heme iron is constant and independent of meal composition, the contribution of heme iron can be readily calculated from dietary records. This is in distinction to marked differences in the availability of nonheme iron.

Dietary iron supply is determined by three main indices: total iron intake, content of heme iron, and bioavailability of the nonheme iron. Models developed to estimate dietary iron supply were based on total intake, proportion of heme and nonheme iron, and content of enhancing factors, such as ascorbic acid and tissue foods (37, 38). More elaborate models will be required to incorporate the dramatic effect of certain inhibiting substances. An important future challenge will be to determine

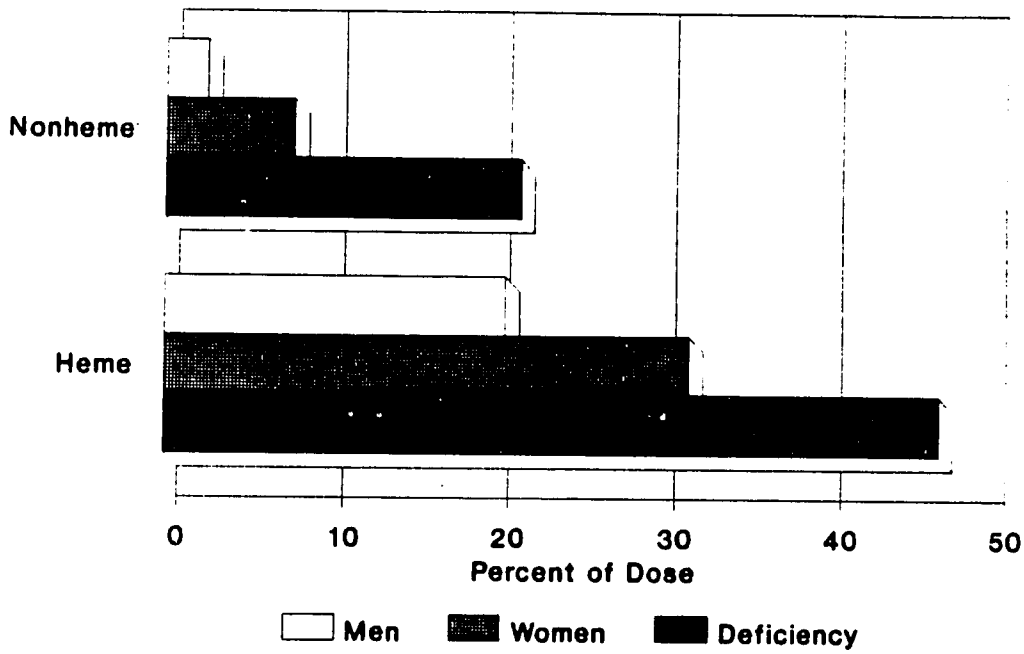

FIG 3. Variation in absorption of heme and nonheme iron from a hamburger meal at different levels of iron status. Percentage absorption from each dietary component is shown for an iron-replete man, for a premenopausal woman, and in iron deficiency with assumed serum ferritin concentrations of 100, 30, and 10 μg/L, respectively.

which, if any, of these dietary indices are determinants of iron status.

The final determinant of iron stores is adaptation or the ability of the mucosal cell to adjust absorption to varying requirements and dietary iron supply. When the recommended dietary intake is calculated, the adaptive response is usually assumed to be uniform, and variability in adaptation among individuals or populations is disregarded. The pronounced day-to-day and subject-to-subject variations have made it difficult to assess variations in adaptive response. There are two quantitative aspects of adaptation that deserve detailed consideration: adaptation to varying requirements for iron and adaptation to variations in dietary iron supply.

Adaptation to variations in iron demand

Let us assume, for this discussion, that the only important determinant of body iron is physiological demand. Because there is a remarkably close relationship between body iron status as measured by serum ferritin and adaptive response as measured by iron absorption (9, 39), we can determine the limits of adaptation from a given diet by measuring absorption in subjects with a wide range in serum ferritin concentrations. We have performed numerous measurements with a standard meal containing a hamburger, french fries, and milk shake (40, 41). The total iron content of the meal is 4.8 mg, of which 1.4 mg is in heme. The heme and nonheme iron components are labeled separately with extrinsic tags of ^{55}Fe and ^{59}Fe. There is a highly significant correlation between serum ferritin and both heme and nonheme percentage absorption. By regression analysis, absorption of heme and nonheme iron can be estimated at different concentrations of iron stores as measured by serum ferritin (Fig 3). In iron-replete men with an assumed serum ferritin of 100 μg/L, absorption of nonheme iron is 2.5% as compared with 26% from heme iron. As the serum ferritin decreases to the iron-deficient range (10 μg/L), heme absorption increase moderately to 47%, but there is a more dramatic increase in nonheme iron absorption from 2.5% to 22%. The adaptive response for nonheme iron is therefore greater than for heme.

If we assume that this hamburger meal is representative of an American diet, this data can be used to estimate iron absorption in absolute terms of mg/d (Fig 4). Because of the greater adaptive response of nonheme iron absorption to decreasing body iron stores, nonheme provides nearly twice as much iron as heme in iron deficiency. The values for iron deficiency shown in Figure 4 assume an average iron intake for men and women of 13.5 mg. With an intake of 16 mg/d, an adult male

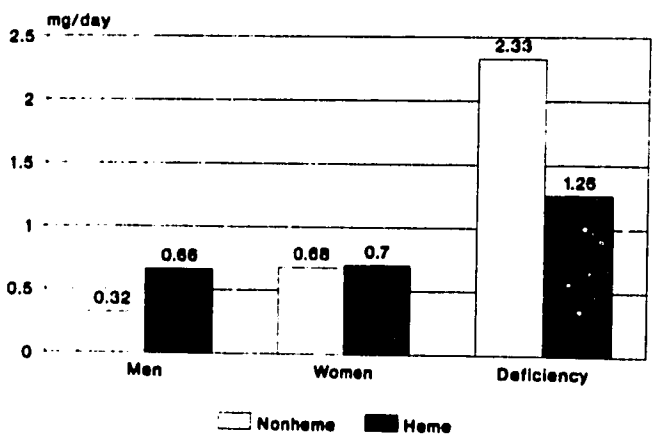

FIG 4. Variation in absolute absorption of heme and nonheme iron at different levels of iron status. Data shown in Figure 3 were converted to mg/d assuming a daily intake of 16 mg iron in men, 11 mg in women (42), and 13.5 mg in iron deficiency.

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could absorb 4.25 mg/d (2.76 mg nonheme, 1.49 mg heme) whereas a premenopausal women ingesting 11 mg/d could achieve a ceiling absorption of 2.92 mg/d (1.89 mg nonheme, 1.03 mg heme).

Studies by the South African group (43) also demonstrated that adaptation in absorption from an isotopically labeled single meal is less for heme than for nonheme iron. They varied the proportion of heme and nonheme iron in a meal containing a total of 6 mg iron and measured absorption from each compartment separately using double extrinsic tags. As the nonheme iron content of the meal was increased from 1.5 to nearly 6 mg, percentage absorption decreased from 18% to 6% and, as a result, there was little variation in the amount of nonheme iron absorbed. On the other hand, mean absorption of heme iron was ~20% from all four meals despite a range in heme iron content from 0.3 to 4.5 mg iron. These observations indicate that heme iron is important in decreasing the prevalence of nutritional iron deficiency but may contribute to an undesirable increase in body iron stores in iron-replete individuals.

It is important to note that these estimates are based on a highly favorable dietary iron supply. The hamburger meal used in our studies contains 30% heme, somewhat higher than the average American diet. The predicted ceilings of adaptive response would be dramatically lower from a diet that contained only nonheme sources of iron. Absorption of heme iron would be eliminated and because no tissue foods are present, absorption of nonheme iron would decrease by 50%. With this diet, ceiling absorption in adult men ingesting 16 mg iron/d would be only 1.73 mg/d and in iron-deficient women ingesting 11 mg, only 1.19 mg/d. These estimates indicate that most women could not meet their basal requirement of 1.5 mg iron/d from a diet that contains no animal foods unless the iron content of the diet is much higher than current averages in the United States.

These calculations also point out the importance of meat intake in determining maximal absorptive response. Indeed, meat consumption is about the only dietary index that has been shown to correlate with iron status in nutrition surveys. This was true in a Finnish epidemiologic study in which an inverse relationship between meat consumption and the prevalence of iron deficiency was demonstrated (44). It was also demonstrated in a recent study showing that iron status is more favorable in elite runners consuming a high-meat diet (45) and in premenopausal women consuming a high-meat or lactoovovegetarian diet (46). In diets containing little or no animal food there appears to be a reciprocal relationship between nonheme bioavailability and total iron intake. Thus, in the absence of heme iron, an inhibitory diet with respect to nonheme iron absorption may be offset to some extent by higher native iron content of the diet. With this type of diet, it is not clear whether nonheme bioavailability, total iron intake, or either is a significant determinant of iron status.

Adaptation to variations in dietary iron supply

It is apparent that the nature of the diet influences the prevalence of iron deficiency in segments of a population where iron demands are high. However, when iron demands do not exceed the maximal absorptive response, there is little convincing evidence that dietary iron supply influences the concentration of body iron. As long as iron demands are not excessive, it is exceedingly difficult to induce iron deficiency by dietary means. For example, the average serum ferritin in a normal adult male of 100 μg/L corresponds to 1000 mg iron stores. Even if the dietary iron supply is totally eliminated, as in a patient on chronic parenteral alimentation, it would take 3–5 y to deplete iron stores if basal iron loss is not increased. With a 50% reduction in dietary iron supply, it would take more than a decade assuming no adaptive response. In this case increased absorption would undoubtedly offset the reduction in dietary iron supply and prevent iron deficiency. It is therefore impossible to study the effect of dietary inhibitors on iron balance in iron-replete individuals although some groups still attempt to use this approach (47).

An alternative way to examine the impact of dietary supply on iron status is to attempt to induce an increase in storage iron by dietary manipulation. We have used ascorbic acid for this purpose because of its profound effect on the absorption of nonheme iron and because vitamin C is used in massive quantities by certain individuals who believe that it reduces the frequency of viral infections. Seventeen subjects were given 500 mg vitamin C with each of the two largest meals of the day for 16 wk (48). Serum ferritin measurements were obtained monthly to monitor iron status. The mean serum ferritin was 46 μg/L at the outset of the study and 44 μg/L after 4 mo of megadose vitamin C administration. Of nine participants who continued in the study for 2 y, five were iron replete (97–250 μg serum ferritin/L) and four had depleted iron stores (3–18 μg serum ferritin/L). In the iron-replete group, serum ferritin concentrations declined slightly after 2 y of megadose vitamin C. In the iron-depleted group there was a modest elevation in serum ferritin from 6 to 35 μg/L after 2 y. However, in three of the four subjects the serum ferritin remained in the iron-deficient range. The apparent increase in this group was due to changes in the only male subject, who had been a regular blood donor before entering the study. It is likely that the progressive increase in his iron stores was related to discontinuing his regular blood donations. This study demonstrates that humans possess a remarkable ability to adapt to differences in nonheme bioavailability and that it is exceedingly difficult to induce changes in iron status by modifying this dietary index.

This study raises questions about the mechanism of the adaptive response. One possibility is that the intestinal mucosal cell adapts to changes in dietary bioavailability over time and becomes less responsive to factors that promote luminal availability. However, direct measurements of absorption in subjects participating in our study failed to demonstrate adaptation to the acute enhancing effects of vitamin C. When studied before chronic megadose vitamin C ingestion, the administration of 1 g vitamin C to a meal containing semipurified dietary ingredients increased iron absorption from 2.6% to 14.4%, a mean absorption ratio of 5.5. After 4 mo of chronic vitamin C ingestion, a repeat absorption study showed an increase from 1.9% to 10.3% when the same amount vitamin C was added to the meal, a relative increase of 5.3. Whatever the nature of the adaptive response that prevents progressive accumulation of body iron in the face of enhanced bioavailability, it appears unrelated to a modification in absorptive response, at least as measured isotopically in the laboratory.

The inability of the intestinal tract to adapt to changes in luminal bioavailability also was observed recently by Brune et

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al (49). They speculated that a high bran and phytate intake may induce changes in the intestine or its microflora over a prolonged period and thereby reduce the inhibitory effect of dietary phytate on iron absorption. They compared the inhibiting effect of bran on iron absorption from wheat rolls with and without bran in a group of strict vegetarians consuming a high phytate diet and a control group. The decrease in iron absorption after the addition of bran was 92% and 93% in the two groups, respectively, indicating that no intestinal adaptation to a highly inhibitory diet occurs. Of additional interest in this study is that the mean serum ferritin was 53 μg/L in the vegetarian group, indicating a highly satisfactory concentration of storage iron in a group that included nine women.

Mechanisms of adaptation

The manner in which the body in general and the mucosal cell in particular achieve the precise regulation of body iron concentrations has perplexed students of iron metabolism for many decades. It is helpful to divide the mucosal handling of luminal iron into two separate phases of mucosal uptake and intracellular transport. There is evidence that some regulation occurs at both steps. Sophisticated kinetic studies in dogs indicated that the mucosal uptake of luminal iron is the rate-limiting step and that the fractional rate of mucosal uptake is < 1% of subsequent intracellular transport (50). Within the cell there are two major pathways by which iron is either transported directly to the circulation or stored within the cell as ferritin. When body demands for iron are high, a large fraction of the iron entering the mucosal cell is transported rapidly to the body and appears in the circulation within 1–2 min from the time that iron is introduced into the lumen. As iron stores expand, an increasing proportion of the iron taken into the mucosal cell is sequestered in mucosal ferritin and subsequently lost to the body at the end of a 2–3-d life span. Within the mucosal cell, regulation of iron absorption may therefore occur at the point at which iron entering from the lumen is partitioned between the throughput pathway to the circulation and the storage pathway within ferritin.

Ferritin synthesis has been considered by some workers to play an active role in regulating iron transport by the mucosal cell since the time that the mucosal block theory was first proposed by Granick (51). It is known that the mucosal concentration of ferritin is closely linked to the absorptive process, but it is unclear whether the protein plays an active or passive role. A recent study in human subjects showed a very close inverse relationship between the absorption of nonheme iron and the intestinal concentration of both L- and H-type ferritin in healthy subjects (40). Interestingly, the concentration of mucosal ferritin in patients with hereditary hemochromatosis is far below the concentration that would be predicted from their serum ferritin, suggesting that impairment in ferritin synthesis may be related to their inherited inability to regulate iron absorption. However, in genetically normal individuals it is more likely that the incorporation of mucosal iron into ferritin is a passive process for iron that is not transported to the circulation.

Chromatographic studies of radiolabeled mucosal homogenates demonstrated the presence of an iron-binding protein that is similar, if not identical, to circulating transferrin. Immunologic measurements of mucosal transferrin in rats showed that the concentration varies directly with both the uptake of mucosal iron and systemic transfer (52). Mucosal transferrin increases sharply in iron-deficient rats whereas there is little or no difference between normal and iron-loaded animals. Thus, over a wide range of induced changes of iron status, a high correlation was observed between iron absorption and the ratio of mucosal transferrin to ferritin.

Identification of mucosal transferrin in small laboratory animals prompted the suggestion that a shuttle mechanism may exist for transporting iron from the lumen to the mucosal cell (53). From measurements of absorption of diferric transferrin in rats, Huebers et al (53) proposed that the absorption of transferrin iron may account for as much as one-third of the iron required to maintain iron balance in normal animals. They hypothesized that apotransferrin is loaded with iron in the intestinal lumen and that the transferrin complex is then taken into the mucosal cell. After releasing its iron, apotransferrin is returned to the brush border for recycling.

The shuttle hypothesis for transferrin-mediated luminal uptake of iron has not been supported by the observations of other workers. There is no evidence for transferrin mRNA in mucosal epithelial cells from the small intestine of either normal or iron-deficient rats (54). Efforts to demonstrate the presence of transferrin receptors on the brush border of mucosal epithelial cells were unsuccessful, although receptors were detected in the basal and lateral portions of the membrane (55). Evidence was presented that mucosal transferrin may be due, at least in part, to plasma contamination (56). In human studies the administration of diferric transferrin labeled with radioiron to achlorhydric subjects failed to promote iron absorption (57). In recent studies we (40) were unable to identify any differences in the concentration of mucosal transferrin and mucosal homogenates in healthy subjects over a wide range of iron absorption and serum ferritin concentrations whereas the concentration of mucosal ferritin bears a striking inverse relationship with absorptive response.

Although there has been some progress in understanding the pathways of iron transport within the mucosal cell, there has been little progress in determining the mechanisms by which the needs of the body are communicated to the mucosal cell. There is no question that the most important factor governing mucosal response is body iron status, but the regulatory signal has never been identified. Manipulations of serum iron, iron-binding capacity, transferrin saturation, and serum ferritin have all failed to demonstrate a significant effect. Crosby and Conrad (58) proposed several years ago that the mucosal cell is conditioned in the crypts of the intestinal villus by the amount of iron supplied by the plasma. This internal iron loading of the cell then in some way determines its subsequent behavior as the cell moves towards the outer portion of the villus. Even more complex kinetic theories have been proposed, but none of these have been substantiated by experiment. In animal studies the only other determinant apart from body iron status that is known to influence iron assimilation is the rate of erythropoiesis (4). However, adaptive mechanisms for controlling the concentration of body iron override this erythropoietic stimulus in hereditary hemolytic disorders as long as maturation of the red cell is normal. Thus, in compensated hereditary hemolytic anemias such as hereditary spherocytosis, body iron stores usually remain within the normal range despite a lifelong

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increase in red cell production, whereas if maturation of the red cell is disordered as in patients with sideroblastic anemia or thalassemia major, excessive absorption of dietary iron continues despite massive body stores. Despite continued investigation the mechanism by which the needs of the body for iron are conveyed to the mucosal cell is still an enigma.

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Serum Transferrin Receptor: A Quantitative Measure of Tissue Iron Deficiency

By Barry S. Skikne, Carol H. Flowers, and James D. Cook

This study was undertaken to evaluate the role of serum transferrin receptor measurements in the assessment of iron status. Repeated phlebotomies were performed in 14 normal volunteer subjects to obtain varying degrees of iron deficiency. Serial measurements of serum iron, total iron-binding capacity, mean cell volume (MCV), free erythrocyte protoporphyrin (FEP), red cell mean index, serum ferritin, and serum transferrin receptor were performed throughout the phlebotomy program. There was no change in receptor levels during the phase of storage iron depletion. When the serum ferritin level reached subnormal values there was an increase in serum receptor levels, which continued throughout the phlebotomy program. Functional iron deficiency was defined as a reduction in body iron beyond the point of depleted iron stores. The serum receptor level was a more sensitive and reliable guide to the degree of functional iron deficiency than either the FEP or MCV. Our studies indicate that the serum receptor measurement is of particular value in identifying mild iron deficiency of recent onset. The iron status of a population can be fully assessed by using serum ferritin as a measure of iron stores, serum receptor as a measure of mild tissue iron deficiency, and hemoglobin concentration as a measure of advanced iron deficiency.

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RELIABLE LABORATORY measurements of iron status are important clinically for detecting iron deficiency and assessing its magnitude. These measurements are also important in epidemiologic surveys to determine the prevalence of iron deficiency and evaluate the efficacy of intervention programs to alleviate it. For both purposes, the most important laboratory parameters are the serum ferritin, which reflects residual iron stores, and the hemoglobin concentration, which measures larger deficits in the functional iron compartment. The greatest difficulty is in assessing mild iron deficiency in individuals with absent iron stores but who have not yet developed overt anemia. This stage of iron deficiency is referred to as iron-deficient erythropoiesis[1] or iron deficiency without anemia.[2] A large array of laboratory measurements have been used to identify this stage, including serum iron, total iron-binding capacity (TIBC), transferrin saturation, free erythrocyte protoporphyrin (FEP), mean red cell volume (MCV), and red cell mean index (RCMI). While each of these measurements have merit, no single determination gives a reliable index of mild iron deficiency in the absence of anemia.

Measurements of circulating transferrin receptor have recently been reported to provide a reliable index of iron deficiency anemia.[3-5] We have developed an enzyme-linked immunoassay (ELISA) for the measurement of serum transferrin receptor using monoclonal antibodies (MoAbs) developed specifically for this purpose.[6] To evaluate the usefulness of this laboratory parameter in the assessment of iron status, serial phlebotomies were performed in normal volunteer subjects. The induced changes in serum receptor levels were compared with established measurements of iron status during the gradual evolution of iron deficiency.

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Submitted August 24, 1989; accepted January 9, 1990.

Supported in part by National Institutes of Health Grant DK-39246, and by AID Cooperative Agreement DAN-5115-A-00-7908-00.

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0006-4971/90/7509-0004$3.00/0

MATERIALS AND METHODS

Volunteer subjects. Serial phlebotomies were performed in 6 male and 8 female normal volunteers ranging in age from 24 to 46 years. The subjects were selected to represent a wide range in body iron status. Several male volunteers had low ferritin levels because of frequent prior blood donations. The diet of the subjects was not altered during the study, but ingestion of iron supplements or vitamins containing iron was not allowed. All subjects gave written informed consent after the nature of the study was fully explained to them. The investigation was approved by the Human Subjects Committee of the University of Kansas Medical Center under the condition that phlebotomies would be discontinued when the initial hemoglobin level had fallen by 2 g from baseline.

Phlebotomy procedure. Twelve-milliliter blood samples were drawn weekly for 3 weeks to obtain reliable baseline values for laboratory measurements before phlebotomy. Because of the uncertain effect of the rate of phlebotomy on induced changes in laboratory values, the subjects were divided into two groups. In the first group, 250 mL of blood was removed once a week, while in the remaining subjects 75 mL of blood was withdrawn twice weekly. Because the changes in laboratory values were similar with the two phlebotomy rates, the subjects were considered as a single group for analysis. Phlebotomies were performed in both groups until the hemoglobin level in each subject had fallen by 2 g from their baseline mean and remained so for at least 3 weeks. Additional phlebotomies were performed if the hemoglobin concentration rose during this 3-week period.

The following hematologic and biochemical measurements were performed on blood removed at each phlebotomy. A complete blood count was performed by an automated cell counter (Ortho Diagnostic Systems, ELT.8/WS, Westwood, MA), which provided measurements of the hemoglobin, hematocrit, MCV, and RCMI, which is equivalent to the red cell distribution width. Reticulocyte counts were performed by a standard chamber method,[7] the serum iron and TIBC were determined by methods recommended by the International Committee for Standardization in Hematology,[8,9] zinc protoporphyrin was measured with a hematofluorometer (Aviv Associates, Bedford, MA), and serum ferritin and serum transferrin receptor were measured with ELISAs using monoclonal reagents.[6,10,11]

Estimates of iron removed. The amount of iron removed with each phlebotomy was calculated from the volume of blood taken and the circulating hemoglobin concentration.[12] Blood removed for laboratory determinations was included in the calculation. Because phlebotomies were performed over several months in some individu-

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als, correction was made for the amount of iron absorbed from the diet. We assumed an absorption of 3 mg iron daily in men and 2 mg iron daily in women once phlebotomies were instituted.[13,14]

Calculation of iron stores. Iron status was defined in relation to the amount of iron contained in the storage compartment.[15] With this convention, iron stores are expressed as a positive value when there is residual storage iron. Negative values denote functional iron deficiency and represent the additional amount of iron removed after stores are depleted, or the amount of iron that must be returned to the body for stores to reaccumulate. Iron status was expressed as milligram per kilogram body weight unless stated otherwise.

Two methods were used to calculate the amount of iron contained in the storage compartment at the outset of the phlebotomy program. The first, termed the hemoglobin method, has been used in many previous studies.[12] The calculation is based on the amount of iron removed during phlebotomy corrected for the decrease in circulating hemoglobin iron and for absorbed dietary iron during the phlebotomy period. In estimating the decrease in circulating hemoglobin iron, total blood volume was estimated as 61.5 mg/kg in men and 58.9 mg/kg in women.[16] One gram hemoglobin was assumed to contain 3.38 mg of iron. The decrement in hemoglobin was calculated as the difference between the mean hemoglobin concentration observed over 3-week intervals preceding and following the phlebotomy program.

The second calculation, termed the ferritin method, assumes that a decrease in serum ferritin level below 12 μg/L represents the point of total depletion of iron stores.[17,18] Iron stores are calculated as the amount of iron removed by phlebotomy at the time when the serum ferritin first falls below 12 μg/L minus the estimated amount of absorbed dietary iron. Although the serum ferritin declined in all subjects, the level did not fall below 12 μg/L in three subjects at the time when a sustained reduction in hemoglobin level greater than 2 g/dL had occurred. Storage iron depletion was defined in these subjects as a persistent decrease in hemoglobin concentration rather than on the basis of a subnormal serum ferritin.

Statistical evaluation. The phlebotomy protocol generated multiple samples in each subject at each 1 mg/kg decrement in body iron, the average ranging from 1.8 to 3.2. Because all samples were obtained at intervals of at least 3 days, they were treated independently when portraying the results graphically. Analysis of variance was used to compare the changes between baseline, complete storage iron depletion, and development of anemia. Only one value in each subject at each of these points was used for this purpose. Scheffe's test was used to determine the significance of the differences.[19]

RESULTS

The baseline hemoglobin concentration was within the normal range in all subjects (Table 1). The average decrease in hemoglobin concentration at the time that phlebotomies were terminated was 2.3 g/dL. There was a wide range in the initial serum ferritin concentration among the 14 volunteer subjects, and by design, the serum ferritin level was in the low normal range in more than half of the subjects. The geometric mean serum ferritin was 33 μg/L in the 6 male subjects as compared with 48 μg/L in the 8 female volunteers. In the three subjects with the highest baseline serum ferritin (150, 150, and 238 μg/L), the level did not fall below 12 μg/L at the time that anemia had developed. The duration of the phlebotomy program averaged 13 weeks with a range from 6 to 22 weeks, longer periods being required in the subjects with the higher initial serum ferritin levels.

The amount of iron removed during the phlebotomy program ranged from 218 to 1,562 mg, with an average of 792 mg (Table 1). Basal iron stores were somewhat lower when calculated with the hemoglobin method (mean 333 mg) than with the ferritin method (mean 453 mg), and the hemoglobin method was less reliable. Based on the latter method, initial iron stores were less than 100 mg in four subjects, although their baseline serum ferritin was within the normal range. In subject no. 8, the calculated decrease in hemoglobin iron was greater than the iron removed by phlebotomy. Therefore, the ferritin method was used in subsequent calculations. The size of prephlebotomy iron stores was similar when predicted from the baseline serum ferritin and that measured by the ferritin method, when the former was estimated using a logarithmic transformation.[20] Iron stores predicted from the initial ferritin averaged 489 mg/L as compared with a phlebotomy value by the ferritin

Table 1. Measurements of Baseline Hematologic Indices, Iron Stores, and Iron Removal in Volunteer Subjects

Subject No.	Sex, Age (yr)	Body Weight (kg)	Phlebotomy Rate (mL/wk)	Hemoglobin (g/L)		Ferritin (μg/L)		Phlebotomy Duration (wk)	Iron Removed (mg)	Iron Stores (mg)		Functional Deficit (mg)
				Initial	Final	Initial	Final			Hemoglobin Method	Ferritin Method	
1	M24	74	250	14.5	12.2	15	6.8	7	483	210	202	281
2	F26	75	250	13.2	10.5	15	1.7	10	620	210	172	448
3	F46	78	150	13.8	11.1	18	7.3	13	566	12	107	459
4	F31	77	250	13.0	10.3	19	5.3	6	336	4	106	230
5	M38	84	250	14.0	11.7	20	5.8	7	443	66	179	264
6	M34	85	150	16.1	13.8	24	6.6	18	1,084	451	337	747
7	M34	95	250	14.8	12.6	26	11	8	613	186	494	119
8	F31	68	150	13.4	11.3	27	11	6	218	−90	174	44
9	M27	93	150	16.1	13.6	44	6.5	19	928	188	335	593
10	F31	85	150	14.6	12.2	70	12	20	1,028	482	646	382
11	F34	66	150	14.7	12.6	81	4.4	15	828	474	408	420
12	F26	63	250	14.6	12.1	150	27	12	885	605	834	51
13	M34	102	150	16.1	14.4	150	19	22	1,489	805	1,102	467
14	F38	111	250	14.2	11.9	238	33	19	1,562	1,054	1,324	238
Mean				14.5	12.2	41*	8.6*	13	792	333	453	339
±2 SD				2.1	2.3	6–271	2–40	11	806	658	750	404

*Geometric mean.

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method of 453 mg/L. An excellent correspondence was observed between the two values when compared on the basis of body weight ($r = .904$, $P < .0001$) (Fig 1). Based on the ferritin method, the induced deficit in functional iron averaged 339 mg, with a range from 44 to 747 mg (Table 1). The final deficit in functional iron was independent of iron stores originally present in each subject.

To examine the changes in the various iron parameters during repeated phlebotomy, mean values were plotted against the level of iron stores over the range of 8 mg/kg to −5 mg/kg. It should be noted that because some of the subjects had low normal ferritins at the outset of the investigation, more observations were available for estimating lower iron stores than higher iron stores. The results for the iron transport parameters are shown in Fig 2. There was relatively little change in the serum iron level during storage depletion, but once stores were exhausted, there was a relatively abrupt decline to below 50 μg/L. The level was further reduced with an increasing functional iron deficit. On the other hand, the TIBC increassd with storage depletion from a baseline mean of 300 μg/dL to approximately 400 μg/dL when stores were absent. This finding is in keeping with the inverse correlation between serum ferritin and TIBC in iron-replete normal subjects reported previously.[21] However, the mean TIBC rose above the normal level only after functional iron deficiency had developed. The changes in transferrin saturation were similar to the serum iron; the mean value fell below the lower cut-off level of 16% only when stores were fully exhausted, and the level continued to fall with increasing functional iron deficiency.

There was surprisingly little effect of phlebotomy on measurements relating to the red cell compartment (Fig 3).

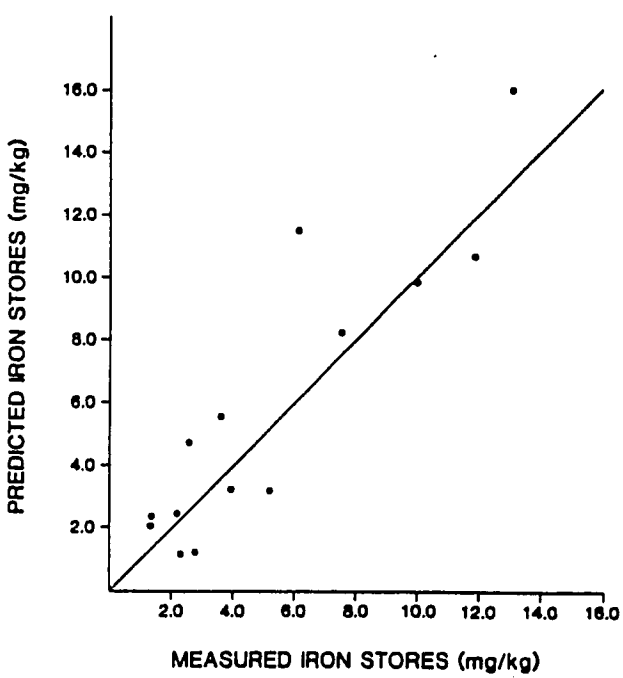

Fig 1. Relationship between iron stores measured by phlebotomy and predicted from the initial serum ferritin value. The solid line represents the line of identity.

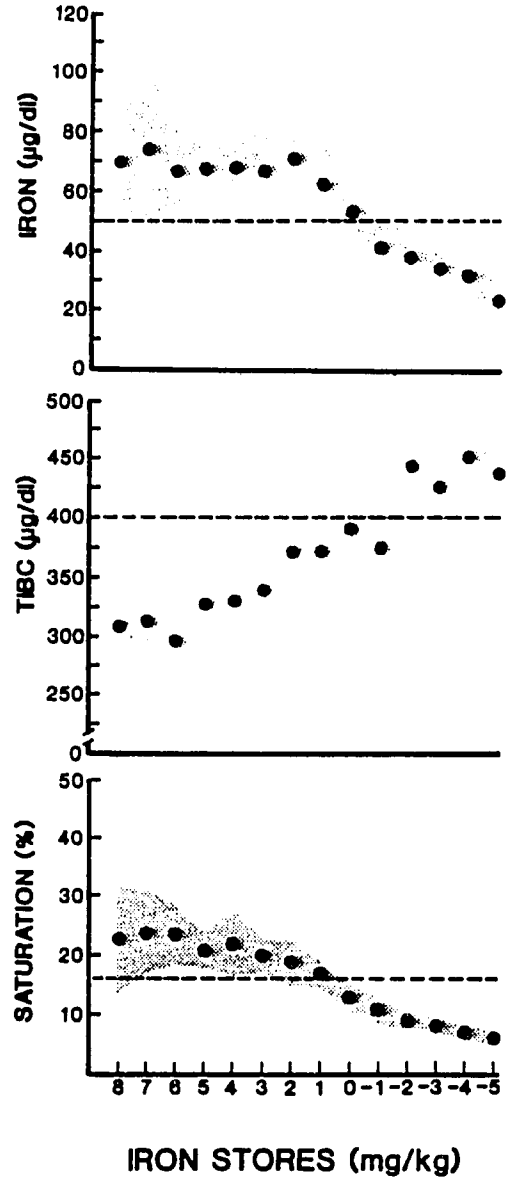

Fig 2. Changes in serum iron, TIBC, and transferrin saturation during phlebotomy. The horizontal dashed lines indicate the limit of the normal range. The shaded areas represent ±2 SE.

A significant elevation in FEP occurred only during the later stage of the phlebotomy program. Similarly, the MCV fell progressively, but remained within the normal range above 80 fl. There was a slight upward trend in RCMI values, but the mean levels remained within the normal range.

Enhanced erythropoiesis and iron deficiency have both been reported to elevate the serum transferrin receptor level.[4,6] It was not known before this study whether the increase in erythropoiesis resulting from phlebotomy would influence the value of the serum receptor as an index of iron deficiency. However, serial determinations in subjects with varying baseline iron reserves indicated that the main determinant was iron deficiency. An example of this is illustrated in Fig 4. In the two subjects with initial iron stores below 500 mg, the receptor level increased sooner in the subject with the lower initial store. In a third subject with an initial store of

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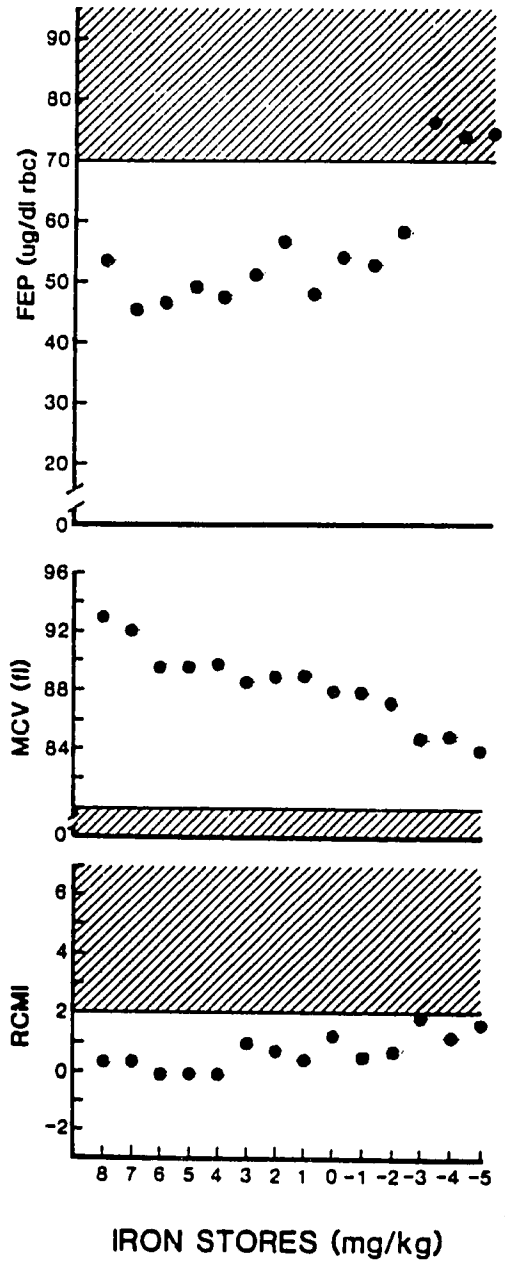

Fig 3. Changes in RCMI, MCV, and FEP during phlebotomy. The cross-hatched areas represent values outside of the normal range. The shaded areas represent ±2 SE.

1,102 mg, there was very little change in serum receptor during the removal of 1,489 mg iron over a 4-month period. Further evidence that the serum transferrin receptor levels were minimally influenced by enhanced erythropoiesis due to phlebotomy was obtained by monitoring the absolute reticulocyte counts (reticulocyte count × red blood cell count). The mean value at baseline of 85,600/mm^3 increased to 113,700/mm^3 when stores were fully depleted, but subsequently fell to 89,400/mm^3 at the end of the study. In contrast, the serum receptor levels did not increase significantly until functional iron deficiency had developed. These findings indicate that at the rate of phlebotomy performed in this study, there is little or no influence of increased erythropoiesis on the serum receptor level.

Serum ferritin and receptor levels during phlebotomy are shown in Fig 5. As anticipated, the serum ferritin changed dramatically during the period of storage depletion. The wide range in serum ferritin levels at higher iron stores reflects the fewer number of observations. There was relatively little change in the serum ferritin after stores were fully depleted. The alteration in serum receptor levels was entirely different in that there was little or no change during storage depletion. As iron stores became fully exhausted, there was a progressive increase in serum transferrin receptor levels with increasing deficiency.

These results indicate that the serum ferritin is the most sensitive index of iron status when there are residual iron stores, whereas the serum receptor is more sensitive when there is functional iron deficiency. Because of the reciprocal relationship between serum receptor and ferritin measurements, the ratio of receptor:ferritin portrays iron status over the entire range examined in this study (Fig 6). When plotted logarithmically, this ratio increased from less than 100 in those with ample iron stores to over 2,000 in those with significant functional iron deficiency. A rise above 500 occurred when stores were fully depleted.

The measurements obtained when iron stores were depleted and when anemia developed were compared with the baseline values (Table 2). When stores were depleted, the hemoglobin, serum iron, transferrin saturation, and serum ferritin were all significantly changed, whereas the TIBC, serum receptor, FEP, RCMI, and MCV were not. When anemia developed, all laboratory measurements differed significantly from baseline with the exception of the FEP. The hemoglobin concentration, serum iron, transferrin saturation, serum ferritin, and serum receptor showed the most significant changes ($F > 24.0$), while the changes in red cell measurements were much less significant. Between storage depletion and the development of anemia, the only significant change occurred in serum receptor levels, which increased from 6.2 mg/L to 8.77 mg/L ($P < .001$).

One objective of this study was to determine whether the serum transferrin receptor is of value in quantifying mild deficits in functional iron. The ratio of serum receptor:

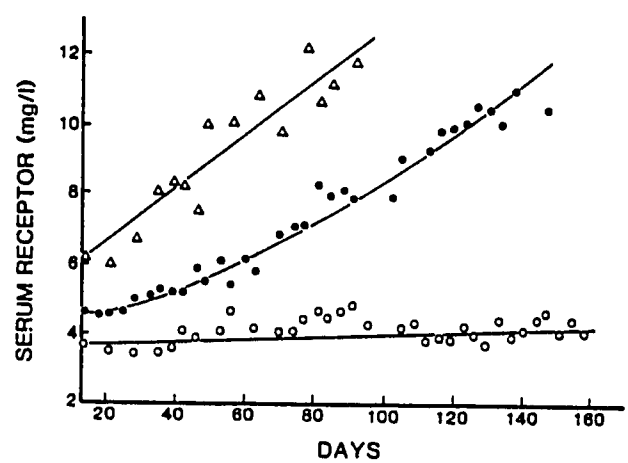

Fig 4. Serial determinations of serum transferrin receptor during phlebotomy in three subjects with initial iron stores of 107 mg (△), 335 mg (●), and 1,102 mg (○).

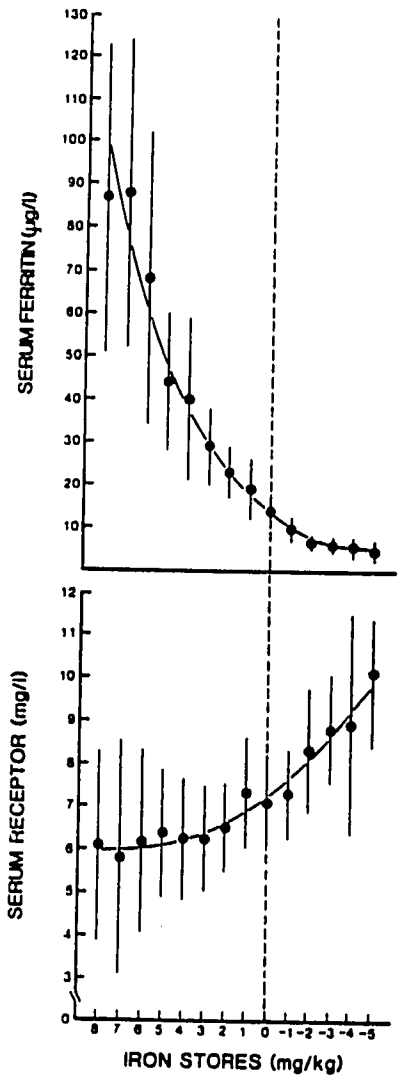

Fig 5. Serum ferritin levels and serum transferrin receptor levels during phlebotomy. The point of depleted iron stores is noted by the vertical dashed line. The vertical bars represent ±2 SE.

ferritin was compared with a previous algorithm based on combinations of the transferrin saturation, serum ferritin, and FEP.[20] In nine subjects with a functional deficit between 50 and 150 mg of iron, mean values of 126 and 121 mg iron were obtained with the ratio and algorithm method, respectively. In eight subjects with a deficit between 150 and 250 mg, the mean values were 215 and 235 mg, respectively. However, the variability was somewhat higher with the ratio method. For example, with functional deficits of 150 to 250 mg iron, the range for the algorithm method was 160 to 240 mg as compared with 95 to 380 mg for the ratio estimate. The variance of 6,646 for the ratio method was also significantly higher than 802 for the algorithm method ($F = 8.29$, $P < .01$).

DISCUSSION

For clinical purposes, the major emphasis in laboratory measurements of iron status is in detecting iron deficiency rather than in estimating its severity. Serum ferritin is particularly valuable in anemic patients because a level below 12 µg/L is diagnostic. The MCV and FEP are also useful, but these measurements are also influenced by inflammation and neither measurement is useful for detecting iron deficiency of recent onset. In general, the identification of iron deficiency in an anemic patient poses relatively few problems, at least in the absence of other diseases that influence iron parameters, such as chronic infection, inflammation, or liver disease.

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 the prevalence of impaired iron status or iron deficiency anemia.[22] Another recent approach is to use algorithms to estimate body iron quantitatively in each sampled individual.[20] 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 anemia, the deficit in body iron can be estimated quantitatively from the decrease in circulating hemoglobin concentration. Between these limits, estimating mild deficits in functional iron is more difficult. In the previous report,[20] 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 anemia.

Because of recent reports that serum receptor levels are

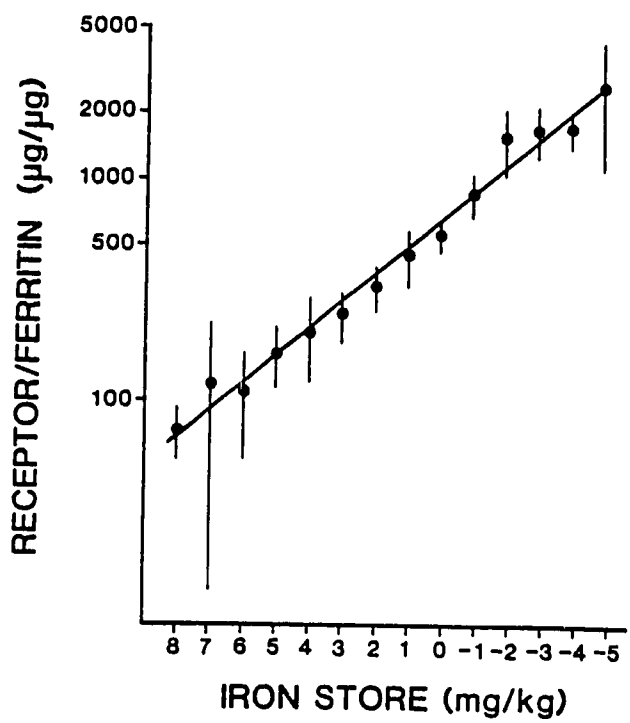

Fig 6. Ratio of serum receptor:ferritin during phlebotomy. The vertical bars represent ±2 SE.

Table 2. Laboratory Measurements Performed Initially and on Developing Storage Iron Depletion and Iron-Deficiency Anemia

Laboratory Measurements	Basal A (N = 14)	Depletion B (N = 11)	Anemia C (N = 14)	F Value	P Value (A:B)	P Value (B:C)
Hemoglobin (g/dL)	14.5 (±2.1)	12.7 (±1.8)	12.0 (±2.2)	32.8	<.001	NS
Serum iron (μg/dL)	78 (±54)	45 (±30)	32 (±22)	25.1	<.001	NS
TIBC (μg/dL)	347 (±117)	382 (±118)	413 (±139)	6.6	NS	NS
Saturation (%)	23 (±18)	11 (±6)	8 (±7)	25.5	<.001	NS
FEP (μg/dL RBC)	59 (±16)	52 (±11)	64 (±18)	NS	NS	NS
MCV (fl)	89 (±7)	88 (±7)	86 (±6)	3.9	NS	NS
RCMI	0.4 (±1.4)	0.9 (±1.2)	1.3 (±2.6)	7.6	NS	NS
Serum ferritin* (μg/L)	40 (16–103)	11 (10–12)	9 (4–18)	24.4	<.001	NS
Serum receptor (mg/L)	5.34 (±2.18)	6.20 (±2.5)	8.77 (±4.54)	30.0	NS	<.001

Abbreviations: RBC, red blood cells; NS, not significant.

*Geometric mean ± 1 SD.

elevated in iron deficiency, we undertook this investigation to examine the potential role of this new assay in quantifying mild degrees of iron deficiency. Cell culture studies indicate that the synthesis of transferrin receptor in erythroid cell lines is closely tied to the iron requirements of the cell.[23,24] Assuming that circulating receptor levels reflect the total body mass of receptors, it is not surprising that elevations occur both in disorders where there is a marked expansion of red cell mass, such as autoimmune hemolytic anemia and sickle cell anemia, and in iron deficiency. We recently reported our initial experience with serum receptor measurements using an ELISA with MoAbs developed against soluble receptor.[6] The mean level in normal subjects was 5.6 mg/L, with a range of 2.8 to 8.5 mg/L (±2 SD). The mean levels in patients with sickle cell anemia and iron deficiency anemia were 33 and 18 mg/L, respectively. In both groups, the receptor level was invariably above the upper normal limit of 8.5 mg/L. These changes are similar to data recently obtained in the rat.[25]

Our present findings indicate that the serum receptor is a reliable index of early tissue iron deficiency. Although serum receptor levels increased significantly during storage depletion, the mean values remained well within the normal range. Our findings also indicate that in normal subjects undergoing graded phlebotomy, tissue iron needs are a much more important determinant of the serum receptor level than induced increases in erythropoiesis (Fig 3). The most important finding is after depletion of iron stores, when increasing deficits in functional iron lead to an increase in serum receptor. With other iron parameters, some changes were observed as the deficit increased from 0 to −5 mg/kg, but none were as brisk or consistent as the serum receptor. Within this range, the transferrin saturation fell from 11% to approximately 8%, and the serum ferritin declined from 11 to approximately 6 μg/L. These changes justify the prior use of these measurements for estimating mild iron deficiency.[20] The FEP and MCV were presumably less useful in the study because sufficient time had not elapsed to replace normal circulating red blood cells with iron-deficient erythrocytes at the time that phlebotomies were terminated. Of all the measurements examined, the serum receptor was the most reliable quantitative guide to a deficiency in the functional iron compartment.

There is some advantage in using a single parameter to estimate body iron in population studies. Because the serum ferritin changed dramatically during storage iron depletion and the serum receptor changed maximally with increasing functional iron deficiency, the ratio of receptor:ferritin appears promising (Fig 6). However, no major advantage in using this ratio was noted. Residual iron stores can be estimated from the serum ferritin when the level is within the normal range, and when the ferritin falls below 12 μg/L, the deficit in tissue iron can be estimated from the serum receptor. The previous algorithm required measurement of the serum iron, TIBC, FEP, and serum ferritin. These laboratory techniques require venous sampling and are cumbersome and costly to perform. One major advantage of using only the serum receptor and ferritin is that both assays can be performed on a few microliters of sample, and therefore are suitable for fingerstick specimens. The technology required for these measurements is identical except for the immunologic reagents.

It will be important to gain further experience with serum ferritin, receptor, and hemoglobin measurements in population surveys. The serum receptor offers a particular advantage in assessing iron status in segments of the population where the prevalence of iron deficiency is high, as in infants, children, and women during pregnancy. In these populations, the serum ferritin is usually close to the iron-deficient range and does not accurately portray differences in functional iron. The serum receptor is more sensitive in detecting iron deficiency in these situations.

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Gastric delivery system for iron supplementation

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A gastric delivery system (GDS) for iron supplementation was evaluated. Radioisotopic studies in 9 volunteers demonstrated a three-fold higher absorption of GDS iron compared with ferrous sulphate elixir. A double-blind placebo controlled trial was done in 200 women to compare the gastrointestinal side-effects associated with 50 mg iron daily given either as GDS or conventional ferrous sulphate. The conventional preparation was associated with a significantly higher frequency of nausea and anorexia, whereas there were no significant differences in reported side-effects between subjects receiving GDS or placebo. A single GDS capsule daily provides the same amount of absorbed iron as conventional ferrous sulphate given three times daily, and does not produce gastrointestinal side-effects.

Lancet 1990; **335**: 1136–39.

Introduction

Iron deficiency continues to be the most prevalent nutrient deficiency in the world.[1,2] Because food fortification is impractical for many developing countries and is not fully effective when iron deficiency is severe, iron supplementation is the preferred intervention in pregnant women. Iron tablets are usually effective in pilot studies but sustained efficacy in applied programmes is uncommon. This may be due to an inadequate distribution system or poor absorption of the iron supplement but the major difficulty is poor compliance related to gastrointestinal side-effects.[3] A variety of preparations are available that delay the solubilisation of iron in the gastrointestinal tract. However, absorption is usually lower[4] because iron release is delayed until the preparation is beyond the area of maximum intestinal uptake. An alternative method of sustaining delivery is to alter the hydrodynamic properties of the delivery system to retain the drug in the stomach.[5] This approach is particularly suited to substances that are more soluble in acid. Iron is one such compound and we have therefore incorporated ferrous sulphate into a gastric delivery system (GDS).

Subjects and methods

Subjects

All subjects gave written, informed consent. The investigation was approved by the Human Subjects Committee at the Kansas University Medical Center.

Absorption studies

Four separate absorption tests were done with ^{55}Fe and ^{59}Fe in each of 9 men. A small pilot study was done in 3 subjects to compare the effect of altering the dissolution rate of GDS. In the other 6 subjects, absorption from varying doses of iron was evaluated. All the men were in good health and denied having had recent infections or gastrointestinal disorders that are known to influence iron assimilation.

GDS contains hydroxypropyl methylcellulose, hydrogenated vegetable oil, crospovidone, microcrystalline cellulose, xanthan gum, talcum powder, magnesium stearate, and colloidal silicone dioxide (P. R. Sheth, J. Tossounian, US patent 4126 672) to which ferrous sulphate is added. When a capsule containing the ingredients is placed in aqueous solution, the gelatin coating dissolves but the matrix remains intact. Buoyancy is conferred by the hydrocolloids which form a hydrated boundary layer that impedes the entry of water into the core. By altering the composition the rate of dissolution can be delayed for several hours. Since the stomach never fully empties, the matrix floats there until final disintegration.

Iron was given as ferrous sulphate either in an elixir or GDS. In the first 3 subjects GDS containing 50 mg elemental iron was prepared in slow, intermediate, and fast release forms; absorption was compared with that from 50 mg ferrous sulphate elixir. In the other 6 men, 10 and 25 mg GDS were compared with equivalent elixir. These preparations were trace-labelled with ^{55}Fe or ^{59}Fe sulphate. The total dose of administered radioactivity for each test was 37 kBq ^{59}Fe or 93 kBq ^{55}Fe. For the first pair of tests, one preparation labelled with either ^{55}Fe or ^{59}Fe was given on days 1 and 3, and the other preparation tagged with the alternative label was given on days 2 and 4. Blood was collected on day 18 to measure the radioactivity incorporated into circulating red cells. Two additional tests with preparations tagged separately with ^{55}Fe or ^{59}Fe, were done on days 19–22. A blood sample was collected on day 36 to measure increase in red cell radioactivity from the second pair of tests.

All test doses were given between 0700 h and 0900 h after a 10-h fast. No intake of food or drink was allowed for the following 3 h. All iron preparations were taken with a breakfast[6] of egg (79 g), muffin (59 g), 40% bran flakes (28 g), sugar (5 g), 2% low-fat milk (227 g), and coffee (170 g). The meal contained 2470 kJ (590 kcal) and 4·7 mg non-haem iron. The radiolabelled elixir or GDS was ingested midway through the meal.

Radioactivity was measured in duplicate 10 ml samples of whole blood with a modification of the method of Eakins and Brown.[7] Sufficient counts were obtained to reduce the counting error to within 2% in subjects who absorbed more than 1% of the test dose. Percentage absorption was calculated assuming that 80% of the absorbed radioactivity was incorporated into circulating red cells 14 days after administration. Total blood volume was estimated from the height and weight of the subject.[8] Serum ferritin was also measured.[9]

Because of the skewed distribution of iron absorption data, the mean and standard deviations were expressed as logarithms and the results retransformed to recover the original units of percentage absorption.[10] Statistical comparisons of any two absorption tests were based on *t* tests to establish whether the mean logarithm of absorption ratios differed from zero.

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TABLE I—EFFECT OF RATE OF DISSOLUTION OF GDS ON IRON ABSORPTION

Subject	Age (yr)	Packed cell volume (%)	Serum ferritin (μg/l)	Iron absorption (% of dose)*				Absorption ratio		
				Slow A	Intermediate B	Fast C	Elixir D	A:D	B:D	C:D
1	24	42	40	1·41	1·35	2·67	1·06	1·33	1·27	2·51
2	24	42	47	0·52	0·40	1·03	0·23	2·26	1·73	4·47
3	26	44	78	4·12	3·33	3·58	0·93	4·43	3·58	3·84
Mean (−1SE, +1SE)	25	43	53	1·44 (0·79, 2·62)	1·21 (0·65, 2·24)	2·14 (1·47, 3·12)	0·60 (0·37, 0·99)	2·37† (1·67, 3·36)	1·99† (1·47, 2·71)	3·51‡ (2·96, 4·18)

*GDS preparations provided slow, intermediate, and fast release. All means are geometric except for age and packed cell volume.
†$p<0·02$, ‡$p<0·001$

TABLE II—ABSORPTION OF GDS IRON AT DIFFERENT DOSES

Subject	Age (yr)	Packed cell volume (%)	Serum ferritin (μg/l)	Iron absorption (% of dose)*				Absorption ratio	
				10 mg		25 mg			
				GDS A	Elixir B	GDS C	Elixir D	A:B	C:D
4	29	45	25	1·03	0·46	1·42	0·58	2·23	2·44
5	23	44	42	0·67	0·37	1·10	0·33	1·81	3·33
6	27	46	44	2·97	0·52	1·13	0·36	5·71	3·13
7	23	46	45	4·36	0·56	2·66	0·67	7·78	3·97
8	32	47	143	1·77	0·51	1·80	1·26	3·47	1·42
9	27	44	168	1·36	0·53	3·22	0·65	2·56	4·95
Mean (−1SE, +1SE)	27	45	60	1·66 (1·25, 2·20)	0·48 (0·45, 0·51)	1·73 (1·44, 2·08)	0·57 (0·47, 0·70)	3·42† (2·71, 4·31)	2·99† (2·51, 3·57)

*$p<0·005$, †$p<0·001$

Evaluation of side-effects

A double-blind, placebo-controlled study was done to compare the gastrointestinal side-effects of GDS iron and conventional ferrous sulphate. Three identical capsules were prepared that could be identified by code number only. One capsule contained 50 mg GDS iron as ferrous sulphate, one contained GDS ingredients without iron (placebo), and one contained 50 mg iron as ferrous sulphate. The code was kown only to the manufacturer of the capsules until the trial was concluded. 200 volunteer women aged between 18 and 48 years were assigned between the three groups with a table of random numbers. 67 subjects were assigned to the GDS group, 67 to the conventional iron group, and 66 to the placebo group.

The women were instructed to take one capsule every morning on arising and to postpone eating for at least an hour. At the end of the first 7 days of the trial, the participants completed a questionnaire that listed symptoms. Several unrelated symptoms were included to reduce the emphasis on gastrointestinal symptoms. The women were first asked about the presence of the symptom. If the reply was positive, they were asked to rate severity increasing on a scale of 1 to 5 and to indicate the number of days that the symptom had been noted. All subjects were interviewed briefly after completing the questionnaire by a single interviewer to provide any clarification. The women were then instructed to continue taking one capsule daily for a further 7 days after which an identical questionnaire was administered. No subject withdrew because of side-effects.

The results were analysed by χ^2 tests. Results at the end of the first and second week were analysed separately. The data were analysed at two levels of severity and duration of symptoms. Level I was defined as a symptom of any severity present for 2 or more days. Level II was defined as a severity of 3 or greater present on at least 3 days. Bonferroni's correction was used to adjust for multiple comparisons.

Results

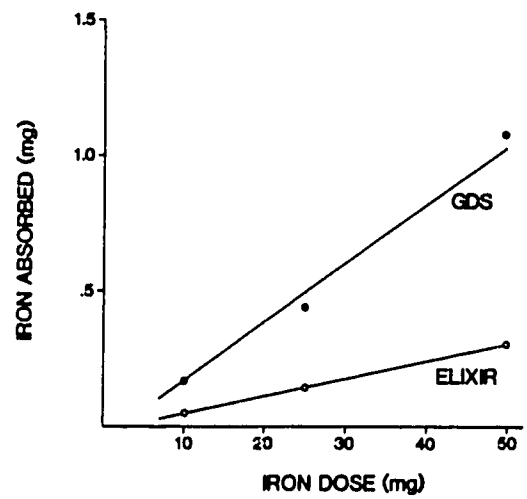
Absorption studies

In the 3 men given GDS formulations with different rates of dissolution, there was no obvious difference between the formulations (table I). However, absorption of GDS iron was invariably higher than that from ferrous sulphate elixir. The most rapidly disintegrating preparation was selected for further study.

Absorption ratios that favoured GDS iron after 10 and 25 mg formulation in 6 men were similar to those observed after 50 mg (table II). GDS containing 16 mg iron provides the same amount of absorbed iron as 50 mg iron administered as ferrous sulphate elixir (figure).

Side-effects study

The frequency of most symptoms did not differ between the three trial groups (table III). Nausea, however, was significantly more frequent at level I during the first week in the conventional group than in either the placebo or GDS groups. At the end of the second week, this symptom had



Iron absorption after GDS or elixir.

TABLE III—PROPORTION OF SUBJECTS REPORTING SIDE-EFFECTS

Side-effects	Week 1			Week 2		
	Placebo	GDS	CONV	Placebo	GDS	CONV
Level I						
Loss of appetite	0·134	0·181	0·283	0·074	0·060	0·194*
Nausea	0·134	0·166	0·358†	0·029	0·060	0·223‡
Belching	0·104	0·090	0·194	0·059	0·106	0·194
Heartburn	0·074	0·090	0·119	0·074	0·060	0·074
Vomiting	0·029	0·0	0·029	0·0	0·0	0·0
Constipation	0·194	0·181	0·328	0·164	0·257	0·238
Intestinal gas	0·194	0·166	0·283	0·104	0·121	0·283*
Black stools	0·029†	0·378	0·492	0·044‡	0·439	0·567
Diarrhoea	0·029	0·136	0·089	0·044	0·060	0·089
Abdominal pain	0·089	0·090	0·119	0·059	0·015	0·089
Drowsiness	0·149	0·106	0·149	0·029	0·090	0·089
Dizziness	0·074	0·015	0·074	0·0	0·030	0·014
Headache	0·194	0·196	0·194	0·164	0·151	0·164
Ringing in ears	0·0	0·030	0·029	0·0	0·0	0·029
Rash	0·014	0·0	0·0	0·029	0·015	0·014
Chest pain	0·044	0·015	0·044	0·024	0·0	0·014
Level II						
Loss of appetite	0·059	0·075	0·119	0·014	0·015	0·089*
Nausea	0·029	0·0	0·179‡	0·029	0·015	0·059
Constipation	0·014	0·015	0·089*	0·014	0·030	0·044
Intestinal gas	0·014	0·045	0·089	0·014	0·030	0·104*

CONV = conventional ferrous sulphate.
*$p < 0·05$, †$p < 0·01$, ‡$p < 0·001$.

virtually disappeared in the placebo and GDS groups, but persisted in about a fifth of subjects given ferrous sulphate capsules. When tested at level II severity, nausea was also significantly more common in the conventional group during the first but not the second weeks. Loss of appetite was more common during the first week in the conventional group compared with the placebo or GDS groups, but the differences were not significant. During the second week, however, a higher proportion of women taking conventional ferrous sulphate complained of mild and severe anorexia. More serious upper intestinal side-effects, such as pain or vomiting, did not develop with these modest doses. Intestinal gas was more common during the second week in the conventional group when tested at both level I and level II. Diarrhoea was uncommon in all groups. The frequency of constipation did not differ among the three groups although the difference approached significance during the first week. When tested at level II, the difference was significant only during the first week, the conventional group being worse.

Discussion

The most troublesome gastrointestinal side-effects associated with iron therapy are epigastric discomfort, nausea, and vomiting.[11] Possible mechanisms include direct irritation of the gastric or duodenal mucosa, alteration in gastric or intestinal motility, or rapid entry of iron to the portal or systemic circulation. The frequency of these symptoms correlates with the concentration of ionised iron in the intestinal lumen and/or the amount of absorbed iron because the addition of ascorbic acid, a strong enhancer of iron absorption, increases symptoms.[12] Lower gastrointestinal symptoms including gas, constipation, and diarrhoea are not dose-related and are less troublesome.

Various pharmaceutical preparations are designed to minimise the peak concentration of iron in the gastrointestinal lumen by slowing dissolution. Side-effects can be reduced significantly with these preparations,[4] but this finding may have been due to a reduction in the amount of absorbed iron. Indeed the efficacy of these preparations cannot be evaluated reliably unless absorption and side-effects are examined together. The use of delayed-release preparations has been criticised because of reduced absorption and increased cost.

Measuring iron absorption from sustained-release preparations is difficult because of the delayed rise in serum iron. Radioisotope techniques are required. It is difficult to prepare these radiolabelled pharmaceuticals because of the potential contamination of the production equipment. Thermal neutron activation can be used to label the final product,[6,13] but this approach is tedious and too expensive for the size of the market. Fortunately these problems do not arise with GDS because no special equipment is needed. Radioactive ferrous sulphate is mixed with the other ingredients, allowed to dry, and encapsulated.

GDS is suited for inorganic iron because it polymerises and precipitates as the gastric contents are alkalinised shortly beyond the pylorus. Ferrous sulphate in the hydrocolloid matrix is gradually released into gastric secretions as the surface layer erodes. Eventually the matrix disintegrates, releasing the remainder of the iron into solution. With ^{57}Co-labelled micropellets and serial gamma camera measurements, GDS is retained in the stomach for 5–12 h in healthy subjects.[5]

We found a striking three to four-fold higher absorption from GDS iron compared with ferrous sulphate elixir. This advantage of GDS is almost certainly related to its prolonged gastric retention because food reduces the absorption of ferrous sulphate.[14] GDS retains iron in the stomach until the bulk of the meal has left. The breakfast used in our study contained several absorption inhibiting foods, such as bran, egg, milk, and coffee, ingredients which highlight the advantage of GDS. Nevertheless this inhibitory type of meal is typical of cereal-based diets in many third world countries. The GDS advantage might not occur if ingested when in the fasting state and might be reduced with meals containing a high content of meat or ascorbic acid, which promote iron absorption.[15]

Because 50 mg iron three times daily is the maximum amount generally recommended for supplementation, we assessed side-effects with 50 mg GDS iron, which provides the same amount of absorbed iron. Symptoms such as vomiting and epigastric pain were not encountered with the low iron doses examined. Nevertheless nearly one-third of women taking 50 mg iron as ferrous sulphate had nausea and anorexia. These milder symptoms will not necessarily reduce compliance in medically supervised pregnant women, but are likely to affect iron supplementation programmes in developing countries. GDS iron, in amounts that will deliver the same dose of iron as the maximum recommended amounts of ferrous sulphate in pregnancy, is not associated with significant side-effects. Another major advantage is that GDS iron is taken only once daily.

The cost of GDS ingredients is low. The material could be sold in bulk and encapsulated regionally. It is therefore possible to implement a GDS iron supplementation programme at no greater cost than that of conventional ferrous sulphate. An extensive field trial of GDS in pregnant women is currently underway in Jamaica.

This study was supported by NIH grant DK39246 and AID Cooperative Agreement DAN-5115-A-00-7908-00.

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American Journal of Hematology 34:193–198 (1990)

Serum Transferrin Receptor Measurements in Hematologic Malignancies

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An enzyme-linked immunosorbent assay using specific monoclonal antibodies was used to measure circulating transferrin receptor (TR) in 87 patients with various hematologic malignancies. The mean serum TR was significantly elevated in patients with myeloproliferative disorders (15.47 ± 12.54 μg/ml), whereas there were no differences in chronic granulocytic leukemia (7.89 ± 3.56 μg/ml), myelodysplastic disorders (9.25 ± 4.73 μg/ml), and acute nonlymphocytic leukemia (3.85 ± 3.50 μg/ml) as compared to normal (5.63 ± 1.42 μg/ml). Among patients with lymphoproliferative disorders, the mean level was normal in lymphoma (5.73 ± 2.59 μg/ml), multiple myeloma (5.47 ± 1.31 μg/ml), and hairy cell leukemia (7.04 ± 3.69 μg/ml). The serum TR was significantly elevated in chronic lymphocytic leukemia (CLL; 14.17 ± 12.29 μg/ml), and the serum levels reflected the clinical stage of the disease. These findings suggest that serum TR measurement may provide a useful laboratory index of disease activity in certain disorders such as CLL, whereas it most likely reflects the intensity of erythropoiesis in the remaining hematological disorders that were evaluated in this study.

Key words: leukemia, myeloproliferative disorders, lymphoproliferative disorders

INTRODUCTION

The transferrin receptor (TR) is a cell-surface glycoprotein that plays an integral role in the incorporation of iron into actively proliferating cells through its binding of the transferrin–iron complex [1–3]. Increased numbers of cell surface TR have been described in a variety of hematologic disorders, including reticulocytes in hemolytic anemias and iron deficiency states [4–6] and malignant cells in a variety of tumors and nonHodgkins' lymphomas (NHL) [7–10]. In 1986, Kohgo et al. [11] described the detection of circulating serum TR in both normal individuals and patients with hematologic malignancies, including acute leukemia, multiple myeloma (MM), and NHL [11]. The TR levels were found to vary widely in these patients, a proportion having markedly elevated values, possibly related to shedding of increased numbers of TR from actively proliferating neoplastic cells into the circulation [12]. We measured the serum TR levels in patients with various hematologic malignancies as well as patients undergoing bone marrow transplantation to determine further in which disorders a deviation from the normal value is seen and to investigate the potential clinical usefulness of serum measurements in the management of these patients.

MATERIALS AND METHODS

The TR content of serum was measured using an enzyme-linked immunosorbent assay (ELISA) [13]. Two monospecific antihuman TR monoclonal antibodies raised in our laboratory were used in a "sandwich" technique, one as coating antibody and the second as indicator antibody after conjugation with horseradish peroxidase as previously described [14]. Purified human placental TR bound to human transferrin was used as reference material to construct a standard curve. Serum unknowns were prediluted to 1:500 in phosphate-buffered saline prior to assay. All samples were run in triplicate [13].

Circulating TR was measured in 87 patients with various hematological malignancies including 11 with acute nonlymphocytic leukemia (ANLL), 12 with myelodysplastic syndromes (MDS), 22 with myeloproliferative

Received for publication November 20, 1989; accepted February 15, 1990.

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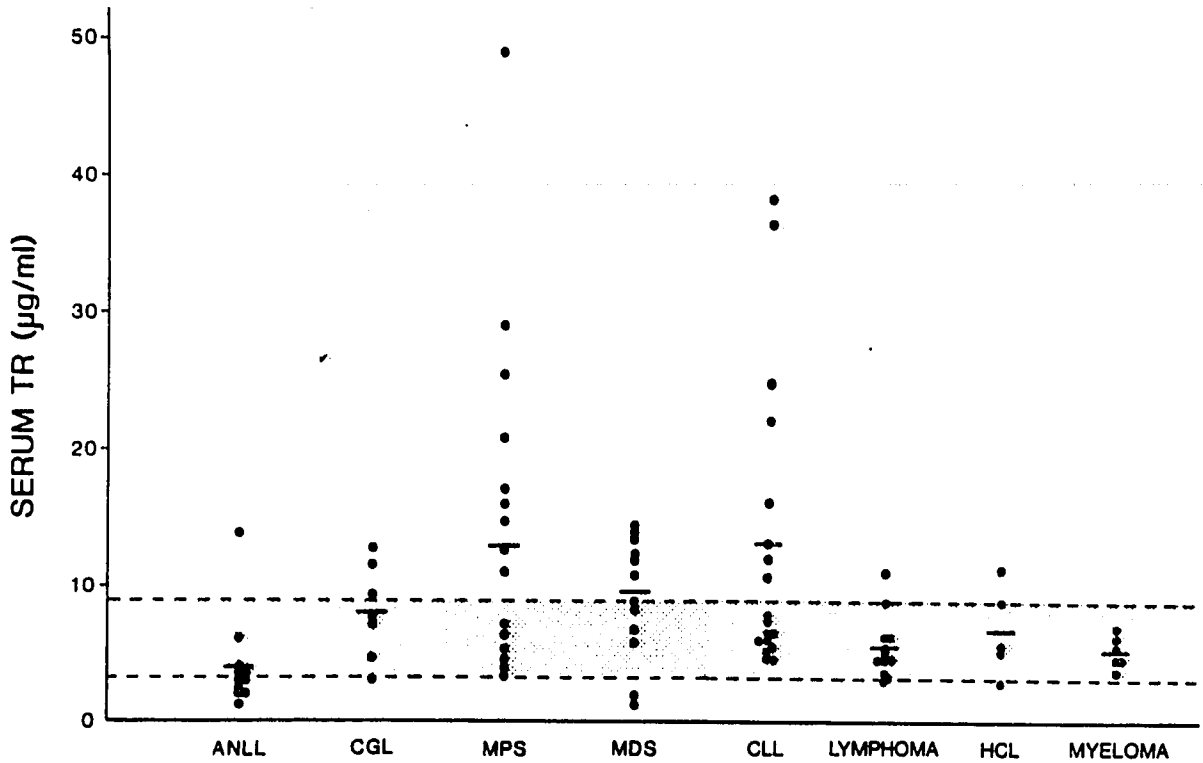

Fig. 1. Serum transferrin receptor levels in myeloid and lymphoid disorders. Normal range indicated by shaded area.

syndromes (MPS), specifically seven with chronic granulocytic leukemia (CGL), three with essential thrombocythemia, three with polycythemia rubra vera, six with idiopathic myelofibrosis, one with DiGuglielmo's disease, one unclassifiable, 19 with chronic lymphocytic leukemia (CLL), five with hairy cell leukemia (HCL), six with MM, and 12 with NHL. All patients, upon entry into the study, had evidence of active disease and had either received no previous chemotherapy or had had no chemotherapy for at least 4 weeks prior to evaluation, with the exception of three patients with CGL who were on chronic maintenance hydroxyurea therapy. The age range of the patient population was 19–82 years, with a mean age of 60 years. A second group comprised nine patients who underwent bone marrow transplantation, including three autologous transplants (two for progressive NHL and one for ANLL in relapse) and six allogeneic transplants (three for ANLL in relapse, two for ALL in relapse, and one for CGL in stable phase). Serial measurements of the serum TR were obtained throughout the peritransplant period. Analysis of variance with Scheffe's test were used to test for significant differences between the means.

RESULTS

The mean serum TR value for normal subjects was 5.63 μg/ml with a range of 2.79–8.47 μg/ml (mean ± 2 SD) [13]. Among the myeloid disorders, the mean TR level was 3.83 ± 3.50 μg/ml in the 11 patients with ANLL, with four of 11 patients having normal levels, six having low levels, and one patient a level above normal (Fig. 1). The mean value for the seven patients with CGL was 7.89 ± 3.56 μg/ml with four patients having levels above normal. Patients with other MPS had high levels (15.47 ± 12.54 μg/ml), the levels rising above normal in nine of the 15 patients ($P < 0.01$). The majority of these patients with raised TR levels had myelofibrosis or polycythemia rubra vera. The mean value for the MDS was 9.25 ± 4.73 μg/ml, with seven of 12 patients rising above the normal range and one falling below it (Fig. 1).

Among the patients with lymphoproliferative disorders, the mean serum TR was highest in the patients with CLL (14.17 ± 12.29 μg/ml, $P < 0.01$). Of 19 CLL patients, nine had TR levels above normal (Fig. 1). The mean value for HCL was 7.04 ± 3.69 μg/ml, with two of five patients rising above normal. All six patients with MM had normal levels with a mean value of 5.47 ± 1.31 μg/ml. Similarly, with the exception of two, all patients with NHL had normal receptor levels, with a mean value of 5.73 ± 2.59 μg/ml.

The patients with CLL were evaluated further in an attempt to ascertain the origin of their elevated TR. The mean TR value in these patients when evaluated by stage [15], spleen size, and degree of lymphocytosis, param-

TABLE I. TR Values in CLL Patients

Parameters of disease activity	Number of patients	Mean TR ± 1 SD	P^a
Stage			
A	9	6.90 ± 3.12	>0.05
B	5	21.80 ± 19.06	
C	5	17.66 ± 8.80	
Spleen size			
No splenomegaly	6	7.13 ± 3.69	>0.05
Splenomegaly	13	16.66 ± 13.60	
Lymphocytosis			
<30,000/mm³	9	8.42 ± 4.59	>0.05
>30,000/mm³	10	18.36 ± 15.01	

[a]Analysis of variance.

eters reflecting degree of disease activity, are shown in Table I. A trend towards higher TR values was noted with evidence of greater disease activity. This was, however, not statistically significant when comparing stage A (mean 6.90 ± 3.12 μg/ml) to stages B (mean 21.80 ± 19.06 μg/ml) and C (mean 17.66 ± 8.80 μg/ml). Patients without splenic enlargement had lower TR levels (7.13 ± 3.69 μg/ml) than those with splenic enlargement (16.66 ± 13.60 μg/ml) and TR levels increased according to the degree of lymphocytosis, levels being 8.42 ± 4.59 in patients with lymphocytosis <30,000/mm³ and 18.36 ± 15.01 μg/ml in those with >30,000/mm³, but these were not significantly different.

Because the serum TR level has been previously described as reflecting the degree of erythropoiesis [16,17], an attempt was made to correlate the degree of reticulocytosis with the circulating TR levels. No correlation between the reticulocyte count and the TR value was found.

The nine patients undergoing marrow transplantation were followed with serial TR levels and white blood cell counts (WBC). The WBC was used as an indicator of bone marrow recovery in view of the slower recovery of erythropoiesis in these patients and the frequent use of red blood cell transfusions in the course of their recovery posttransplantation. There was a close correlation between the TR value and total WBC, both following bone marrow ablation and in the posttransplant period of marrow recovery (Fig. 2). The mean time to half the initial TR level was 15 days after ablation therapy. The TR level decreased to a nadir of approximately 45% of the baseline level. Recovery of both the WBC and TR value occurred concomitantly on approximately day 12 posttransplantation, both parameters rising in a parallel fashion, with the exception of minor fluctuations in the TR value. Comparison of the mean TR values in eight transplant recipients to one patient who underwent autologous transplantation for high-grade NHL resistant to chemotherapy is shown in Figure 3. The TR value was elevated at the time of his disease relapse and subsequent transplantation. His TR level declined slower, reaching a nadir 21 days posttransplantation. The patient subsequently died with resistant NHL 3 months later.

The potential clinical usefulness of the serum TR is illustrated by two patients who were followed with serial measurements throughout the course of their illness.

Case 1

A 50-year-old white male presented with a hemoglobin 10.9 g/dl, a platelet count of 48,000/mm³, and a WBC of 2,400/mm³, with a differential of 10% neutrophils, 77% lymphocytes, 6% monocytes, and 1% basophils. Bone marrow aspirate was consistent with the diagnosis of refractory anemia with excess blasts in transformation (RAEBIT). The initial TR value was 13.34 μg/ml. During the next 3 weeks the TR value increased to 36.10 μg/ml. Two weeks later the value dropped to 17.11 μg/ml, without obvious change in his clinical status or peripheral blood counts. A repeat bone marrow examination at this time was consistent with transformation to ANLL. It was not until 8 weeks later that the peripheral counts reflected evidence of disease progression with a myeloblast count of 16%. The receptor value during this time continued to decline to 5.09 μg/ml at the time of leukemic peripheral blood involvement. The clinical status remained unchanged throughout this time.

Case 2

A 52-year-old white female was diagnosed with a MPS when she was found to have a hemoglobin of 8.9 g/dl; a WBC of 42,500/mm³, with 60% neutrophils, 7% eosinophils, 1% basophils, 5% myelocytes, 7% promyelocytes, and 3% myeloblasts; and a platelet count of 550,000/mm³. The TR was elevated at 11.20 μg/ml. Eight months after initial presentation, the level declined to a nadir of 5.70 μg/ml. The hemoglobin was 7.2 g/dl, platelet count was 298,000/mm³, and WBC was 48,400/mm³, with 28% neutrophils, 53% myeloid precursor cells, and 5% blasts. Chromosomal analysis of the bone marrow now revealed the appearance of a new 5q−,11p+ abnormality, not previously noted at presentation. The bone marrow revealed features of myelodysplasia and the myeloid series was left shifted, with 12% blast forms. The patient subsequently developed ANLL with intractable lactic acidosis and expired 1 month later.

Both patients displayed elevated TR values during the stable phase of their disease. A progressive fall in TR value heralded the transformation to ANLL prior to any appreciable change in clinical status or peripheral blood counts by nearly 2 months in the first patient and concomitantly with these changes in the second patient.

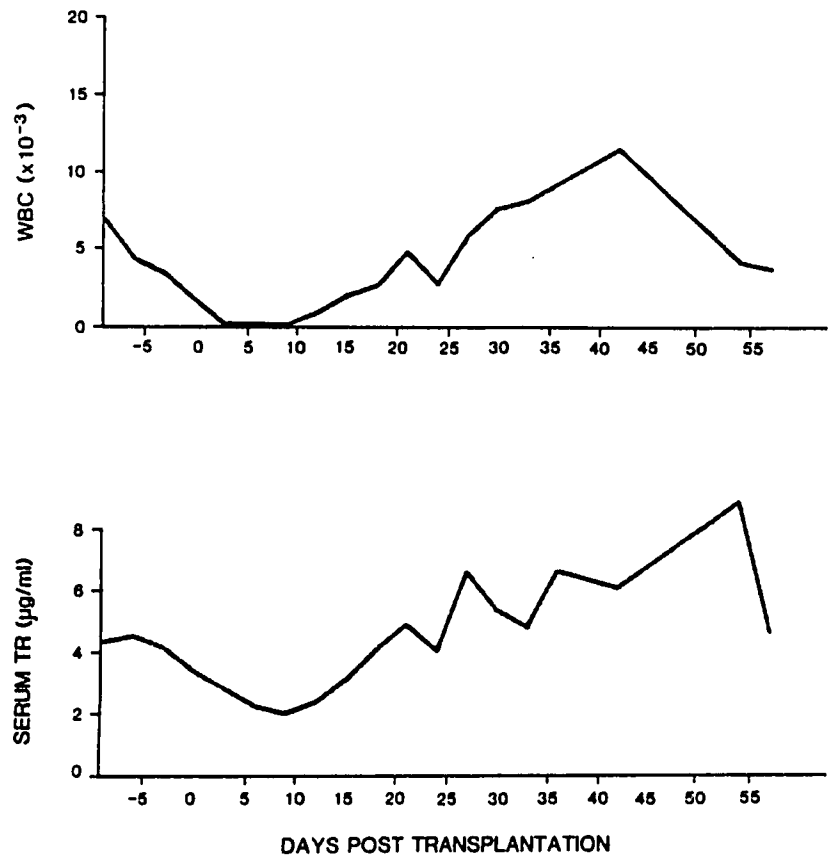

Fig. 2. Serial measurements of white blood cell counts and serum transferrin receptor levels during the ablative and recovery phases of bone marrow transplantation. The mean and 1 SD are indicated by the solid line and shading, respectively.

DISCUSSION

The TR's role in iron delivery to actively proliferating cells has been well characterized [1–3,18]. The number of cell-surface TR on reticulocytes has been reported to be increased in a variety of nonmalignant hematologic disorders characterized by increased erythropoiesis [19]. Increased numbers of TR occur in lymphomatous tissue from patients with NHL, and the higher grade lymphomas tend to have greater numbers of surface receptors than low-grade lymphomas [9,10]. Circulating TR both in normal subjects and in patients with various malignant disorders were recently described [11,17,20]. Patients with ANLL, MM, and NHL were found to have elevated levels of serum TR, and it was suggested that these receptors were shed from actively proliferating malignant cells [12].

The major contributing source of circulating TR in normal subjects and in patients with hemolytic anemia is thought to be the erythroid mass, since these cells require large amounts of iron for hemoglobin synthesis. It is likely that expanded erythropoiesis was the major source of the serum TR seen in our patients with MPS and MDS. However, the active proliferation of other marrow elements in these disorders should also be considered as possible contributing sources. In contradistinction to prior findings, we found no correlation between the circulating TR value and the reticulocyte count in our patient population [20]. In the patients undergoing bone marrow transplantation, the serum TR closely paralleled the WBC, both during the periods of bone marrow suppression and recovery, thus suggesting that a significant proportion of the circulating receptor is derived from bone marrow activity. Interestingly, the TR concentration leveled off between 2 and 3 µg/ml during the aplastic phase following transplantation. Similarly, serum TR levels were approximately 50% of normal in aplastic anemia patients [20]. These findings suggest that sources other than the bone marrow are likely to contribute to the circulating TR.

In the present study, the mean serum TR was three times the normal value in the CLL and the MPS, the highest value in this latter group being in patients with polycythemia vera and idiopathic myelofibrosis. The mean TR level was nearly two times normal in the MDS, and it was only modestly elevated in HCL and CGL. In

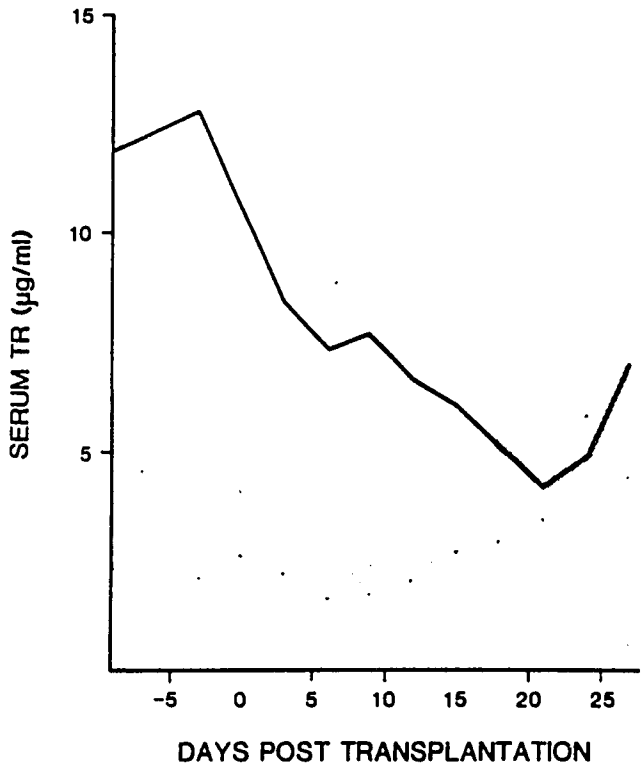

Fig. 3. Serial measurements of serum transferrin receptor (solid line) in a patient with high-grade lymphoma during bone marrow transplantation. The range of receptor levels during transplantation for the remaining patients is shown by the shaded area.

contrast to prior findings [11], patients with ANLL had reduced levels of circulating TR. The etiology of the reduced levels was not determined but presumably this reflects diminished erythropoiesis.

The elevated receptor values found in CLL may have significant clinical impact in the staging and management of patients. Studies were done to determine the source of the serum TR in these patients. The circulating leukemic lymphocytes were excluded as a source, because only negligible amounts of receptor were found when lymphocytes from five CLL patients were assayed after isolation by Ficoll-Hypaque separation, freeze-thawing, and sonication in the presence or absence of detergent. This finding is in keeping with that of Barnett et al. [21], who found only small concentrations of TR on CLL lymphocytes. Shedding of TR from expanded erythropoiesis as the source of the raised serum TR is unlikely, since quantitative marrow erythroid activity was not increased in four of five of our patients whose bone marrows were reviewed. No significant difference was found when comparing patients on the basis of disease activity, although patients with a greater leukemic burden (stages B and C) had higher TR levels than those patients with less aggressive disease (stage A). We could not exclude elaboration of TR into the circulation from diseased lymph nodes, spleen, or lymphocytes in the bone marrow as a source for the raised levels in CLL. It is possible that the raised serum level is derived from sites where the CLL lymphocytes are produced. Typically, circulating CLL lymphocytes are quiescent and do not undergo proliferation or differentiation in the circulation, under most circumstances. However, cell replication occurs during the growth phase of the lymphoid mass, and this is a likely source of circulating TR elaboration, since TR expression is a prerequisite for the cell to proceed through the cell cycle [22,23]. The two patients described with progression to acute leukemia highlight the potential value of TR measurements in the management of MDS and MPS. A progressive decline in the serum TR from high levels into the normal range may signify transformation of the disease prior to any change in the peripheral blood counts.

In conclusion, serum TR levels are disturbed in a variety of malignant and nonmalignant hematologic disorders. However, serum TR levels appeared to be increased only out of proportion to expected erythropoiesis in the CLL patients in this study. Changing TR levels may indicate disease progression as well as provide a useful index of bone marrow proliferative activity. The clinical usefulness of these observations has yet to be clearly defined.

ACKNOWLEDGMENTS

This work was supported by NIH grant DK39246 and AID Cooperative Agreement DAN-5115-A-00-7908-00.

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British Journal of Haematology, 1990, 75, 603–609

Serum transferrin receptor as an index of iron absorption

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Received 9 January 1990; accepted for publication 2 April 1990

Summary. Recent studies indicate that serum transferrin receptor levels are a quantitative index of tissue receptor mass. To determine whether the latter plays a role in the regulation of iron absorption, we examined the relationship between serum receptor, serum ferritin and iron absorption in healthy subjects. Using radioisotopic techniques we measured absorption of inorganic iron in 174 subjects and dietary nonhaem iron in 60 subjects. With both forms of iron, the correlation with absorption was far lower for serum receptor than for serum ferritin and was no longer significant when subjects with depleted iron stores were excluded. These results indicate that in normal subjects the iron store is the main physiological determinant of iron absorption and that in the absence of iron deficiency, tissue receptor mass, reflected by serum transferrin receptor levels, has no discernible influence.

Disturbances in the mechanisms which control iron absorption from the gastrointestinal tract may play an important role in the pathogenesis of iron deficiency and iron overload. Despite several decades of investigation, the nature of the regulatory process is still poorly understood. Body iron stores are known to be important because of the close inverse relationship that has been demonstrated between serum ferritin and iron absorption in normal subjects (Cook *et al.* 1974; Walters *et al.* 1975; Baynes *et al.* 1987). There is also some evidence that the rate of erythropoiesis and/or tissue receptor mass participates in the regulation of iron absorption (Bothwell *et al.* 1979). Using sensitive immunologic techniques, soluble transferrin receptor has recently been detected in the circulation (Kohgo *et al.* 1986, 1987; Beguin *et al.* 1988; Flowers *et al.* 1989). Both animal and clinical studies indicate that serum receptor levels reflect the total mass of tissue receptor. The present investigation was undertaken to examine the interrelationships between iron absorption, serum receptor as a measure of tissue receptor mass, and serum ferritin as a measure of storage iron.

METHODS

Measurements of iron absorption, serum ferritin and serum transferrin receptor were performed in 234 normal volunteer subjects. Absorption of ferrous sulphate was measured in 174 individuals and of nonhaem dietary iron in 60 subjects. The mean age of the participants was 25·2 and 26·1 years, respectively, with a composite range of 18–46 years. All of the subjects were in good health and denied a history of recent infections, haematologic diseases, or gastrointestinal disorders which are known to influence iron absorption. None of the subjects was anaemic as defined by a haematocrit above 38% in women and 42% in men. No exclusions were made on the basis of iron status. Written informed consent was obtained from each volunteer and all of the experimental procedures were approved by the Human Subjects Committee at the Kansas University Medical Center.

The iron absorption measurements reported here were one of four separate tests performed in each subject in our ongoing studies of dietary factors affecting iron absorption. All test meals were fed between 07.00 and 09.00 hours following a 10 h fast. No further food or liquid was allowed for 3 h. On the day preceding the first pair of test meals, 15 ml of blood was drawn for measurements of haematocrit, serum ferritin, serum receptor, and background blood radioactivity. Test meals labelled with either 37 kBq $^{59}FeCl_3$ or 111 kBq $^{55}FeCl_3$ were fed on the next two consecutive mornings. 25 ml of blood were drawn 14 d after the second test dose for repeat measurements of serum ferritin and receptor and for measurement of incorporated red cell radioactivity. Two further test meals were then fed on the following two mornings and a final blood sample was drawn 2 weeks later for measurement of serum ferritin, serum receptor, and increase in red cell ^{55}Fe and ^{59}Fe radioactivity. Consequently, two separate measurements of serum ferritin and receptor were obtained 2 weeks apart. Food iron absorption was measured in the first pair of tests, and inorganic iron in the second pair of tests. In subjects studied with food iron, 15 ml

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Table I. Measurements of serum ferritin, serum receptor and iron absorption

	Men	Women	Composite
Inorganic iron			
No. of subjects	87	87	174
Iron absorption (% of dose)	18·3 (8·7–38·2)	33·9 (33·9–67·6)	24·8 (11·4–53·9)
Serum ferritin (μg/l)	56·4 (24–135)	20·1 (9–45)	33·7 (13–90)
Serum receptor (mg/l)	5·90 ± 1·50*	5·57 ± 1·38	5·73 ± 1·45
Food iron			
No. of subjects	19	41	60
Iron absorption (% of dose)	3·9 (1·7–9·0)	10·3 (4·3–24·6)	7·6 (2·9–19·9)
Serum ferritin (μg/l)	80·0 (36–178)	25·0 (11–56)	36·2 (14–95)
Serum receptor (mg/l)	5·48 ± 0·80*	5·59 ± 1·33	5·56 ± 1·18

* ± SD.

of blood was obtained 2 weeks before the second set of ferritin and receptor measurements, whereas in those studied with inorganic iron, 15 ml blood was drawn 4 weeks before and 25 ml blood 2 weeks before the second set of measurements. ^{59}Fe and ^{55}Fe radioactivity was measured in duplicate 10 ml samples of whole blood using a modification of the method of Eakins & Brown (1966). Percentage absorption was calculated on the basis of blood volume estimated from height and weight (Wennesland *et al.* 1959; Brown *et al.* 1962) and an assumed red cell incorporation for absorbed radioactivity of 100%.

Absorption of dietary nonhaem iron was measured from a standard hamburger meal (Lynch *et al.* 1985, 1989; Whittaker *et al.* 1989) containing 4·8 mg iron of which 3·4 mg was nonhaem. The meal included ground beef (113 g), a bun (53 g), French fries (68 g) and a milk shake (145 g). The meal was tagged extrinsically by adding 0·1 mg iron as $^{59}FeCl_3$ in 0·5 ml 0·01 N HCl to the bun (Cook *et al.* 1972). The measurement of inorganic iron absorption was performed as a reference test to characterize the absorptive response of each subject and thereby facilitate comparisons of food iron availability performed on different occasions (Layrisse *et al.* 1969; Magnusson *et al.* 1981). The test dose consisted of a freshly prepared solution containing 3 mg iron as $FeSO_4.7H_2O$ and 18·9 mg of ascorbic acid (molar ratio of 2:1) in 50 ml water.

A two-site ELISA was used to measure the concentration of serum ferritin and serum transferrin receptor. The two assay procedures were identical except for the immunologic reagents. The serum ferritin method was established with monoclonal antibodies developed against purified basic isoferritin (Flowers *et al.* 1986) and the serum transferrin assay with monoclonal antibodies prepared against soluble transferrin-saturated receptor isolated from human placenta (Flowers *et al.* 1989). The receptor assay was standardized from the protein concentration of transferrin-free receptor isolated from human placenta by the method of Turkewitz *et al* (1988). The sensitivity of receptor assay is 0·5 μg/l and the between-assay variability less than 3·0%. Sera was harvested on the same day that blood was obtained and stored at −70°C until the day of analysis. The initial and 2-week follow-up measurements in each subject were always performed in the same microtitre plate.

Percentage iron absorption and serum ferritin levels were analysed as logarithms because of their skewed distribution (Cook *et al.* 1969). The results were reconverted as antilogarithms to recover the original units. All statistical analyses including *t*-tests, correlation coefficients and regression parameters were calculated on logarithmically transformed data.

RESULTS

Since iron stores are much higher in men, the influence of iron status was first assessed by comparing the results in male and female volunteers. The mean serum ferritin was 56·4 and 20·1 μg/l, respectively, in those given inorganic iron and 80·0 and 25·0 μg/l in those given dietary iron (Table I). The composite mean serum ferritin levels were similar in the two absorption groups. The serum ferritin mean − 1 SD was 13 and 14 μg/l in the two absorption groups indicating that iron stores were essentially absent in about 15% of our subjects. The sex difference in serum ferritin was mirrored precisely by iron absorption values. Absorption of inorganic iron averaged 18·3% in men and 33·9% in women whereas absorption of food iron averaged 3·9% and 10·3% respectively. The pronounced inhibitory effect of food on iron absorption is reflected in the composite means of 24·8% with inorganic iron and 7·6% with food iron.

In contrast to serum ferritin and iron absorption data, there was no appreciable sex difference in serum transferrin receptor levels. In subjects studied with inorganic iron, the mean receptor level was slightly higher in men, but in neither study group was the sex difference statistically significant ($P > 0·10$ for both inorganic iron and food iron). The composite mean receptor levels of 5·73 and 5·56 μg/l in those given inorganic and food iron respectively, were also similar. The composite mean receptor level in the 234 subjects was 5·69 ± 1·38 μg/l, indicating an upper 95% confidence limit of 8·5 μg/l.

Measurements of both serum ferritin and serum receptor were highly consistent within the same subject when repeated after a 2-week interval. The correlation coefficient

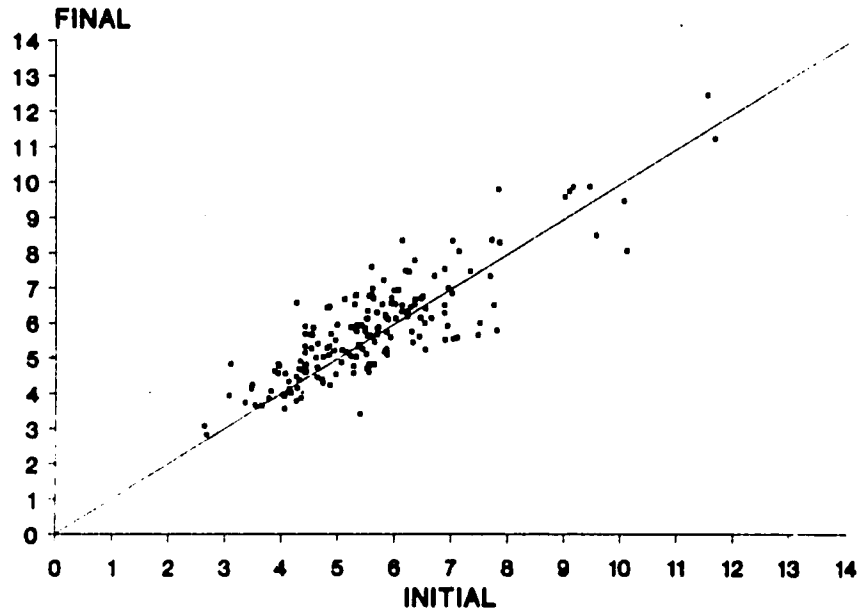

Fig 1. Measurements of serum receptor (mg/l) obtained 2 weeks apart in subjects studied with inorganic iron.

Table II. Correlations between iron absorption, serum ferritin, and serum receptor

		Ferritin: receptor		Absorption: ferritin		Absorption: receptor	
	n	*r*	*P*	*r*	*P*	*r*	*P*
Inorganic iron							
All subjects	174	−0·269	<0·001	−0·683	<0·0001	0·243	0·001
Ferritin > 15 μg/l	133	0·112	ns*	−0·554	<0·001	0·038	ns
Food iron							
All subjects	60	−0·388	<0·002	−0·803	<0·0001	0·369	0·004
Ferritin > 15 μg/l	44	0·202	ns	−0·729	<0·001	0·017	ns

*Not significant.
n = Number of subjects.

for serum ferritin levels was 0·970 in both study groups indicating that 94% of the variance (coefficient of determination) was explained by biological differences. The correlation coefficient between initial and repeat receptor measurements was also highly significant, 0·853 in those given inorganic iron and 0·797 in those given food iron (Fig 1). Thus, roughly two-thirds of the variance in receptor levels reflected biological differences. In subjects tested with food iron, nearly identical initial and repeat serum receptor levels of 5·55 and 5·57 mg/l were observed ($P > 0·20$). In contrast, a small but highly significant increase in receptor concentration from 5·61 μg/l initially to 5·83 μg/l on repeat measurements was seen in subjects tested with inorganic iron ($t = 3·90$, $P < 0·0001$). This difference presumably reflects the greater amount of blood removed during the preceding month from subjects tested with inorganic iron as compared to those tested with food iron.

A significant correlation was observed between serum receptor and ferritin levels in both the inorganic iron ($r = -0·269$) and food iron group ($r = -0·388$) (Fig 2 and Table II). However, if iron-deficient subjects defined as a serum ferritin ≤ 15 μg/l were excluded, the correlation was no longer significant. In the subjects studied with inorganic iron, only nine subjects had an elevated serum receptor above 8·5 mg/l and only one of these had a serum ferritin > 20 μg/l.

A highly significant inverse relationship was observed between serum ferritin and the absorption of both food iron ($r = -0·803$) (Fig 3) and inorganic iron (Table II). Significant correlations were also observed between serum receptor and absorption of food iron ($r = 0·369$, $P = 0·004$) (Fig 4) and inorganic iron ($r = 0·243$, $P = 0·001$) (Table II). To determine whether these correlations were due to the effect of iron deficiency which increases both iron absorption and serum receptor (Kohgo *et al*, 1986; Flowers *et al*, 1989), correlation

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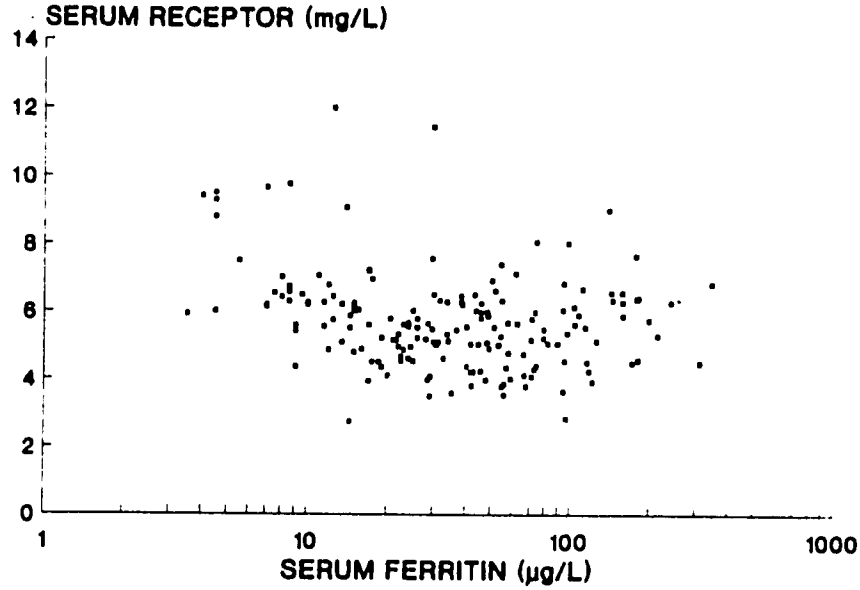

Fig 2. Relationship between serum ferritin and serum receptor measurements.

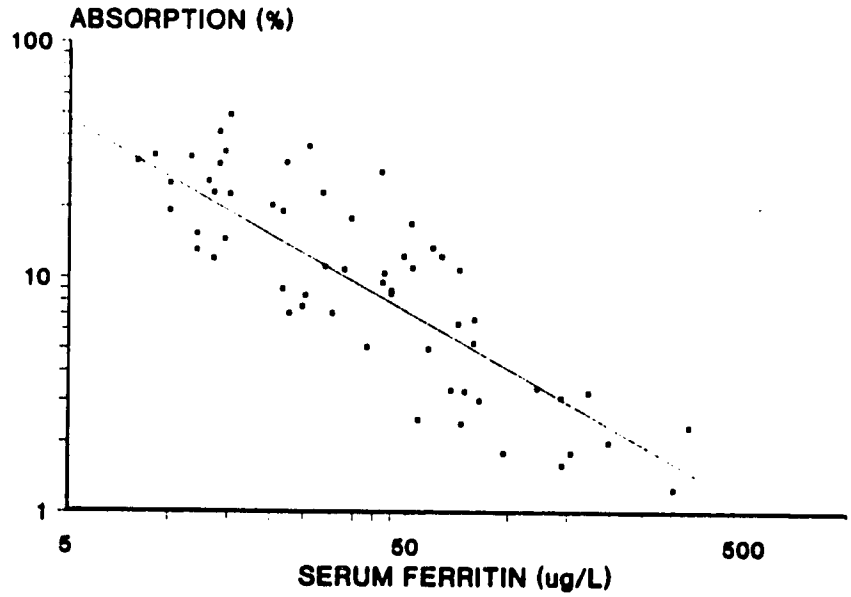

Fig 3. Relationship between food iron absorption and serum ferritin.

analysis was repeated after excluding subjects with a serum ferritin ≤ 15 µg/l (Table II). In both study groups the correlation between absorption and receptor was no longer significant. The lack of a relationship between serum receptor and iron absorption in iron replete subjects was also supported by multiple regression analysis. When iron absorption was treated as the independent variable and serum ferritin and receptor levels as dependent variables, the regression slope for serum receptor was not significant either in those given inorganic iron ($b=0{\cdot}034$, $t=1{\cdot}10$, $P=0{\cdot}27$) or dietary iron ($b=0{\cdot}056$, $t=0{\cdot}80$, $P=0{\cdot}43$).

DISCUSSION

Studies over the past several decades in both animals and humans provide convincing evidence that iron status is the key determinant of iron absorption (Bothwell *et al.* 1979). The only other physiological regulator that is often mentioned is the rate of erythropoiesis or tissue iron uptake. Cavill and coworkers proposed that iron absorption is a function of both the size of the labile pool of iron in various body compartments and the rate of tissue iron uptake (Cavill *et al.* 1975). They suggested that at any given level of tissue iron, iron absorption can be enhanced by an increased outflow of plasma iron which is in turn determined primarily by the mass of transferrin receptors in the erythroid compartment. This hypothesis was examined in rats by performing exchange transfusions with high reticulocyte blood to produce an abrupt increase in transferrin receptor mass without altering plasma iron concentration or body iron stores (Finch

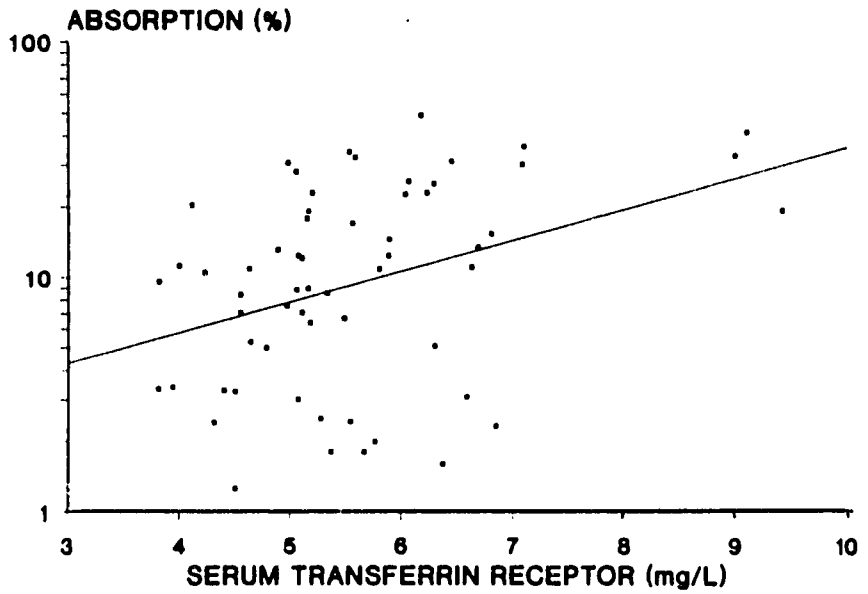

Fig 4. Relationship between food iron absorption and serum receptor.

et al. 1982). Within 1 h, and for at least 6 h following exchange, iron absorption was increased by 50–130% over control animals, suggesting that tissue receptor mass plays a significant role in regulating iron absorption.

Much of the evidence that erythropoiesis influences iron absorption in humans is derived from the clinical observation that certain patients with pronounced erythroid hyperplasia develop progressive iron overload. In an earlier study, iron absorption was shown to be increased in patients with enhanced erythropoiesis, but the increase was far greater when erythropoiesis was ineffective, as in patients with thalassaemia major, than when red cell production was effective, as in patients with hereditary spherocytosis, acquired haemolytic anaemia, or glucose-6-phosphate dehydrogenase deficiency (Eriandson *et al.* 1962). In anaemic patients with varying degrees of erythroid hyperplasia, a high correlation was observed in a recent study between iron absorption and plasma iron turnover, a quantitative ferrokinetic measure of total erythropoiesis (Pootrakul *et al.* 1988). As in the earlier report, the increase in iron absorption at any given level of plasma iron turnover was far greater in patients with ineffective erythropoiesis than in those with compensated haemolytic anaemia. In haematologically normal subjects there is no conclusive evidence that erythropoiesis or the rate of tissue iron uptake influences iron absorption significantly. The increase in iron absorption that occurs when subjects are transported to high altitude has been attributed to enhanced erythropoiesis (Reynafarje & Ramos, 1961), but more recent studies have shown that hypoxia *per se* can increase iron absorption independent of erythropoiesis (Mendel, 1961; Raja *et al.* 1986). It is known that phlebotomy produces a brisk increase in iron absorption in normal subjects (Bothwell *et al.* 1958), but this effect can be due either to enhanced erythropoiesis or a reduction in body iron stores.

The recent development of serum transferrin receptor measurements has provided an important new laboratory measurement of erythropoiesis (Trowbridge, 1989). There is now convincing evidence that the serum receptor level is a reliable index of tissue receptor mass of which the erythroid marrow is the major component (Kohgo *et al.* 1986; Flowers *et al.* 1989; Huebers *et al.* 1990). The serum receptor level is sharply elevated in patients with chronic haemolytic anaemia including autoimmune haemolysis (Kohgo *et al.* 1987; Huebers *et al.* 1990), hereditary spherocytosis (Huebers *et al.* 1990) and sickle cell anaemia (Flowers *et al.* 1989). An even more dramatic elevation in serum receptor levels occurs in patients with ineffective erythropoiesis such as β-thalassaemia/haemoglobin E or haemoglobin H disease (Huebers *et al.* 1990). The serum receptor is derived in part from nonerythroid tissues because the level falls by only 50% in patients with complete marrow aplasia (Kohgo *et al.* 1986; Flowers *et al.* 1989; Huebers *et al.* 1990). In a recent study of 140 patients with a broad spectrum of haematological disorders, a high correlation was observed between serum receptor levels and erythron transferrin uptake, an accurate measure of erythroid precursor mass (Huebers *et al.* 1990). The only other disorder that is associated with a significant elevation in serum receptor levels is iron deficiency (Kohgo *et al.* 1986; Flowers *et al.* 1989). This elevation presumably is due to a higher density of transferrin receptors on individual normoblasts and perhaps nonerythroid cells as well. Serial measurements in normal subjects undergoing repeated phlebotomy indicate that the serum receptor level is a sensitive quantitative measure of early tissue iron deficiency (Skikne *et al.* 1990). Thus, clinical experience with serum receptor measurements to date indicates that it is a reliable indirect measurement of tissue receptor mass and is sensitive to changes in erythropoiesis or the development of iron deficiency.

The present study was undertaken to examine the possible relationship in normal subjects between iron absorption

and tissue receptor mass as measured by serum receptor levels. Although there is no conclusive evidence that the serum receptor reflects the level of erythropoiesis in normal subjects, such a relationship is suggested by the high correlation observed between measurements in the same subject after a 2-week interval. Moreover, the removal of a relatively small amount of blood (40 ml) during the preceding month resulted in a small, but highly significant, increase in receptor levels. Our results indicate that iron absorption in normal subjects is highly correlated with serum ferritin, but only modestly correlated with serum receptor. This latter relationship was due entirely to the effect of iron deficiency on serum receptor because it disappeared completely when iron deficient subjects were excluded on the basis of serum ferritin measurements. Our findings therefore indicate that the rate of tissue uptake in normal subjects as reflected by serum receptor levels is not a determinant of iron assimilation in normal individuals.

The assay used for serum receptor measurements in the present study is similar to that described originally by Kohgo and coworkers (Kohgo *et al.* 1986). The major difference is that our monoclonal antibodies were developed against soluble transferrin receptor rather than against receptor on the surface of intact cells. The relative increase in serum receptor levels in patients with enhanced erythropoiesis or iron deficiency are virtually identical with the two assay systems. However, the values obtained by the assay used in the present study are roughly 20-fold higher than values obtained by Kohgo and coworkers. The difference appears to be related to the material used for standardization (Flowers *et al.* 1989).

The biochemical nature of circulating receptor protein is not yet fully defined. It has been reported that the electrophoretic mobility of the circulating protein is similar to intact placental receptor and that the serum protein can be fully removed from plasma by adding an excess of anti-transferrin antiserum (Benguin *et al.* 1988; Huebers *et al.* 1990). On the other hand, Chitambar & Zivkovic (1989) reported that soluble transferrin receptor isolated from the culture media of HL60 cells is significantly smaller in molecular mass (78 000 Da) than intact cell surface receptor (94 000 Da) when analysed by gel electrophoresis under reducing conditions. They concluded that soluble receptor represents the extracytoplasmic domain of membrane receptor released by proteolytic cleavage. We also observed that the immunoreactive material isolated from human serum using a monoclonal affinity column is a cleavage fragment of intact transferrin receptor (Shih, Bayne, Hudson, Flowers, Skikne & Cook, unpublished observations). These observations are similar to studies of the interleukin-2 receptor which has been shown to exist in soluble form, the truncation occurring just beyond the transmembrane region of the molecule (Rubin *et al.* 1985). It is not clear whether these soluble products arise from proteolytic cleavage of the intact peptide or whether they are truly secretory forms of the protein lacking transmembrane regions and produced by either multiple genes (Kress *et al.* 1983) or alternative mRNA splicing pathways (Early *et al.* 1980).

ACKNOWLEDGMENT

This work was supported by NIH grant DK39246 and AID Cooperative Agreement DAN-5115-A-00-7908-00.

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Annu. Rev. Nutr. 1990. 10:133–48
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IRON DEFICIENCY

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KEY WORDS: requirements, bioavailability, food fortification, work performance, diet composition

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0199-9885/90/0715-0133$02.00

INTRODUCTION

A nutritional deficiency of iron develops when the amounts absorbed from the diet by the gastrointestinal tract are insufficient to meet normal requirements. Food iron absorption is a function of dietary composition. While the overall iron content of diets remains relatively constant, differences in composition account for wide variations in bioavailability. This review discusses several topics, including the assessment of iron storage status, iron losses and requirements, food iron absorption, and the prevalence, biologic consequences, and prevention of iron deficiency.

ASSESSMENT OF IRON STATUS

There are different degrees of iron deficiency (11). The mildest manifestation is a reduction of iron stores. When stores have become totally exhausted, less iron is delivered to the transferrin of plasma and, as a consequence, the erythroid marrow becomes depleted of iron. The final and most severe manifestation is an anemia in which the red cells are microcytic and hypochromic. Diagnostic measurements can be divided into those that assess iron stores and those that reflect the availability of iron to the erythroid marrow. The former measurements include the histologic assessment of reticuloendothelial iron stores in the bone marrow and the serum ferritin and transferrin concentrations; the latter include the saturation of transferrin, free erythrocyte protoporphyrin content, mean red cell volume, hemoglobin concentration, and serum concentration of circulating transferrin receptors.

Size of Iron Stores

The absence of bone marrow hemosiderin in reticuloendothelial cells is regarded as the most accurate indicator of iron deficiency (21); such measurements, however, are clearly not appropriate for population studies. Of much more practical value is the serum ferritin concentration, which bears a semiquantitive relationship with body iron stores in the concentration range of 20 to 200 μg per liter; 1 μg per liter serum ferritin is roughly equivalent to 8 mg storage iron (21, 114). A low serum ferritin concentration (< 12 μg per liter) is significant, since it is virtually diagnostic of iron deficiency (101). The serum ferritin loses its diagnostic value in the presence of infection, inflammation, ineffective erythropoiesis, malignancy, and liver disease, all of which result in higher serum concentrations (76).

While the serum transferrin concentration rises in people with iron deficiency as a consequence of enhanced hepatic synthesis (90), it is not a sensitive indicator of iron deficiency, since one third of anemic patients responding to iron treatment have transferrin concentrations in the normal range (2).

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Measurement of Iron Supply to the Erythroid Marrow

The serum iron concentration is low in iron-deficient people. It is, however, a relatively unreliable index of iron nutrition, since it is modified by a number of other factors (11). As a result, the degree to which transferrin is saturated with iron also has limitations when used on its own. A saturation of less than 16% is nevertheless useful when interpreted in combination with other iron measurements. It is noteworthy that iron-deficient red cell precursors express greater numbers of receptors for transferrin on their surfaces, and fragments of these receptors circulate in the plasma in increased quantities in iron deficiency (49, 68). The relative diagnostic value of this exciting finding awaits elucidation.

As the iron supply to erythroid precursors becomes progressively curtailed, there is a corresponding rise to > 70 μg per deciliter in the concentration of free erythrocyte protoporphyrin in maturing red blood cells. Such a rise, however, is not specific to iron deficiency and is found also in lead poisoning, the anemia that accompanies chronic disease, and thalassemia. The reduction in the iron supply to the erythroid marrow leads to a progressive drop in the hemoglobin concentration. To define the cutoff value for normality in population studies poses a problem, because there is significant overlap between iron-deficient subjects and normal subjects with hemoglobin concentrations at the lower limits of normal (22). In established iron-deficiency anemia the red cells are small (< 80 fL). Similar reductions in the mean red cell volume occur in other conditions associated with impaired hemoglobin synthesis, including the anemia of chronic disorders, thalassemia, and sideroblastic anemia.

Use of a Combination of Measurements

While single measurements are of limited value as predictors of iron-deficiency anemia, their specificity is significantly increased when two or more are abnormal (23, 43). A more sophisticated approach has been developed recently for population studies (30). Several iron measurements are used jointly to estimate the total body iron content in individual subjects and to assess changes in the iron status of population samples. The usefulness of this approach has recently been confirmed in a fortification trial in South Africa (4).

Quantitative Aspects of Iron Losses

THE INFANT A normal full-term infant has a minimal daily dietary requirement of 0.3 mg iron, and a premature infant requires significantly more iron (14, 20). Growth in the second year of life increases the daily requirement in infancy to 0.4 mg. Thereafter, iron requirements steadily rise to about 1.6 mg per day during the adolescent growth spurt. In late adolescence and early adulthood the daily requirement drops to 1.2 mg in men.

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THE ADULT MAN Basal obligatory losses of iron in the adult man occur predominantly from the gastrointestinal tract, skin, and urinary tract (0.6 mg, 0.3 mg, and <0.1 mg per 24 hr, respectively) (54). These losses are regulated in relation to iron storage status but within a narrow range (11). Claims that significant amounts of iron can be lost in sweat have not been confirmed in a recent study (13).

THE ADULT WOMAN Median monthly menstrual blood loss in the adult woman is between 20 and 30 ml. This loss increases median daily requirements from a basal figure of about 0.8 mg to 1.3 mg in a 55-kg woman. The ceiling iron loss for 95% of normal menstruating females is approximately 1.6 mg per day, which translates into a total daily iron requirement of 2.4 mg per day (11). Methods of contraception markedly influence iron losses. Oral contraceptives reduce losses by about 50%, whereas intrauterine devices increase losses by up to 100%.

PREGNANCY Approximately 1000 mg iron is needed by a 55-kg woman during a normal pregnancy. This requirement includes 230 mg for basal losses, 450 mg for an increased red cell mass, 270 to 300 mg for the fetus, and 50 to 90 mg for the products of conception (11, 56, 65). Since the greatest increase in fetal and erythropoietic requirements occurs later in gestation, the major requirements (5 to 6 mg per day) are in the second and third trimesters. The iron present in the expanded red cell mass is returned to stores postpartum, but this recovery is partially nullified by iron lost with peripartum blood loss (56). The total iron loss during pregnancy may be reduced substantially in developing countries, where the increase in red cell mass is limited by lack of available iron and where maternal and fetal weights are lower than in more highly developed countries. A period of amennorhea occurs postpartum, but iron losses via lactation are roughly equivalent to losses incurred via menstruation.

PATHOLOGIC LOSSES Epidemiologically, the most important pathologic losses of iron occur with hookworm infestation, which affects as many as 450 million people. It has been calculated that infestation with *Necator americanus*, (~5000 eggs per gram of feces) increases the daily iron requirement by 3–4 mg (75). It should, however, be emphasized that the hookworm load is small in the majority of subjects living in endemic areas and that other factors, such as the poor bioavailability of dietary iron, contribute to the widespread prevalence of iron-deficiency anemia in endemic areas.

Recommended Daily Intakes of Dietary Iron

Recommendations are that normal infants receive 1 mg iron per kg per day and that low-birth-weight infants receive 2 mg iron per kg per day (20). Such

requirements can be met only by fortification of infant formulas. The recommended amounts for children and male and female adolescents are 10, 12, and 15 mg per day, respectively (64). For women during the reproductive years; 15 mg per day is recommended and adult men and postmenopausal women require only 10 mg per day. The requirements of women in the second and third trimesters of pregnancy (5 to 6 mg per day) cannot be met by any diet, and supplemental elemental iron (30 mg per day) is required.

While these are useful guidelines, they do not allow for the considerable variations in the bioavailability of iron in different diets. In this context, significant numbers of apparently well people in more highly developed countries consume significantly lower amounts of iron than the recommended daily intake (5).

FOOD IRON ABSORPTION

The amounts of iron absorbed from the gut are determined by the food iron content, the composition of the ingested food, and the ability of the gastrointestinal tract to absorb iron.

Food Iron Content

Typical Western diets have a remarkably consistent iron content of approximately 6 mg per 1000 kcal. The major determinants of the absorption of this iron are the particular sources of iron within the meal. The iron in food is present either as heme or nonheme compounds. Heme iron is efficiently absorbed via specific, high-affinity, mucosal brush-border heme-binding sites (53). While absorption of heme iron is not influenced by the promoters and inhibitors of nonheme iron absorption (70, 81), it is enhanced by dietary protein, digestion products of which prevent the formation of poorly absorbed heme polymers (81). The bioavailability of the heme iron in meat is excellent. In contrast to heme iron, the absorption of the nonheme iron in food is profoundly influenced by the interplay of a number of enhancing and inhibitory iron-binding ligands in different diets (17). While most nonheme iron in the diet enters a "common pool" insofar as absorption is concerned, certain dietary components, such as ferritin and hemosiderin, are very poorly absorbed. Various forms of iron that often contaminate the diet in developing countries are also poorly absorbed (57, 74). As a result, the dietary iron content in these regions does not necessarily reflect the amount of bioavailable iron.

Food Composition and Iron Bioavailability

The absolute iron content of a diet is less important in terms of bioavailability than is the composition of the diet. This section highlights the major factors that modify nonheme iron bioavailability.

ENHANCERS OF NONHEME IRON ABSORPTION Not only does meat contain highly bioavailable heme iron, but it also can enhance nonheme iron absorption even from meals of low bioavailability (8, 72). This important property, which relates specifically to meat protein (8, 26), may involve the release of cysteine (73), cysteine-containing peptides (73, 105), and other peptide digestion products (67), or interaction with the carboxyl groups of amino acids (100). Current data suggest that 1 to 1.5 g meat is equivalent to 1 mg ascorbic acid (61, 89) in its ability to promote nonheme iron absorption.

Ascorbic acid is the most powerful promoter of nonheme iron absorption (3, 41, 52, 58, 99). It both reduces and binds dietary nonheme iron. A plot of the absorption of food iron against the molar ratio of ascorbic acid to iron yields a biphasic curve; a relatively greater ascorbate effect occurs with ratios <7.5 (80). The absolute effect of ascorbic acid in a particular meal depends on the relative proportion of ligands that promote or inhibit iron absorption (58). Prolonged heating leads to the inactivation of ascorbic acid. Nonheme iron absorption is also promoted by citric, malic, tartaric, lactic, and other organic acids. Citric acid promotes iron absorption from a number of fruits, including citrus products (3), and lactic acid promotes iron absorption from fermented cereal beers (42) and sauerkraut (52). Certain spices (69) and fermentation products of the soybean (85) have recently been shown to have promoting activity.

INHIBITORS OF NONHEME IRON ABSORPTION Polyphenols are secondary plant metabolites that are rich in phenolic hydroxyl groups (15). Both the hydrolyzable and nonhydrolyzable (condensed) polyphenols inhibit nonheme iron absorption (44, 52). The first polyphenols shown to have such an effect were the tannins in tea (44). Subsequent studies showed polyphenols in vegetables, such as sorghum and legumes, and in certain condiments to be powerful inhibitors of nonheme iron absorption (52, 97). The effect is dose related, with a near maximal inhibition of absorption (± 75%) at doses of ~50 mg.

Phytates constitute 1 to 2% (by weight) of many cereals, nuts, and legumes and represent the physiologic phosphorous storage mechanism of these plants (19). The bulk of in vivo evidence indicates that phytates inhibit dietary iron bioavailability (52, 62); 50 mg of phytates cause a reduction in dietary iron absorption of ~70% (60, 62).

Unlike proteins in meat, the plant proteins in soybeans, nuts, and lupines inhibit iron absorption (27, 40, 83-85). In vitro data suggest that high-molecular-weight peptides may be the inhibitory factors (67). Calcium and phosphorous, when fed together, inhibit food nonheme iron absorption (88). While data show that fiber components can inhibit iron bioavailability, there is no evidence that the intact dietary fiber complex inhibits iron absorption (1, 28, 46, 51).

Nutritional Implications of Dietary Composition

Individual foods can be divided into three categories based on their effects on the bioavailability of iron in the diet: low, medium, or high bioavailability (Table 1) (9, 89). The fact that the ultimate absorption of iron from any particular meal is the result of a complex interplay of promoting and inhibitory factors makes accurate predictions of the bioavailability of iron from a particular meal difficult. Certain general conclusions can be reached, however. A diet of low bioavailability (5% absorption in a subject with depleted iron stores) is a simple monotonous diet of cereals, roots, or tubers with negligible quantities of meat, fish, or ascorbic acid. This diet inhibits

Table 1 Relative bioavailability of iron in the presence of various dietary components

Dietary components	Bioavailability of iron		
	Low	Medium	High
Cereals	Maize Oat meal Rice Sorghum Whole wheat flour	Corn flour White flour	
Fruits	Apple Avocado Banana Grape Peach Pear Plum Rhubarb Stawberry	Cantaloupe Mango Pineapple	Guava Lemon Orange Papaya Tomato
Vegetables	Aubergine Butterbean Broadbean Lentil Spinach	Carrot Potato	Beetroot Broccoli Cabbage Cauliflower Pumpkin Turnip
Nuts	Almond Brazil Coconut Peanut Walnut		
High-protein foods	Egg Isolated soy protein Soy flour		Fish Meat Poultry

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BIOLOGIC CONSEQUENCES OF IRON DEFICIENCY

The most obvious result of iron deficiency is anemia, with all its well-known sequelae. Increasing evidence suggests, however, that iron deficiency also adversely affects other metabolic processes, including electron transport, catecholamine metabolism, DNA synthesis, and several enzyme systems (11, 25, 31, 66). Impairments in work performance, neurologic function, the immune response, and epithelial tissues have been attributed to iron deficiency.

Work and Exercise Performance

Data from animal studies indicate that iron deficiency, independent of anemia, produces specific enzymatic changes that lead to impaired endurance exercise performance (36, 47, 94). The endurance muscle fibers are selectively affected (86). The extent to which these findings are applicable to humans, however, is still not clear. Although iron-deficiency anemia has been shown to impair work performance in studies of both short-term exercise (7, 18, 50, 109) and more long-term manual labor (6, 45), it is difficult to distinguish between the effects of anemia per se and those of iron deficiency. Two attempts have recently been made to separate the two components. In one study, subjects were venesected to induce iron deficiency and then received transfusions to reverse the anemia rapidly without affecting the tissue iron deficiency (16). In the other study, work performance was assessed in subjects with polycythemia vera who had been rendered chronically iron deficient by venesection therapy (98). Neither study was able to demonstrate a disturbance of work performance that could be ascribed to a specific muscle defect.

Neurologic Function

Animals rendered iron deficient at an early age exhibit a depletion of brain iron and behavioral defects that are not totally corrected by iron administration (33, 34, 48, 111). Behavioral disturbances have also been noted in human infants with iron-deficiency anemia and are not reversible by use of either short-term (78, 79, 92) or more long-term treatment (78). In addition, follow-up studies of such children years later have reported the persistence of some behavioral abnormalities (38, 93, 102). In summary, reviews of current data indicate that iron-deficiency anemia has deleterious effects on neurologic function (77, 95) and that these effects may be only partially reversible. Iron deficiency without anemia has not been found to be associated with behavioral abnormalities (37, 78).

The Immune Response and Infection

Certain components of the immune response are disturbed in the presence of iron deficiency. Although the humoral component of the inflammatory response appears to be intact (25, 32), abnormalities have been described in cell-mediated immunity (32, 82, 96), in neutrophil function (91, 110), and in the secretory response of macrophages (63). The clinical significance of these findings is unclear, however. Indeed, whether predisposition to infection is the result of iron deficiency or iron-replacement therapy is not clear. Various explanations are given for these conflicting findings: inadequate diagnosis of iron deficiency, nonphysiologic forms of iron administration, the dependence of certain parasites such as malaria on adequate red cell numbers, nonrepresentative patient and control sampling, and socioeconomic variables (25, 32). Controlled clinical evaluation is urgently needed to resolve this issue.

Epithelial Tissues

The tissues of the gastrointestinal tract have been reported to be extremely sensitive to iron deficiency. However, glossitis, stomatitis, esophageal webs, and chronic gastritis may partly result from other coincidental deficiencies. Such manifestations are now rare, but atrophic gastritis has possible pathogenetic significance, since the resultant hypochlorhydria in itself limits nonheme iron absorption (11, 25, 31, 66).

PREVENTION OF NUTRITIONAL IRON DEFICIENCY

There are two approaches to iron deficiency: supplementation and fortification. Supplementation with pharmacologic amounts of oral iron is used to induce a rapid improvement in symptomatically deficient subjects and to prevent anemia in pregnancy. Fortification, on the other hand, seeks to improve the iron nutritional status of population groups on a long-term basis. Small amounts of iron are added to some staple dietary constituent and thus the compliance of individual subjects is not required. Since iron fortification represents the only potential way to address the global problem of iron-deficiency anemia, we briefly consider some of the principles involved in its application.

Iron Fortification

The choice of a suitable iron compound presents difficulties, since iron is highly reactive and can cause food discoloration and oxidative reactions. Ferrous sulphate is widely used in bread and bakery products because the storage time is usually brief. Ferrous salts are also employed in infant formulas. Elemental iron powder of small particle size has also been used in bread and flour fortification, but recent evidence indicates that one such

compound (carbonyl iron) is of low bioavailability (59). The chelator NaFeEDTA is stable and less susceptible to the inhibitory ligands present in cereal-based diets (71, 87). Another approach uses a relatively inert form of iron, such as iron orthophosphate, together with an enhancer of iron absorption (112).

The ideal vehicle should be processed centrally and consumed by the target population in fairly uniform quantities (29). It should have a brief storage period and should lend itself to the unobtrusive and inexpensive addition of the iron compound. The most commonly fortified vehicles in more developed countries are flour, infant cereals, and milk powder. For adequate absorption of iron these two latter vehicles should contain adequate amounts of ascorbic acid (42, 104).

Iron fortification is difficult in developing countries for a number of reasons. These include cost considerations, decentralized food production, and staple foods of low iron bioavailability that do not lend themselves to fortification. Several strategies have been employed in pilot fortification programs, including the use of NaFeEDTA as the iron compound and the use of sugar and condiments such as fish sauce, fish paste, and curry powder as the vehicles (4, 69, 71, 87, 108). In one novel approach, dried animal hemoglobin was baked into chocolate biscuits as part of a school lunch program (103).

Depending on circumstances, fortification programs can be applied nationally or targeted specifically at vulnerable groups (e.g. infants) (29). If a centrally processed dietary staple cannot be identified, then multiple regional programs may be necessary. Before embarking on such programs, the bioavailability of the fortificant iron from typical diets must be tested. Storage characteristics and consumer acceptability of the fortificant and the vehicle must also be established. If all preliminary tests are satisfactory, a pilot study can begin. In one recent study a targeted approach was successfully applied in which curry powder was the vehicle and NaFeEDTA the fortificant (4). Curry powder was chosen because it was widely consumed by the iron-deficient Indian population but not by blacks, in whom iron overload occurs commonly. In a double-blind trial conducted over two years, the prevalence of anemia in the group of women receiving fortification dropped from 22 to 5% (Figure 1).

Results of several field trials suggest that carefully planned iron-fortification programs are effective and safe (4, 29, 107). At a national level, however, pinpointing the individual reasons for improvements in iron nutrition is still difficult. For example, in Sweden hemoglobin levels of women have risen significantly in the recent past (55), despite evidence that the iron fortificant used in the diet is poorly bioavailable (59). Other factors that may have contributed include improvements in socioeconomic status, widespread

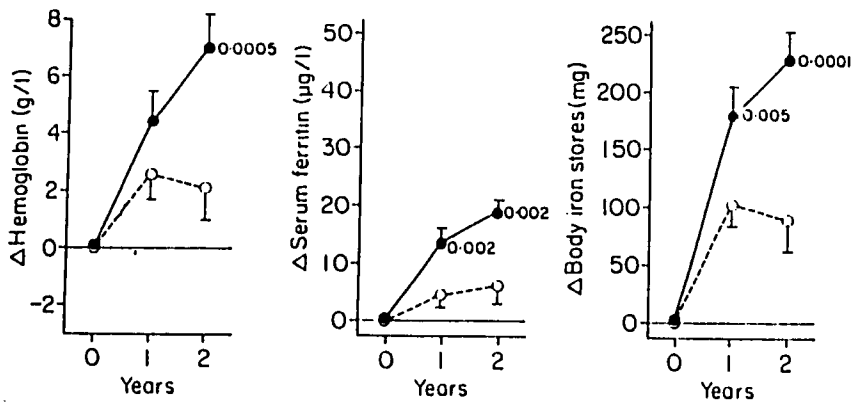

Figure 1 Changes in hemoglobin, serum ferritin, and calculated body iron stores (30) after one and two years in iron-fortified (*filled circles with solid line*) and control (*open circles with dashed line*) groups of Indian women. The fortificant was NaFeEDTA, and the vehicle was curry powder. Individual points represent mean changes ± SE. The probability (one tailed) using Student's *t* test that individual changes were greater in the fortified group than in the control group is also shown [data from D. E. Ballot and co-workers (4)].

consumption of over-the-counter iron and ascorbic acid tablets, and decreased menstrual losses due to the use of birth-control pills (55).

Questions remain about the potential hazards of increased iron fortification in whites because of the high gene frequency of the HLA-linked iron-loading gene, which can predispose homozygous carriers to clinical hemochromatosis (10). These doubts emphasize the need for careful monitoring in developed countries where iron fortification programs are in operation. While application of national fortification programs is widespread in more highly developed countries, where they are least needed, the absence of such programs in the developing world, where iron deficiency anemia is far more prevalent, is cause for concern.

ACKNOWLEDGMENTS

Supported in part by NIH grant DK-39246 and by AID Cooperative Agreement DAN-515-A-00-7908-00.

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APPENDIX B

JAMAICAN ANEMIA PROJECT

January 16, 1990

The following summarizes an interim evaluation of a project being conducted at the Caribbean Food and Nutrition Institute (CFNI) to assess the efficacy of a gastric delivery system (GDS) for iron supplementation in pregnancy. This evaluation was performed by James D. Cook as a consultant to the project. The following summarizes discussion held with Dr. Bill Simmons and Dr. Wynette Patterson from January 9 - 12, 1990. The study is being funded by the International Center for Research on Women (ICRW); funding will terminate on April 30, 1990. The final report for the project is due on June 15, 1990.

Briefly stated, the objective of this study is to examine the efficacy of GDS for supplying iron in pregnant women attending health clinics in Kingston, Jamaica. The subjects are randomized into three groups consisting of a control group, a conventional treatment group, and a GDS group. The three groups are given a placebo, 65 mg of elemental iron as ferrous sulphate twice daily and 50 mg elemental iron as GDS once daily, respectively. All women are given 0.4 mg folicin daily. The main objective of this review was to identify any problems arising during the final stage and to finalize plans for completing biochemical and statistical analysis.

Personnel

There have been some changes in personnel participating in this project since last reviewed in August of 1988. Maria Jackson continues as one of the two field supervisors and has remained in the study since its inception. The second field supervisor is Jean Jackson who has been engaged in the project for several months and is well acquainted with the conduct of the study at the health clinic level. Diane Mitchell, the prior laboratory technologist, was replaced by Marjorie Thomas in June 1989 and has performed the vast majority of the biochemical determinations. Adesh Singh has been replaced by Keith Bingham who has undertaken the statistical analysis. The latter is being performed using SPSS on the UWI (University of West Indies) mainframe computer. All of the current personnel will remain on the project until its conclusion at the end of April. In addition, it is hoped that Marjorie Thomas will be supported by later projects and remain as a technologist at CFNI for anemia studies. It is anticipated that she will spend 3-4 months in the summer of 1990 at Kansas University Medical Center for further training in enzyme-linked immunoassay techniques.

Study Progress

The initial study objective was to perform complete biochemical analysis after three months of iron supplementation in a total of 300 pregnant women, 100 in each of the three study arms. At this time 357 women have been entered into the trial and approximately 200 have completed the three month trial and given blood for the final biochemical measurements. Of the 157 women who have entered

the trial but not yet completed it, approximately 100 have dropped out of the study while an additional 50 women are at some point in the 3 months period of supplementation. The biochemical determinations are relatively current. However, all data has been entered for only about 80 women and there are a large number of completed questionnaires ready for data entry.

A persistent concern throughout this project has been the relatively high drop out rate. The cause of this was discussed in some detail. The main reason appears to be reticence to have the 6 week and 12 week blood sample drawn. Many women remain in the study until time for this blood sample and then simply do not return. Additional causes include reluctance to continue visiting the health clinic every two weeks. There is little hard data on the cause of drop out because of the difficulty in locating those who do not return to the health clinic. However, the field supervisors were strongly encouraged to document the precise causes for drop out in at least a subsample of those who did not return. Tracking of these women is difficult, but it is hoped that perhaps as many as 50% of those who did not return to the health clinic can be located and questioned. Apparently no one has withdrawn from the study because of side effects. Incidentally, it has not been difficult to recruit women for the study; the participation rate is in excess of 90% of those asked. The daily incentive apparently has little to do with this high participation rate. Rather, the women are aware of the importance of taking iron and participate because it is supplied at no cost. The travel incentive is

obviously not a strong incentive for remaining in the study given the high drop out rate.

Because there are no more funds after April, no additional women will be entered in the study after January 1990 to allow for the 12 weeks of supplementation. Thus perhaps an additional 10-15 women will be entered into the trial and the total number of participants completing the three month study will be in the order of 250 women. It was suggested during this review that it may be useful to continue recruiting women during February and early March and obtain only the 6 week follow-up sample. This would increase the number of women supplemented for at least 6 weeks.

Compliance Measurements

One of the objectives of the study was to determine whether there is any difference in compliance in women given two conventional ferrous sulphate tablets as compared to one GDS tablet daily. Thus, one questionnaire (B) was administered every two weeks during the three month supplementation. At the present time a total of 1656 of these questionnaires have been entered into the computer and are being analyzed. There are two independent objective measures of compliance in this study. First, the bottle containing the two week supply of tablets is returned for a tablet count. Secondly, the women are asked to state on how many days they took the medication. Preliminary statistical analysis indicates an extremely high correlation of approximately 0.9 between these two independent compliance measurements. None of the symp-

toms that were queried at these two week intervals have been analyzed.

Biochemical Determinants

Just prior to this review, biochemistry on approximately 80 women entered into the trial was analyzed at ICCNA. The preliminary results indicated a very satisfactory degree of intercorrelation between iron status measurements. Each of the main biochemical determinants were discussed during this review. The quality control sera for the ferritin assays are giving an acceptable degree of variability which ranges about ±20% between assays. Both a high and low quality control numbers are being entered to permit correction to a common quality control value at the end of the study if this seems desirable. Some interim analysis based on repeat measurements with and without a quality control correction will be undertaken during the next 4-6 weeks to determine the desirability of correcting results for quality control.

The measurement of erythrocyte protoporphyrin has posed some problems during this study. The major difficulty has been the breakdown of the hematofluorometer which was not available for several months during the conduct of the study. A large number of the protoporphyrin measurements were performed on samples that were frozen for several weeks prior to analysis. Protoporphyrins were also performed by the Piomelli extraction technique on numerous samples and there is an excellent correlation between these two values (approximately 0.85). It was decided that because of the

difficulty in performing the extraction technique (must currently be performed at UWI and 2-3 weeks advance notice is required to obtain laboratory facilities) and lack of protoporphyrin standards for this technique, no further determinations by the extraction technique are required. However, a special study is needed to determine whether hematofluorometer measurements on frozen samples give the same result on fresh specimens. This study will be initiated within the next two weeks.

An interesting finding during the preliminary analysis was a relatively low correlation between hemoglobin and hematocrit measurements on the initial biochemical sample. It should be noted that there are actually two hemoglobins available for comparison. The first was done with the portable instrument at the health clinic and second with the Coulter counter. The relatively poor correlation between hemoglobin and hematocrit were apparently based on data generated by the Coulter counter.

Finally, some discussions were held as to the desirability and timing of transferrin receptor measurements. Samples have been stored at -20°C for this purpose and will now be shipped to Kansas University in batches to undertake this determination. Some of these analyses may be performed by Marjorie Thomas during her visit to KU in the summer of 1990. Samples on each patient drawn at time 0, 6 weeks, and 12 weeks will be placed together in the shipment so that all can be performed within the same assay. Serum ferritin analysis may be performed on some of these samples as

well and this information will assist in determining whether the data should be corrected by quality control results.

Data Analysis

Data analysis is well underway and Keith Bingham is providing outstanding statistical support for this project. Much of this review was spent in discussing possible approaches to analysis and identifying the key biochemical determinants of efficacy. Mr. Bingham has had extensive experience with SPSS and in fact, is currently giving a course at CFNI in its use. Arrangements were made to send all of the biochemical results on questionnaire C to Kansas University at monthly intervals. The major purpose will be to identify algorithms that may assist in condensing the hematologic and biochemical determinants of iron status.

Extensive discussions were held as to the key objective of this study. Some consider the compliance data are the main objective whereas others believe that the focus is on the biochemical results. The biochemical and hematologic measures of efficacy should be the key parameters. Because a recent evaluation of GDS has shown that there are no gastrointestinal side effects associated with taking a single tablet daily in nonpregnant women, it is hoped that one GDS tablet will be as effective as two ferrous sulphate tablets daily. The statistical analysis should focus on the 6 week follow-up samples in particular. The 12 week sample will determine whether any benefit achieved after 6 weeks has been sustained. On a small subsample of approximately 60 women divided

into three groups, there has been an impressive hemoglobin response at 6 weeks in both groups receiving iron. This averages approximately 0.7 g% in those given GDS and 0.9 g% in those given ferrous sulphate. A slight decrease in hemoglobin levels in controls was observed (from 9.64 g% to 9.59 g%). Thus, based on hemoglobin levels alone, a relatively crude measure of iron status, a clear benefit of iron supplementation is being realized.

It will also be important to determine any differences in side effects among the three treatment groups and associated differences in compliance. However, this data, which was obtained at two week intervals, may not prove to be of major benefit because of repeated questioning on the same points. Nevertheless, the compliance data may assist in explaining any differences in hematological response among the three treatment arms.

Summary and Conclusions

In the present review, it has been found that the study is preceding well and that preliminary results indicate a highly satisfactory hematological response with either GDS or conventional ferrous sulphate. The total number of women completing three months of trial will be somewhat less than the 300 projected initially. No major problems in the biochemical or hematologic analysis have been identified. The high drop out rate appears to be similar in the three study groups and is largely due to reluctance to continue visiting the health clinic and submitting to further venous sampling. The major focus in these final months of the study will

be on the data analysis and this task is well in hand by the statistical consultant.

GRENADA FORTIFICATION PROJECT

SITE VISIT

MAY 13-18, 1990

The following is a brief outline of a site visit conducted in St. George's, Grenada, West Indies, from May 13-18, 1990 for the purpose of evaluating a proposed study of wheat flour fortification. The purpose of this visit was to revise a final protocol that will be submitted to AID for funding. The project will be conducted in Grenada under the direction of personnel from the Caribbean Food and Nutrition Institute (CFNI), Kingston, Jamaica. The funds will be administered by the Pan American Health Organization (PAHO), Washington, D.C. This evaluation was conducted by Dr. James Cook, ICCNA, and Dr. William Simmons, CFNI, who drafted the original proposal and will draft a revised proposal in response to our evaluation.

The starting point for this proposal was an assessment of iron status performed in the Grenadian population in 1986 which showed a disturbingly high prevalence of nutritional anemia in virtually all segments of the population. A survey of nearly 1000 subjects in the country revealed the following prevalence of anemia based on accepted WHO criteria: age 0-5 - 44%, age 6-14 - 37%, males aged 15-44 - 14%, females aged 15-44 - 33%, males over 45 - 35%, females over 45 - 24%, anti-natal - 63%, and post-natal - 47%. Serum ferritins were performed in this survey and established that the cause of this anemia was iron deficiency. At a workshop held in Grenada in 1986, a strategy for the control of anemia was

developed that listed the fortification of wheat flour as one of the potential interventions. At that time, approximately 60% of the wheat flour used within the country, so-called "counter flour" was not fortified with iron whereas the remaining 40% of "bakers flour" was fortified at a level of 17 mg iron/lb of flour. Because of the extensive use of counter flour by the lower socioeconomic segments of the population, it was felt that fortification of this product would reduce the prevalence of nutritional anemia significantly.

Key individuals contacted during this visit included Dr. D. Murray, Medical Officer of the Ministry of Health, Maria Noel, Ministry of Agriculture (Chairman, Food and Nutrition Council) and Mr. Cecil Hypolite, Operation Manager for the Caribbean Agro Industries, the milling company where flour fortification is performed. Meetings were also held with the Minister of Health who supports this project fully and will direct correspondence to Dr. Patterson, Director of CFNI, stating this fact.

Based on production figures for the years 1987 and 1988, sufficient flour is supplied within the country to give 145 g/person or 530 kcal, roughly 25% of the caloric intake. Production figures at Agro Industry indicate that there has been a gradual shift since the 1986 study to the greater use of bakers flour with the result that only 45% of the flour produced within the country now remains unfortified. Thus, based on a nationwide average, 65 g of unfortified flour/person/day is now consumed. If this flour were fortified to a level of 44 mg/kg (20 mg/lb) each person would increase

their iron intake by 2.86 mg/day. The potential absorption of this added iron is difficult to estimate. However, if one assumes that 10% is assimilated by an iron deficient anemic individual, this should produce an increase in hemoglobin concentration of approximately 0.5 g/yr or 1.5 g% over the three year life of the study. Absorption will be less in individuals with residual stores as reflected by a serum ferritin level >12 ug/L, but if they absorb roughly half the above amount an increase in serum ferritin of 5-10 ug/L could be expected over three years.

The general design of the study is as follows. During the first 6 months, an island wide survey of wheat intake will be performed, on a household basis, to estimate per capita intake of flour and the proportion of unfortified, or counter flour, within each household. This survey should permit realistic estimates of the nutritional impact of the fortified product. It is then planned to conduct a baseline household survey at the start of the fortification project and again 18 months and 36 months following the institution of fortification. These surveys will be performed on a household basis; a total of 600 households are projected. Some estimate within each household of wheat consumption will be made and capillary blood samples will be obtained for measurement of hemoglobin, ferritin, and transferrin receptor. It is anticipated that 2000 subjects will be sampled at each time. No effort will be made to monitor the same families longitudinally. Rather, a random sample will be performed at each of the follow-up times. No final decision was made in regard to whether a preliminary survey in addition to the baseline household survey is required since there

is obviously some duplication here.

Additional information will be needed on whether the firm that presently provides premixes to Agro Industries (Agro Chem Inc., Huntington, NY: Phil Schamberger) can provide the necessary premix. Reduced iron is currently used to fortify bakers flour at a level of 17 mg/lb. We would like to obtain a premix that would provide 20 mg/lb of flour as ferrous sulphate. It is hoped that Agro Chem can provide this product, but we were assured by Mr. Hypolite that they would be happy to use any premix supplied that was recommended as part of the protocol.

Government officials expressed some concern about the potential increase in price of flour due to fortification. Mr. Hypolite predicted that the cost would be less than $0.01/lb or roughly 0.5% increase in cost. He thought that it was quite likely that this cost would be absorbed by Agro Industries, but whether it will would depend on the stability of wheat prices. He felt that it was unlikely that they will have to increase the cost of counter flour. The feeders required for fortification are all installed and are presently being used for fortification of bakers flour.

A meeting was also held with personnel in the Ministry of Agriculture who may conduct iron analysis of the fortified product to verify fortification at the desired level. A new facility is planned with an anticipated completion date of January 1991. They are planning to obtain all of the equipment that will be required for chemical analysis of iron in fortified wheat. It was also men-

tioned by Mr. Hypolite that samples could be sent to their sister company in Puerto Rico where there is a lab equipped to perform these iron analyses. Thus, there are at least 2, if not more, options for performing quality control measurements on the fortified wheat and this again seems to present no problems.

One important feature of this visit was the enthusiasm of all those in Grenada for this project. Maria Noel has acquired a great deal of experience during the nutritional survey conducted in 1986 and is obviously capable of directing the work in Grenada. The CFNI laboratory is well equipped to perform the serum ferritin and probably transferrin receptor measurements. The laboratory monitoring will be simplified by using capillary samples and curtailing the number of iron measurements performed. The total duration of the study is expected to span 4 years leaving 6 months at the start for preliminary surveys and 6 months at the end for collating and analyzing the data. The major uncertainty at this point is when money will become available from AID in support of this project.

REVISED RESEARCH PROPOSAL ON

"INVESTIGATION OF THE IMPACT OF AN IRON SUPPLEMENTATION STUDY ON THE PROUCTIVITY OF WOMEN WORKING ON AN INDUSTRIAL ESTATE IN KINGSTON, JAMAICA"*

INTRODUCTION

Studies in Guatemala by Viteri[1], *et al,* have suggested that even mild anaemia in males results in decreased near maximal physical work performance, which can be returned to normal with iron supplementation. Viteri found that physical fitness varied inversely with the degree of anaemia. There is some evidence that mild to moderate anaemia may limit performance of less demanding tasks such as are involved in many occupational pursuits. In Indonesia, a correlation was shown between the work output of latex tappers and the haemoglobin concentration. The work output was significantly less in anaemic tappers than in non-anaemic tappers. Following treatment of the anaemia with iron the output of the anaemic group increased to that of their non-anaemic colleagues.

The possibility that iron deficiency might cause abnormalities in brain function was first investigated in schoolchildren. Webb and Oski[2] working with junior high schoolchildren in Philadelphia suggested that anaemia is associated with disturbances in attention and perception. Investigations conducted by Pollitt[3] and his coworkers in Central Java have shown a significant increase in haematological indices in iron deficient schoolchildren given iron supplementation over a three-month period. Changes in the iron status of iron-deficient anaemic children were also associated with significant changes in their performance on the school achievement and concentration tests. However, while the iron deficit was fully reversed with the iron supplementation, this did not occur in the case of the deficits in the achievement test.

In the proposed study the target population will be women engaged in the manufacture of garments. These tasks require dexterity and concentration, attributes similar to those investigated in schoolchildren.

*Prepared by Dr. A.W. Patterson, Director, CFNI; Mr. K. Bingham, Consultant Statistician, University of the West Indies, Jamaica; and Mrs. H. McKenzie, Senior Lecturer, Department of Sociology, University of the West Indies, Jamaica.

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It has been estimated by Hallberg et al[4] that Swedish women have an average basal obligatory iron loss of 0.8 mgms daily. It is suggested that women in the reproductive age group may have difficulty maintaining adequate iron nutriture from a non-iron fortified diet. This is especially so when dietary iron is mainly of the non-haeme variety. The non-pregnant, non-lactating adult female population in Jamaica has been reported[5] to have a 13-15% prevalence rate of iron deficiency; so it is expected that there will be a number of anaemic and iron deficient persons in the sample. It is to be noted that work output is profoundly influenced not only by health considerations but also by motivation, by environmental conditions such as lighting and ventilation, and by the availability of raw materials.

Although the relationships between iron status and physical work output have been demonstrated in other parts of the world, the results have not received attention in the Caribbean especially not at the level of the workplace. Moreover there is little, if any, research on the relationships between iron status and "intellectual" work in adults mainly because of the difficulty in establishing a reliable productivity measure. In this instance, one is in-built in the "equivalence system" devised for salary determination.

The Free Zone is an industrial estate developed in response to the Caribbean Basin Initiative, a collaborative trade programme between the USA and some of its neighbours.

In Kingston, Jamaica, the Zone comprises 14 companies, all but two of which are engaged in garment manufacturing. The companies each employ between 30 and 3000 workers who are paid on an incentive basis. Salary is computed on price per unit of output times quantity using the concept of the "Standard Hour", which establishes equivalences between tasks. Ninety-nine percent of workers are female, most are between 18-30 years of age.

The presence of the Free Zone in Kingston, with well-organized administrations capable of providing reliable records of workers, their output in "standard hour" equivalences, payments made, as well as a well functioning and competently staffed health facility, offers a remarkable opportunity for operational research of the nature suggested. The intervention can be readily and continuously monitored on site, as one of the benefits provided to the workers is free primary health care, delivered on site. The health clinic is staffed by a visiting medical doctor, a senior public health nurse, a registered nurse, and an office attendant. The clinic is open for service throughout the working day. The main health

problems relate to gynaecology, allergies and trauma. Pre-and postnatal, family planning and curative services are offered. The Caribbean Food and Nutrition Institute, also situated in Kingston, has an excellently equipped iron monitoring facility where the blood analyses can be speedily and competently carried out. The efficiency with which the study could be conducted is very attractive given the benefits which could accrue.

The study of a Caribbean female labour force also is of importance. The employees are mainly young women, a population group which contains the highest unemployment levels in the country. Some have major financial responsibility as heads of single-parent households. The Five-Year Regional Plan of Action on Women in Health and Development notes that women carry an increasingly heavy burden in maintaining the family while working outside the home, and that their socio-economic and health conditions are becoming increasingly important as crucial determinants of the prospects of development. Jobs in the Free Zone are eagerly sought and demand rapid acquisition and maintenance of a high productivity level. The physical fitness of such workers should be an important social and medical concern, and the results of this study will be of importance to political, economic, health and other social interests.

The results of this study also will be of interest to both the management and the workers, since it is expected that the iron supplementation intervention could result in higher productivity and an opportunity for workers to increase their earnings.

PURPOSE OF STUDY

This study seeks to explore the relationships between iron deficiency anaemia and work output and to examine the impact of an iron supplementation intervention on task performance among a group of women workers in the Free Zone who are engaged in garment manufacturing tasks which require dexterity, skill and attention.

RESEARCH HYPOTHESES

The central hypothesis is that iron deficiency can adversely affect productivity in a group of female Free Zone workers. The following consequences of the hypothesis will be examined by the research:

1. Anaemic workers will exhibit lower levels of productivity than their non-anaemic counterparts.

2. The level of productivity will have a direct relationship to the level of iron status.

3. Workers provided with iron supplementation will exhibit increased productivity.

4. Workers provided with iron supplementation will exhibit improved haematological indices.

5. Any increased productivity noted during the period of the treatment will be retained during the six-week follow-up period. The study will also explore relationships as follows: (1) supplementation and job satisfaction; (2) supplementation and improvement in iron status indices; and (3) supplementation and compliance.

METHODOLOGY

Two to three companies will be selected for the project: Jamtex which manufactures jeans, and Jamaica Needlecraft which manufactures female underwear and possibly one other.

The site has been visited and the concept introduced and discussed with management. The workers will be approached when funding is secured and consent to participate obtained in writing (Appendix 1).

After the definitive study, the results will be presented and discussed with participants. Should the results of the intervention be positive, discussions concerning the sustainability and possible replication of the intervention will be undertaken. Given the low cost of the intervention, it is anticipated that management will consider it cost-effective to provide the supplement as a benefit and incentive to workers.

The preliminary results will be presented at the Commonwealth Caribbean Medical Research Council (CCMRC) Meeting in 1991, and published. Subsequently other papers will be prepared for publication.

The study seeks to investigate the relationship of iron deficiency anaemia and work output among a group of low income women. The work output in these circumstances is dependent not only on a level of physical wellness but particularly on dexterity, attention span, and skills' learning ability, all of which are cognitive functions thought to be influenced by iron nutriture. The study participants are women in the child bearing age group. Pregnant or lactating women will be excluded. There are several aspects of the study e.g. level of training, experience on-the-job, which will require strict controls.

The research can be characterized as a randomized, double-blind, tightly controlled clinical trial. The central feature is the comparison of changes in work productivity in two equal sized groups, one of which will receive a placebo while the other will receive 60 mgms. of bioavailable iron supplementation daily over a 12-week period. Historical control is provided by a two-week observation period prior to the experiment so that baseline levels of productivity can be ascertained. Concurrent controls, as well as controls for the Hawthorne effect, are provided by a smaller group of workers drawn from each experimental factory who will not be aware that they are involved in the study. A six-week follow-up period will be used to observe the extended effects of the treatment on

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productivity. Production data on workers from all three groups will be recorded over the 20-week period of the study. The design may be tabulated thus:

Time (Weeks)	Instrument or Supplementation	Control Group (N=60)	Placebo Group (N-170)	Fe Supplementation (N=170)
0	Demographic Data Sheet	x	x	x
2.	Pre-questionnaire Pre-supplementation haematology and body mass index (wk 0-2)	x	x	
4				
6				
8	Daily supplementation with weekly compliance measures and haematology (twice) during study week 2-14		x	x
10				
12				
14	----------Post Questionnaire--------		x	x
16	Observation of extended effects of supplementation			
18				
20	----------Debriefing----------			

(Productivity data from companies' records will be obtained on all subjects throughout the 20 weeks.)

SAMPLE SIZE

The total number of workers under study will be 500 taken from two factories to allow for a 25% attrition rate. The various target subsamples are as follows:

	Control Group	Placebo Group	Iron Group	
Factory 1	30	85	85	
Factory 2	30	85	85	
Totals	60	170	170	400

Each of the factories to be studied has over 350 female workers on piece-rates, which will permit clear productivity documentation. Experimental, Placebo and Control Groups will be randomly selected out of these numbers of workers. Haematological data will be collected from the Experimental and Placebo Groups at the beginning, halfway mark, and end of the observation period.

The rationale for the choice of sample size is three-fold. First, samples must be large enough so that any experimental effect that exists can be observed. While we may prove an effect of supplementation on productivity, we cannot prove a lack of effect. Hence the need exists to have a 'large enough' sample, and generally 'the larger the better'.

Second, groups must be large enough so that inferential tests can be applied to subgroups as required. For example:

1. The placebo group within each factory (85 workers) is large enough to allow comparison of the productivity of anaemic and non-anaemic subgroups.

2. The number (170) of workers within a factory is large enough to allow comparison of the two experimental groups within the subsample of experienced workers.

Third, one must allow for some dropouts from the study, for any of the following reasons: (a) leaving factory or changing job; (b) unwillingness to tolerate needle; (c) clear lack of interest in iron supplementation; (d) real or imagined side effects; (e) sickness, absenteeism, etc. Since iron supplementation is generally regarded as beneficial we expect a low dropout rate. The initial sample size will therefore be 500 to allow for a possible 25% attrition. **Data for workers who fail to persistently register 80% compliance (80% of required pills) or who otherwise drop out will be excluded from the analysis.**

Significance tests will include two-way analyses of variance which will test differences in average productivity levels among four groups, before, during and after treatment:

1. Anaemic Experimental Group (receiving iron)

2. Non-anaemic Experimental Group (receiving iron)

3. Anaemic Placebo Group

4. Non-anaemic Placebo Group.

It is expected that there will be a substantial homogeneity of variance within these samples because management states that low productivity performers (below 85% efficiency) are not usually kept on the job.

In addition, differences between the mean productivity levels of the Experimental, Placebo and Control Groups will receive significance testing. All results will be judged at a 95% confidence level.

INSTRUMENTS

The following instruments will be required:

1. Socio-demographic data sheet - data to be collected from company records.

2. Weekly productivity data sheet - data from company records.

3. Pre-questionnaire - will elicit background information on socio-economic status, dependents and health status (Appendix 2).

4. Post-questionnaire - this will provide useful cognitive and affective information on subjects in the treatment groups (Appendix 3).

5. Compliance form. Compliance will be estimated by two methods: (1) questioning the participant and (2) counting pills in returned pill boxes. Method (2) is the method of choice, but if the pill box is not returned, Method (1) will be used as back-up.

6. Blood chemistry form - this will be completed three times and will include the following haematological values obtained from venous blood samples: haemoglobin, haematocrit, serum ferritin. It will also include measures of weight and height from which body mass index will be computed.

7. Productivity will be measured from the companies' records relating to targets set and identified tasks turned in and judged to be accomplished satisfactorily by the supervisor. Identified tasks will be those with equivalency ratings, as workers are expected during the 20-week period to be shifted from one location on the production line to another.

EXPERIMENTAL CONTROLS

To succeed in demonstrating an effect of iron supplementation on productivity, where an effect indeed exists, tight controls and a powerful intervention will be required. The measures to be taken in this regard include:

1. The allocation of an adequate time to the supplementation;

2. The utilization of an appropriate level of iron supplementation;

3. Attempts to measure and ensure compliance by weekly monitoring through the Free Zone health post.

4. Complete equivalence in the handling of groups: (a) double blind strategy, (b) concurrency, (c) equivalent pill format;

5. Attempts to ensure low drop out rates;

6. Randomized allocation to groups, followed by careful check for equivalence;

7. Obtaining management's cooperation to ensure minimal environmental changes, and monitoring this by observation of the productivity in the control group;

8. Giving careful instructions to participants regarding the purpose of the study - making sure that both treatment groups receive the same information;

9. Tracking dropouts to determine reasons.

A local pharmaceutical company (Federated Pharmaceuticals) has agreed to manufacture the iron supplement and placebo. The Regional Drug Testing Laboratory in Kingston will be responsible for the quality control of the supplementation.

Participants will be instructed that this is an investigation of the effect of iron supplementation on health improvement. A brief statement of health indices, as measured, will be provided to each participant at the end of the study. Subjects will not be informed initially that productivity is being investigated.

Approval for the conduct of the study will be sought from the University of the West Indies Ethical Committee on Medical Research.

TESTING THE HYPOTHESES

Having established the equivalence of experimental groups as well as the stability of the control group productivity throughout the 20-week period, we will be in a position to test the consequences of the central research hypothesis (see page 3).

1. Within each of the treatment groups we can compare the productivity of anaemic and non-anaemic workers.

2. Within each treatment group we can look for correlations between productivity and the various haematological indicators.

3. The productivity of placebo and iron groups will be compared at various stages of the 12-week treatment period.

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4. We will compare the haematological indicators across the two treatment groups.

5. We will look for trends in both productivity and the haematological indicators, especially during the 6-week follow-up period.

Further, using data from the post-questionnaire, we will try to determine whether workers are aware of any changes that have taken place in either body chemistry or productivity during the period of the experiment. How do they feel (during the follow-up period) about iron supplementation?

TIME FRAME

1. Pilot study to test some aspects of the design and to prepare and test instrument (pre-study)

2. Preparation and selection of sample: 4 weeks

3. Pre-intervention study: 2 weeks

4. Intervention/analysis of data/analysis of blood - 12 weeks

5. Post-intervention study - 6 weeks

6. Analysis of blood/analysis of data 12 weeks. Sub-total: 36 weeks

7. Report - 10 weeks

Total 46 weeks.

BUDGET (US$)

PERSONNEL	
Interviewers and blood collection (Free Zone Nurses)	5,300.00
Biochemist	6,000.00
Lab assistant	1,200.00
Data entry (6,875 records)	3,000.00
Data analysis (2 months)	4,000.00
Part-time secretary	1,000.00
SUPPLIES	
Ferrous sulphate	375.00
Placebo	368.00
Lab and field supplies	3,500.00
Scale for clinic	550.00
OTHER	
Cost to link CFNI microcomputer to UWI main frame	2,000.00
Attendance at CCMRC:	
Graduate medical student	1,000.00
Free Zone public health nurse	1,000.00
DIRECT COSTS	2,929.00
Total	32,222.00

Note: Program Support Cost of 13% not included.

BUDGET CLARIFICATION

1. Study Groups

Complete data on 400 subjects:

Placebo - 170
Iron - 170
Control - 60

Assuming 25% dropout rate:

Placebo - 213
Iron - 213
Control - 75

Tablet Calculations (12 Weeks)

7 tablets/week - 84 tablets/patient; 84 x 425 = 23,004 tablets/study group - purchase therefore 25,000.

Calculation of Length of Study

20 samples/day; 4 days/week implies 80 samples/week. Time required to recruit 500 patients = 3 weeks. Add 12 weeks for last recruit to complete implies 15 weeks. Add 8 weeks to complete laboratory and statistical analyses 23 weeks. Therefore, proposed length of haematologic aspect of study is 6 months.

2. Lab and Field Supplies

Ferrous sulphate	-	25,000 @ $4.30/1,000	=	US$375.00
Placebo	-	25,000 @ $43.56/2,000	=	US$368.00

EDTA tubes	
Serum tubes	
Haematocrit tubes	
Critoseal	
Pasteur pipettes	
Micro centrifuge tubes	
Plastic storage tubes	US$3,500.00
Pipette tips	
Vacutainer needles	
Reagents for cyanomethaemoglobin Method	
Reagents for ferritin	
Acid for acid baths	
Examination gloves	
Gloves for acid bath	
Miscellaneous	

Salaries (6 Months)

Biochemist	-	US$1,000/month
Technician/lab helper (4 hours/month)	-	US$ 200/month

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3. Interviewers

		Encounters	Price J$	Total (J$)
Patients	- 500			
Interviewers	- 2	1,000	$5.00	5,000.00
Blood	- 3	1,500	5.00	7,500.00
Weigh	- 1	500	1.00	500.00
Distribute and check pills	- 12	6,000	4.00	2,400.00
				37,000.00

J$37,000.00 = US$5,300.00

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APPENDIX 1 - CONSENT FORM

I (name of person) consent to participate in a health study being done by the Caribbean Food and Nutrition Institute (CFNI) at the Free Zone where I am employed.

I agree to give a blood samples three times during the study for testing of my blood for anaemia and understand that I shall be given a report of the results. I also agree to answer the questions put to me in the questionnaire..

I understand that I can refuse to answer any question to which I object and that my answers and blood results will be kept confidential.

Signed:

Date:

APPENDIX 2: SOCIO-ECONOMIC

Request consent to participate. If obtained, proceed.

1. Name _____

2. Date of Birth _____

3. Address _____

_____

SOCIO-ECONOMIC

4. Did you complete primary school? Yes [ ] No [ ]

5. How many persons live in your household?

   One person [ ] Two to three persons [ ] Four to five persons [ ]

   Six to seven persons [ ] Eight to nine persons [ ] Ten or more persons [ ]

6. How many rooms does your household occupy? (Not including bathroom and kitchen)

   One [ ] Two [ ] Three [ ] Four [ ] Five or more [ ]

7. What do you use to cook your meals?

   Electric stove [ ] Gas stove [ ] Kerosene stove [ ] Coal pot [ ] wood [ ]

Which of the following do you have?

8. Fridge Yes [ ] No [ ]

9. Radio Yes [ ] No [ ]

10. Television Yes [ ] No [ ]

11. Telephone Yes [ ] No [ ]

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MOTIVATION TO WORK

12. Do you have children? Yes [ ] No [ ]
    If yes, how many are you financially responsible for? _____

13. How many dependents do you have:

    None [ ] One or two [ ] Three or four [ ] Five or six [ ] Seven or more [ ]

14. Apart from you, is there any other member of your household who works?

    Yes [ ] No [ ]

15. Do you have any source of income apart from your salary? Yes [ ] No [ ]

16. Are you able to save any money from your salary? Yes [ ] No [ ]

17. Do you work overtime? Yes [ ] No [ ]
    If no why not?

    No overtime is available [ ] I don't have time [ ]
    I feel too ill [ ] I'm not interested [ ]
    Other specify _____

    _____

18. Are you satisfied with the rate of pay you receive? Yes [ ] No [ ]

19. In general, do you like the conditions at your workplace? Yes [ ] No [ ]

PERCEPTION OF HEALTH STATUS

Please complete the following by putting an (x) in the appropriate space.

At work:	Very Often	Often	Sometimes	Seldom	Never
20. I get headaches	[ ]	[ ]	[ ]	[ ]	[ ]
21. I feel dizzy	[ ]	[ ]	[ ]	[ ]	[ ]
22. I get palpitations	[ ]	[ ]	[ ]	[ ]	[ ]
23. I feel tired	[ ]	[ ]	[ ]	[ ]	[ ]
24. My limbs ache	[ ]	[ ]	[ ]	[ ]	[ ]
In general:					
25. I have heavy menses	[ ]	[ ]	[ ]	[ ]	[ ]
26. I often go to the doctor	[ ]	[ ]	[ ]	[ ]	[ ]
27. I feel well	[ ]	[ ]	[ ]	[ ]	[ ]
28. I do exercise	[ ]	[ ]	[ ]	[ ]	[ ]

29. Do you suffer from any disease? Yes [ ] No [ ]

    If yes, what disease? _____

30. Have you had any infection during the past month? Yes [ ] No [ ]

31. During the past month did you use any antibiotics? Yes [ ] No [ ]

APPENDIX 3: ATTITUDINAL QUESTIONNAIRE

ATTITUDINAL QUESTIONNAIRE

Please respond to the following questions in the following way:

ATTITUDE TOWARDS SUPPLEMENTARY IRON	Strongly Agree	Agree	Undecided	Disagree	Strongly Disagree
1. I do not like iron tablets	[ ]	[ ]	[ ]	[ ]	[ ]
2. I would use iron tablets often if they are supplied.	[ ]	[ ]	[ ]	[ ]	[ ]
3. I take iron tablets often	[ ]	[ ]	[ ]	[ ]	[ ]
4. Even if I am healthy, I would take iron tablets.	[ ]	[ ]	[ ]	[ ]	[ ]
5. I get enough iron from foods so I do not need iron	[ ]	[ ]	[ ]	[ ]	[ ]
ATTITUDE TOWARDS THE JOB					
6. I feel that my job is very important	[ ]	[ ]	[ ]	[ ]	[ ]
7. I do not like my job.	[ ]	[ ]	[ ]	[ ]	[ ]
8. I would do any other job if I have the chance to.	[ ]	[ ]	[ ]	[ ]	[ ]
9. My job is very hard for me	[ ]	[ ]	[ ]	[ ]	[ ]
10. I enjoy doing my job.	[ ]	[ ]	[ ]	[ ]	[ ]
11. I get bored doing the same job everyday	[ ]	[ ]	[ ]	[ ]	[ ]
12. I am never late for work	[ ]	[ ]	[ ]	[ ]	[ ]
13. I put every effort into my job	[ ]	[ ]	[ ]	[ ]	[ ]
ATTITUDE TOWARDS INCENTIVES					
14. Compared with other jobs the pay here is very good	[ ]]	[ ]	[ ]	[ ]	[ ]
15. I am looking forward to a promotion	[ ]	[ ]	[ ]	[ ]	[ ]
16. If pay rises I would produce more	[ ]	[ ]	[ ]	[ ]	[ ]

ATTITUDE TOWARDS INCENTIVES (CONT'D)	Strongly Agree	Agree	Undecided	Disagree	Strongly Disagree
17. Health care is a good benefit to workers here	[ ]	[ ]	[ ]	[ ]	[ ]
18. I *like* piecework because I can earn more	[ ]	[ ]	[ ]	[ ]	[ ]
ATTITUDE TOWARDS MEALS					
19. Meat is expensive but I always buy it	[ ]	[ ]	[ ]	[ ]	[ ]
20. I do not like kidney and liver	[ ]	[ ]	[ ]	[ ]	[ ]
21. I often eat oranges with my meals	[ ]	[ ]	[ ]	[ ]	[ ]
22. Eating callaloo is healthy	[ ]	[ ]	[ ]	[ ]	[ ]
23. I eat bread or other flour products almost every day	[ ]	[ ]	[ ]	[ ]	[ ]
24. I eat meat at least once every day	[ ]	[ ]	[ ]	[ ]	[ ]
25. I would like to eat meat every day	[ ]	[ ]	[ ]	[ ]	[ ]

Project Proposal

Etiology and Epidemiology of Anaemia in Ethiopia

ENI

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Etiology and Epidimiology of Anaemia

Introduction

Anaemia is commonly defined as a state in which the individuals' concentration of Haemoglobin or packedcell volume is below normal for the age sex and physiological status of the patient.

The causes of anaemia are multiple but deficient diet and infectious diseases are most common. It is particularly prevalent in young children, lactating women, women of reproductive age group and during pregnancy.

Anaemia is one of the World's Public Health problems and the problem is more severe in Developing countries. The available data have indicated that the prevalence in Temperate Zones ranges from 10-15% of menstruating women, and from 20-30% of pregnant women due to iron deficiency. In infants, the frequency of anaemia may be as high as 40% in the first year of life. In Tropical areas, because of parasitic infections, prevalences of between 20% and 40% have been found in men. The prevalence in women ~~are~~ is, of course, even higher and the anaemia often more severe.

The three broad categories of anaema, namely nutritional anaemia, anaemia associated with chronic infections and anaemia associated with heredity abnormalities of the Haemoglobin molecule are wellknown. Surveys in many countries have indicated that nutritional anaemia is of paramount public health importance. Because some infestations increase the requirement for haemopoietic nutrients and accelerate the development of nutritional anaemia deficiency. Besides, in a community with a high prevalence of nutritional anaemia, there may also be a high prevalence of anaemia due to haemoglobinopathy. (WHO 1978)

Thus nutritional anaemia is a condition in which the haemoglobin content of the blood is lower than normal as a result of a deficiency of one or more essential nu nutrients, regardless of the cause of such deficiency (WHS 1982)
The main haemopoietic mutrients are Iron, folate,

Vitamin B_{12} and proteins, less important nutrients include Ascorbic Acid, copper, cobalt, pyridoxine and vitamin E. However, Iron deficiency in the diets is most frequent.

Children, pregnant and lactating women and women in the reproductive age group are vulnearble with regard to nutritional anaemia. They are vulnerable primarily because of their great nutritional needs for Iron. The amounts needed to cover the requireemnts for growth during childhood or of the feotus during pregnancy and to replace the losses of Iron via the breast milk during lactation and haemorrhage at delivery and Iron losses in the blood due to menstruation, usually exceed the quantities of Iron available from the daily diet. This is specially true when the diet contains few animal products or is entirely vegetarian.

The prevalence of folate deficiency as a public health problem is uncommon. In developed countries, surveys in apparently elederly populations have shown low folate concentrations in 10-15% of the population, but there is no evidence that these levels were related to any ill-health. When folate deficiency of pregnancy is complicated by other factors such as for example malaria , seckll cell diseases, that increase folate requirements, severe folate deficiency may occur that require appropriate publich Health measures, vitamin B12 deficiency is also uncommon in most parts of the world except in areas where populations live on a very strict vegetarian diet:-

Protein deficiency is a major problem and protein is essential for the formation of haemoglobin. The shortage of protein can be expected to play some part in the wide spread of anaemia when protein lack is sufficient enough to cause anaemia. Over and above, P.E.M. depresses the immune system and is commonly complicated by anaemia resulting from infections and deficiencies of Iron and folate.

Past works in nutrition indicated that nutritional anaemia is uncommon in Ethiopia because of high Iron content of the diet (ICNND). But Hofvander '1968) in his Haematological studies in Addis Abeba showed that the prevalence of Anaemia was 5.3% in preschool age children, 7.8% in school age children, 30% in adolescents 7% in adult-males and 8.2% in adult females, with the prevalence of anaemia in Rural village, Ijaji, was 80% in infants, 48.1%, in preschool age children, 68.6% in school age children, 69.2% in adolescents, 44.6% in adult males and 53.7% in adult non pregnant females, and the overall prevalence in the area was 60% out of which 17% was of Iron deficiency origin. The difference in the prevalence of anaemia in Rural village has been described to be due to food pattern mainly of milk and bulky dietary gruel for the age group 1 1/2 - 2 years and high diarrhoeal incidence responsible for low dietary Iron intake and absorption respectively.

Nutritional anaemia is also a reflection of the communities dietary situation and consumption pattern which in turn is a function of production. The major dietary sources are cereals and plants (Ensete-false banana), and the percentage of animal and vegetable sources in some parts of Ethiopia has been practically nil except in nomadic areas.

However, dietary surveys carried out in the past showed that the iron intake was more than adequate level, but in most tropical countries the dietary iron intake could be equal to or greater than that recommended for adequate diets, but because of bulky carbohydrate foods and high contents of phosphates and phytates with low calcium lavels that the iron bioavailability from cereals is low.

Anaemia is caused by infectious diseases. Malaria schistosomiasis and hook worm infectious are known as the major causes. In Ethiopia, communicable diseases account for about 80% of the causes of morbidity and mortality, out of which malaria and schistosomiasis are among the top leading causes.

Malaria has a prevalence of 30% and is found below altitudes of 1600 meters. Schistosomiasis is also highly prevalent in altitudes of 1000-2000 meters with a prevalence of 20-80%. It is most common in the northern part of the country.

Hook worm infections are relatively high in the south western regions where there is high moisture and warmth which supports the transmission of the disease. Hence, as all evidences indicate that anaemia might be a public health problem in the population. To sum up, the prevalence of anaemia has not yet been studied in the general population. Although, the irion content of the diet is high, the poor bioavailability of dietary iron from cereals compounded by meagre or practically absent animal products in the diet and high prevalence of diseases known to be of major causes of anaemia in the whole country explains the potentialities for high prevalence of anaemia in the rural community; particularly Nutritional Anaemia in those population groups of great nutritional needs. Thus, the purpose of this study is to describe the Etiology and to measure the incidence and distribution in the general population with the objective of designing appropriate intervention programme for all affected.

2. Objectives

2.1 To collect information on population groups with respect to the prevalence, severity and distribution of anaemias in selected communities.

2.2 To establish specific causes of anaemias in different population groups.

2.3 To design appropriate intervention strategy based on the outcome of the study.

3. Methodology

3.1 Study population

The requirements of dietary iron differs according to the physiological status of individuals. Infants, because of their rapid growth, the requirement of dietary iron is high beyond the age of 6 months, as breast milk cannot meet the requirement.

Low birth weight infants require also greater amount of exogenous Iron due to a more rapid postnatal growth since breast milk alone will not meet the demand. The highest prevalence of iron deficiency is most likely to occur between the ages of 6/12 - 3 years of age as bulky carbohydrate diets are unfavourable for iron absorption. Iron requirements of children are closely related to growth, and increase during periods of rapid growth both in preschool and school age children. There is also a further increase of iron requirements at the onset of menstruation, pregnant and lactating mothers have increased physiological demands for iron for the growing featus and to replace iron lost in the breast milk.

Thus, as staples of cereals and legumes are poor sources of bio-available iron, dietary iron deficiency is expected to be relatively common among those groups whose iron demands are high.

Infants, preschool and school children, pregnant and lactating aswell as fertile mothers are also vulnerable to a variety of infectious disease. The study will therefore consider these sectors of the population in the following groupings:- Adults of both sexes are included in the study primary to establish a full spectrum of the condition.

1. Infants - 1 year of age
2. preschoolers - 1 - 4 years of age
3. School age children - 5 - 9 years of age
4. Adolescents - 10 - 14 years of age
5. Male adults - 15 - 44 years of age
6. Female adults - 15 - 44 " " "
7. Pregnant women -
8. Lactating mothers -

3.2 Sampling Design and Site selection

In this study design, information on the geographic distribution of major dietary sources has been obtained and the dietary iron content of major sources has been computed using Ethiopian Food Composition Table, for all regions. Obviously, there are regions of "high and low" available dietary iron. Additional information collected on the prevalence of diseases that are associated in the cousation or aggravating anaemia has also indicated high and low prevalence rates in all regions. Based on the available information to date, all Administrative Regions have been stratified with reference to the amount of dietary iron assumed to be obtainable.

More on, further stratification has also been done with the purpose of forming a more homogeneous regions from the point of view of dietary iron availability and prevalence rates of associated diseases. Each substratum is exclusively characterised by one of the following categories:-

- High Iron intake and high disease prevalence
- High Iron intake and low disease prevalence
- Low Iron intake and low disease prevalence
- Low iron intake and high disease prevalence.

Making into account the prevalences of the different infectious disease and and dietary iron availability, a study site will be identified in accordance with the following matrix.

Dietary	Iron	High intake of Iron	Low intake of Iron
Malaria	High	x	x
Prevalence	Low	x	x
Schisto-somiasis	High	x	x
Prevalence	Low	x	x
Hook Worm	High	x	x
Prevalence	Low	x	x

As from the matrix the maximum number of survey sites is twelve However, the possibility that a site represents more than one site of variables can reduce the number of survey sites. The selection of sites will be made at two stages:

Firstely, Regions iwth High and Low prevalences of the diseases mentioned above and with staple foods of "High and Low" iron content will be selected. From preliminary information available from various sources 5 regions namely Gojjam, Wollo, Shoa, Wollege, and Gamu Gofa have thus been identified.

Secondly, specific localities that meets the discription given above will be chosen. This will be possible after reconnaissance visits to the Regions to collect data Required:

3.3. Sample Size

The sample size (n) for each survey site is fixed to be 400. If a strata variances differ, the Optimum sample allocation among strata is given by

$$n_h = \frac{N_h \sigma_h}{\Sigma N_h \sigma_h} n$$

Where Nh= Total elements in the population in stratum h

h= Standard Deviation in stratum h

n= The total sample size

nh= Optimum Sample size in stratum h.

The Etiological investigations will be carried out in all cases wity Homoglobin concentrations lower than the standard for the specific population group and altitude of the survey area.

The study subjects will be selected by Systematic Random Sampling Technique and an individual is considered as the unit of sampling.

3.4. Clinical Exams:

In this study, only apparently healthy subjects will be considered fit and clinical exams will be done on each subject to screen those with chronic abnormalities or diseases.

3.5. Nature of Indicators

3.5.1. Haemoglobin:- This is used to measure prevalence of anaemia. Cyanmthhaemoglobin method is used because of its stable solution of Cyanmthhaemoglobin that can be standardised accurately. The accuracy is automated photometers is from ± 1-2% and 2-5% in standard manual techniques. The Haemoglobin concentration values defined (WHO. 1968) below which Anaemia could be considered to exist of persons residing at sea level is as follows:-

- Children aged 6 months to 6 years = 11 gms%
- Children aged 6-14 years = 12 gms%
- Adult males = 13 gms%
- Adult females - nonpregnant = 12 gms%
- Adult females - pregnant = 11 gms%

N.B. An approximate correction for altitude can be calculated by increasing the values by 4% per 1,000 meters elevation (INACG 1979)

3.5.2. Haematocrite:- Haematocrit determination will be carried out as a reliable alternative to the analysis of Haemoglobin concentration in screening for Anaemia.
The micro method is used and it is relatively more efficient. The normal Haematocrit values for males and females is as follows.

Males is 47±7;

Females 42±5

A value below the normal range for age and sex indicates Anaemia.

3.5.3. Blood Film:- It is used to determine the morphologic type of anaemia. The morphlogical picture of a well developed Iron deficiency anaemia has a marked Hypochromic and microcytic erythrocytis. A blood picture of anaemia caused by folate and or vit. B_{12} deficiencies is characterised by Macrocytosis Sickle-all Anaemia is also recognised by observing Sickle-all forms in the film.

3.5.4. Serum Iron:- It is used when diagnosis on clinical grounds is not possible to establish Iron deficiency anaemia. It reflects short term balance and has a considerable diurinal variation in the Iron level which is high in the early morning and low in the evening. To this effect, all blood samples will be collected between 9 a.m. - 8 p.m. The normal values range from 50-150 ug/100 ml and values below this range indicate Iron deficiency anaemia.

3.5.5. Serum Ferritin:- Reflects the size of Iron stores and serum concentration of less than 10 ug/ml is a characteristic of Iron deficiency Anaemia, and values of 10-20 ug/ml are considered to be presumptive but not diagnostic of Iron deficience anaemia. It is determined by Radio-Isotopic assay.

3.5.6. Serum and RBC Folate:-

This is also determined by Radio-isotopic assay and the normal level below which folate deficiency is characterised is as follows:-

- Serum folate:- Adult normal values = 5-21 ug/ml
- RBC folate:- Adult normal values = 160-640 ug/ml

3.5.7. Serum Vit B_{12}:-

Vitamin B_{12} deficiency is determined by Radio-immuno-assay and the normal serum value ranges from 150-1000 pg/ml and values less than 100 pg/ml is indicative of deficiency.

3.6. Parasitology

3.6.1. Blood film:- This will be carried out to observe blood parasites such as malaria.

3.6.2. Stool Exams:- Stool exams will be done for possible observation of Hook worms in the subjects sampled.

3.7. Transport of Specimens:- The observations of blood films and stool exams will be complete at each study site.

However, Haematological specimens will be collected in heparinized test tubes and Biochemical specimens will also be collected in sterill test tubes. Eventually, all specimens will be kept in deep freeze and will be transported frozen to the Central Laboratory, Addis Ababa.

3.8. General Information:- A format will be organized to collect information on major dietary sources, major constituents of the diet, method of preparation, food taboos and intrafamily food distribution systems. These formats shall be answered by a group of 3.5 of known women and men in the locality.

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