IRON DEFICIENCY AND SUSCEPTIBILITY TO INFECTION:

A prospective clinical study of the effects of iron deficiency and iron prophylaxis in infants in Papua New Guinea

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SUMMARY

Investigation of the relationship between iron deficiency, iron supplementation and susceptibility to infection, was suggested by the author's initial observations of an association of anaemia with serious bacterial infections in infancy in Papua New Guinea. The bulk of previous longitudinal clinical intervention studies in infancy showed beneficial effects of iron supplementation. However, defects of control and design and recording in these studies and contradictory anecdotal reports left the question unresolved.

A prospective, placebo controlled, randomised, double blind trial of iron prophylaxis (3ml intramuscular iron dextran = 150mg Fe) to two month old infants was carried out on the North coast of Papua New Guinea where there is high transmission of malaria.

A literature review, pilot studies, protocol, demography, geography and laboratory methods developed are described.

Findings indicate that the placebo control group became relatively iron deficient over the first year of life and that the iron dextran group had adequate, although not excessive, iron stores and a higher mean haemoglobin; however, the prevalence and effects of malaria recorded in the field were higher in the iron dextran group. Analysis of field and hospital infectious morbidity in the trial indicated a deleterious association with iron dextran for all causes including respiratory infections (the main single reason for admission). Total duration of hospitalisation was significantly increased in the iron dextran group. Analysis of other factors showed (1) a higher admission rate associated with low weight-for-height recorded at the start of the trial; (2) a significant positive correlation between birth haemoglobin and hospital morbidity rates; (3) increased malaria rates in primiparous mothers of the cohort

infants who received iron infusion during pregnancy; (4) lower relative risk of malaria associated with iron prophylaxis in individuals with alpha thalassaemia, which was found to be highly prevalent in this region.

In conclusion, it is suggested that policies of iron supplementation, total dose iron injection and routine presumptive iron therapy for anaemia which are widely in practice in malaria endemic areas should be closely reviewed.

CHAPTER 1

BACKGROUND LITERATURE REVIEW

THE CLINICAL EFFECTS OF IRON DEFICIENCY AND IRON SUPPLEMENTATION

INTRODUCTION

Iron is indispensable as a micronutrient to almost all living organisms (Anon: Nutrition Reviews, 1979). Its ready ability to exchange electrons gives iron a key role in biological oxidative and reductive processes which is enhanced by combination with a variety of complex molecules which are classified as haem or non-haem. Competition for iron could be a controlling factor in the relationship between vertebrates and micro-organisms. Both groups have developed powerful iron binding proteins (transferrins, lactoferrins and siderophores which may play a role in this relationship (Neilands, 1981, 1982; Weinberg, 1971, 1974, 1978; Bullen et al, 1978b; Kochan, 1973)).

Iron deficiency may be relatively recent in human evolutionary history, as hunter gatherers turned to agriculture (Fleming, 1982), but is now the commonest micronutrient deficiency in the world (Baker and DeMaeyer, 1979). The lowest non-haem iron values in humans have been recorded in India (Charlton et al, 1970) coastal Papua New Guinea (Disler, 1973) and Burma (Aung-Than-Batu, 1972), but iron deficiency is not confined to the third world. Recent reports confirm that it is still the most important nutritional deficiency in America particularly in infancy (Lane and Johnson, 1981; Andelman and Sered, 1966).

The simplistic approach that any possible iron deficiency should be treated may not necessarily be in the best interests of the patient or community. The need for intervention should be determined by the degree of iron deficiency in the individual group and knowledge of its

effects on quality of life, morbidity and mortality. If it could be shown that latent or mild iron deficiency had no adverse effects or, as some claim, that it might even protect against infection, then a programme of community prophylaxis would at best be costly interference and at worst potentially harmful. Conversely if iron deficiency was shown to prejudice intellectual development or increase mortality from infection there would be an obligation to provide routine prophylaxis to those at risk. For these reasons it is crucial to define and quantify under controlled conditions, the physiological effects of iron deficiency.

Certain clinical findings such as anaemia, angular stomatitis, glossitis, dysphagia, hypochlorhydria, koilonychia and pica are well known and accepted effects of iron deficiency. There are many other symptoms which clinicians associate with the condition but which remain unproven effects of iron deficiency. These "soft signs" include, loss of energy, easy fatiguability, lack of concentration, irritability, poor school performance, anorexia and increased susceptibility to infection. Some of these effects are not directly related to haemoglobin level.

Definitions of Iron Deficiency

There is no single discriminating test for iron deficiency. This is partly due to intrinsic variations in the tests themselves and also variation in the biological functions they represent.

A normal adult contains between 3 and 5 grams of elemental iron. The term newborn has 75 mg/kg (Widdowson and Spray, 1951). 65-70 per cent is in the form of haemoglobin; 25% is in the form of non-haem iron stores (ferritin and haemosiderin) ; myoglobin accounts for about 4%; less than 1% is contained in the cytochromes, catalases, peroxidases and other iron containing enzymes (Oski, 1979).

A three stage scheme for evaluating iron deficiency has been proposed (Cook and Finch, 1979). The first stage is a reduction of iron stores, often called iron depletion or prelatent iron deficiency. In this stage serum ferritin falls roughly in proportion to non-haem iron stores. In the second stage , called iron deficient erythropoeisis , iron stores are completely exhausted and haemoglobin synthesis is impaired, erythrocyte protoporphyrin levels increase and transferrin saturation falls (Charlton and Bothwell, 1982). Circulating haemoglobin levels however remain in the normal range although iron treatment will raise the levels slightly. In the third stage restriction of iron supply to the marrow causes haemoglobin levels to fall below the accepted normal range for the population producing "iron deficiency anaemia". The main feature is a reduction in the concentration and amount of haemoglobin in individual red cells. The stained blood film shows hypochromia, microcytosis, anisocytosis and poikilocytosis and sometimes abnormal cells including elliptocytes, pencil shaped cells, and target cells (de Gruchy, 1978). The mean corpuscular haemoglobin concentration (MCHC) falls below 30g/dl (Dacie and Lewis, 1975), mean cell volume (MCV) below 75fl (Herschko et al, 1981) and mean corpuscular haemoglobin (MCH) below 25pg (Hershko et al, 1981). The fall in MCV tends to show greater relative deviation from normal than haemoglobin (Koerper et al, 1976).

Although it is convenient to classify iron deficiency in these three stages the profile of tests in individual patients, may not conform; e.g. patients with iron responsive anaemia and some abnormal tests may have normal serum ferritin levels (Dallman et al, 1981; Saarinen and Siimes, 1978a; Krause and Stolc, 1980).

Tests for Iron Deficiency: The following tests are used in assessing the iron status of individuals or populations : haemoglobin (Hb); packed cell volume (PCV); mean corpuscular haemoglobin concentration

(MCHC); mean cell volume (MCV); free erythrocyte protoporphyrin (FEP); serum iron (SI); total iron binding capacity (TIBC); transferrin saturation (TS); serum ferritin (SF) and stainable iron in bone marrow (Cook and Finch, 1979; Cook et al, 1976; Cavill, 1982).

Haemoglobin as measured by cyanmethaemoglobin method is a simple and accurate test (Dacie and Lewis, 1975), but has disadvantages; (a) a significant fall only occurs in the third stage of iron deficiency; (b) it is affected by age, sex, haemoglobinopathies, deficiency of other haematinics, chronic infection, altitude and possibly by race (Saarinen and Siimes, 1978b; Lundstrom and Siimes, 1980; Dallman and Siimes, 1979; Bentley, 1982; Dallman et al, 1979; Reeves et al, 1981b; Cartwright, 1968).

Haematocrit (PCV) Estimation of packed cell volume has the advantages of simplicity, can be done on capillary samples, is subject to fewer technical errors than haemoglobin estimation in unskilled hands and is suitable for low budget laboratories in the third world.

MCHC, being a derived ratio, from Hb and PCV, is subject to great variation. It has the advantage that after the first few months of life it is less affected by age than any other red cell index (Matoth et al, 1971; Guest and Brown, 1957). In iron deficiency the fall in MCHC may be exaggerated due to plasma trapping (England et al, 1972). In laboratories with electronic counters, other red cell indices for iron deficiency have superseded the MCHC.

MCV. With the advent of the electronic counter MCV has become a practical, accurate test. Its theoretical advantage over haemoglobin is that a fall is specific to the hypochromic anaemias including the thalassaemias (Kwaku and Schwartz, 1980). The disadvantage of MCV is the developmental change that occurs in infancy and throughout childhood (Saarinen and Siimes, 1978b; Hows et al, 1977; Dallman and Siimes, 1979).

FEP. Protoporphyrin accumulates in developing red cells when there is insufficient iron available to form haem. FEP is measured by a fluorescent test (Piomelli et al, 1976). FEP levels tend to rise above 100 ng/dl of red blood cells in stage 2 iron deficiency (Bothwell et al, 1979). Advantages of FEP estimation are that it is rapid, simple, can be performed on capillary samples and discriminates between iron deficiency and thalassaemia in patients with microcytosis (Stockman et al, 1975). Disadvantages are that it is raised in lead exposure (Stockman et al, 1975) and early infancy (Koenig et al, 1977).

Serum Iron (SI), total iron binding capacity (TIBC) and percentage transferrin saturation (TS). SI and TIBC are measured photometrically (ICSH, 1978). TS is obtained by dividing SI by TIBC and the result expressed as a percentage. TIBC tends to change reciprocally with iron stores in iron deficiency (Morton and Tavill, 1977). TS is now commonly used as a screening test. It tends to fall below 16% in iron deficient erythropoesis (Cook and Finch, 1979). Serum iron, however, shows wide diurnal and day to day variations (Schwartz and Baehner, 1968; Cavill, 1982) and tends to fall in acute infections, (Beresford et al, 1971; Torrance et al, 1978; Fernandez, 1980). TIBC is a more stable measurement, but TS is subject to the same variation as SI. TIBC can discriminate between the low TS of chronic inflammation and that of true iron deficiency. It falls in the former and tends to rise in the latter (Bentley, 1982). TS appears to be lower in infants and young children and increases gradually throughout childhood (Koerper and Dallman, 1977).

Serum Ferritin. SF is measured by immuno-radiometric assay (Miles et al, 1974) or by enzyme linked immuno-assay ELISA (Anaokar et al, 1979). Capillary methods have been developed (Segall et al, 1979; Pintar et al, 1982). The logarithm of SF appears to correlate in

health with the logarithm of non-haem iron stores (Bezwoda et al, 1979; Blunden et al, 1981). The logarithmic transform is necessary because of the non-normal distribution of serum ferritin values. A serum ferritin of less than 10 µg/L represents reduced or absent stores in any circumstances (Worwood, 1979). The converse is not true. Serum ferritin levels may be normal or elevated in iron deficiency. Levels may be raised in inflammatory disease particularly of the liver and in malignancies (Worwood, 1979). Saarinen and Siimes (1978a) found that in infants signs of iron deficiency could occur before serum ferritin reached subnormal levels. In rapidly growing infants iron deficient erythropoesis correctable by oral iron may occur owing to inadequate mobilisation of iron stores (Lundstrom, 1980).

Bone Marrow Iron Stores. The presence of stainable iron is strong evidence against depleted iron stores, but absence is not conclusive evidence of iron deficiency since iron may still be available in the form of histochemically unstainable ferritin (Ludin et al, 1964).

In view of the relative unreliability of individual tests for iron deficiency various proposals have been made to sharpen discrimination. One method is to use a battery of the tests available (Cook et al, 1976). Distribution analysis based on the assumption of normal distribution of haemoglobin values in a non-anaemic population, has been used to determine the proportion of anaemic individuals in that population (Cook et al, 1971). From a theoretical point of view the absolute discriminator should be the response of individuals to iron supplementation (Garby, 1970).

Dallman et al, (1981) gave oral iron to 188 infants with haemoglobins below 11.5 g/dl. Thirty-five percent had a rise of 1 g/dl Hb or more. The use of any additional test criteria (abnormal

MCV, FEP, TS or SF) as a discriminator for therapy would have excluded a large number of responders. In a similar study on Eskimo children it was found that of four pre-treatment tests (Hb, SF, TS and FEP), a haemoglobin one SD below the mean for age and sex was the most effective predictor of response to therapy. Using combinations of tests improved specificity at the expense of sensitivity (Margolis et al, 1981).

Physiological Effects of Iron Deficiency

Physiological effects of iron deficiency may result from lowered haemoglobin or may be unrelated to haemoglobin level. In practice it can be difficult to separate these.

Effects due to Low Haemoglobin. A low haemoglobin impairs the oxygen carrying capacity of blood for which there are various compensatory mechanisms. Ventilation rate is increased at rest but maximum ventilation cannot be increased (Davies et al, 1973). Cardiac output is increased mainly by tachycardia (Davies et al, 1973) and there is shunting of peripheral blood flow to vital organs. However, maximum cardiac output is no greater than in normals (Viteri and Torun, 1974). Oxygen delivery to tissues may be more efficient owing to a shift to the right of the oxygen dissociation curve. Possible mechanisms are reduced buffering capacity of the blood with an increased "Bohr" effect as CO_2 is released from tissues, and raised 2, 3 - DPG content of red cells of anaemic patients (Viteri and Torun, 1974).

The effectiveness of these compensatory mechanisms is proved by the observation that subjects with a mean haemoglobin of 6.7 g/dl, when required to perform at maximal capacity, achieved 66% of the maximal oxygen uptake of normal subjects (Davies et al, 1973). The relationship between chronic anaemia and physical work capacity has been studied using the Harvard Step Test (HST). There appeared to be nearly a linear relationship between log HST score and Hb over a wide

range of haemoglobins from 4 g/dl to 16 g/dl; r = 0.72 (Viteri and Torun, 1974).

Edgerton et al, (1981) studied maximum physical work in iron deficient subjects with haemoglobins ranging from severely anaemic to normal. Maximum physical work correlated positively with haemoglobin levels between 2.5 and 10 g/dl. Work capacity improved 83% after transfusions to the most severely anaemic patients. They concluded that haemoglobin per se, rather than non- haemoglobin related biochemical effects, was mainly responsible for decreased work capacity. In the Third World the earning capacity of manual workers may be seriously affected when Hb falls below 7.9 g/dl (Vaughan et al, 1973). Edgerton et al, (1979) showed that improvement in productivity was greater in the more anaemic subjects when workers were supplemented with iron.

Anaemia due to iron deficiency in pregnancy is a major cause of morbidity. Even moderate maternal anaemia as in β thalassaemia trait may cause foetal growth retardation (Fleming, 1974; Singla et al, 1978), an increased risk of preterm delivery and foetal distress. When the maternal haemoglobin is below 7 g/dl there is a large increase in foetal mortality. In the untreated severe anaemias of pregnancy more than 30% of infants may die of hypoxia (Fleming, 1982).

Maternal morbidity increases when haemoglobin is less than 7.0 g/dl and breathlessness occurs even at rest. Cardiac failure which is often fatal may supervene when maternal haemoglobin falls below 4.9 g/dl. Before the introduction of exchange transfusion and rapidly acting diuretics the death rate in Nigerian mothers presenting with a PCV less than 13% and engorged neck veins was 55 percent (Fullerton and Turner, 1962).

The effect of maternal iron deficiency anaemia on iron metabolism in the newborn and infant is fourfold. 1) Maternal anaemia is a

cause of low birth weight and total body iron is closely related to birth weight (Widdowson and Spray, 1951). 2) There is a higher incidence of preterm delivery with maternal anaemia and percentage total body iron progressively increases with gestational age (Widdowson and Spray, 1951). 3) Infants of low birth weight tend to have a high postnatal growth rate and use up iron stores more quickly than normals (Dallman et al, 1980). The fourth factor which is more controversial, relates to the effect of maternal iron status on foetal iron stores and total body iron. That there is an active transplacental concentration gradient is inferred by higher foetal serum irons and transferrin saturations (Lanzkowsky, 1976). Lanzkowsky (1976) reviewed the literature and his own experience and found no convincing evidence of a depressive effect of maternal iron deficiency on this process. Several other groups have found no significant relationship between maternal and cord serum ferritins (Hussain et al, 1977; Rios et al, 1975 and Bratlid and Moe, 1980) and Jansson et al, 1979 found no difference in serum ferritin of infants born to mothers with serum ferritins above or below 12 ng/ml. Murray et al, (1978a) found no differences in cord haemoglobins and transferrin saturations between babies of 19 iron deficient mothers (mean Hb 9.2 g/dl) and 24 normal mothers. Ferritins were not measured. In contrast, Singla et al, (1978) found highly significant correlations between maternal haemoglobin, cord haemoglobin, and serum iron in 85 mothers grouped from severely anaemic (mean Hb 4 g/dl) to normal. MacPhail et al, (1980) found significant differences between serum ferritins of infants born to mothers with serum ferritins above or below 50 ng/ml. A proportion of the mothers had had iron supplements during pregnancy. Fenton et al, (1977) found cord serum ferritins to be significantly lower in infants of mothers with serum ferritins less than 12 ng/ml.

Many of the studies which failed to find a relationship between maternal and foetal iron status were undertaken on small numbers or involved mothers who were not severely iron deficient.

Non-haematological effects of iron deficiency. A small amount of iron is involved in non-haematological processes. The biochemical and ultrastructural correlates of tissue iron deficiency are poorly understood (Jacobs, 1969, 1982; Dallman et al, 1978). Apart from its effect on iron containing enzymes and proteins, iron deficiency may have a direct effect on DNA synthesis and cell division (Robbins and Pederson, 1970; Hershko et al, 1970; Hoffbrand et al, 1976).

The tissue effect of iron deficiency on muscle is hard to separate from the effect of anaemia, and, for obvious reasons, experimental work in humans is very limited. In iron deficient rats, muscle oxidative power is reduced (Hagler et al, 1981; Mackler et al, 1978). Finch et al, (1979) noted lactic acidosis in exercising iron deficient rats and suggest it was due to a deficiency in α-glycerophosphate oxidase. In an elegantly controlled series of experiments Finch et al, (1976) separated haemoglobin effects from tissue effects by exchange transfusion. At a haemoglobin compatible with normal work performance, iron deficient rats showed impairment of running ability which could be corrected by iron therapy in 4 days.

In a controlled study of iron supplementation in tea pickers in Sri Lanka, Edgerton et al, (1979) concluded that there was an effect on work tachycardia within 4 days of starting iron therapy which could not be explained by haemoglobin changes.

Epithelial tissues. Iron deficiency affects many epithelial structures. In the epidermis loss of hair, poor hair growth, and dry fissured skin have been noted (Heilmeyer and Harwerth, 1970). Nails become ridged and fissured and in the more marked cases koilonychia occurs. Beveridge et al, (1965) found koilonychia in 30% of iron

deficient adults. Koilonychia also occurs in iron deficient infants (Hogan and Jones, 1970) but should be differentiated from congenital koilonychia.

Angular cheilitis, stomatitis and glossitis are all features of iron deficiency. In a quantitative study Rennie et al, (1982) showed deficiency in the maturational compartment of human buccal epithelium. Jacobs (1961) noted a decrease in cytochrome oxidase content of buccal epithelium.

There is an association between edentia, glossitis and stomatitis (Beveridge et al., 1965). Fletcher et al., (1975) noted an association between the buccal lesions and candida infection. Saliva from their iron deficient patients supported candida growth better than controls.

Dysphagia has been reported in from 5% (Jones, 1961) to 20% of adult iron deficient patients (Lundholm, 1939). The dysphagia is characteristically at the post cricoid level. In Jones' series (1961) half the patients investigated by barium swallow had post cricoid webs. Dysphagia with or without a post cricoid web in iron deficiency anaemia is variously known as the Paterson-Kelly (Paterson, 1919; Kelly, 1919) or Plummer-Vinson syndrome (Vinson, 1922).

Gastritis, hypo- and achlorhydria, are well known associations with iron deficiency in adults, (Beveridge et al., 1965). Uncertainty remains as to whether iron deficiency is the primary aetiological factor or arises secondary to the gastritis (Jacobs, 1982).

In infancy and early childhood, gastrointestinal pathology caused by iron deficiency may be difficult to differentiate from pathology due to cow's milk protein intolerance with secondary iron deficiency. Naiman et al., (1964) described 14 infants and young children with iron deficiency anaemia, associated with prolonged excessive milk ingestion, among whom there was a high incidence of achlorhydria,

malabsorption, occult blood in stools and evidence of chronic duodenitis and mucosal atrophy on intestinal biopsy. These abnormalties were infrequent in a control group of 8 children with anaemia from other causes. All the abnormalities in the iron deficient children improved on iron therapy while still on a milk containing diet. The authors concluded that their patients had primary iron deficiency and not a milk induced enteropathy. Guha et al., (1968) also noted structural and functional changes in the gut in iron deficient children which recovered on iron therapy.

Less clear cut results were reported by Lanzkowsky et al., (1981) who studied 29 children under 30 months of age with severe iron deficiency anaemia which was associated with exudative enteropathy in seven, malabsorption in ten and positive stool guaiac tests in fourteen. All children were treated with iron for six to ten weeks with no change in their milk intake. All guaiac tests became negative with haematological recovery and all cases of malabsorption returned to normal on iron therapy alone. Only two of the seven children with exudative enteropathy showed complete haematological and biochemical recovery on iron therapy. The remaining patients required milk free diets in addition to iron before they showed complete recovery. Some patients with lactose deficiency improved on iron therapy alone. Wilson et al., (1974) using a radio labelling technique showed significant intestinal blood loss in infants fed whole cow's milk which did not diminish on iron therapy and was directly related to the quantity of milk ingested. Kuitunen et al., (1973) have demonstrated mucosal damage to a milk challenge, similar to that observed with gluten challenge in coeliac disease, in children with cow's milk intolerance.

Iron deficiency may impair iron absorption from the gut. Kimber and Weintraub (1968) demonstrated impaired Fe^{59} labelled haemoglobin

absorption in severely iron deficient children and dogs. Cytochromal oxidase and lactase levels were decreased in the small bowel mucosa of the iron deficient dogs. Gross et al., (1976) reported iron malabsorption in iron deficient children that was corrected by parenteral iron administration.

The polar view is taken by Woodruff (1981) and Woodruff & Clark (1972), who suggest that cow's milk is solely responsible for malabsorption and exudative enteropathy and that iron has no specific beneficial effect on intestinal function.

Central Nervous System. An adverse effect of iron deficiency on the central nervous system has long been suspected but systematic study of possible effects has only recently begun.

Pica, the eating of unusual or non-food stuffs occurs quite commonly in under 3 year-olds and pregnant women and an association with iron deficiency has been suggested. Mengel et al., (1964) described geophagia (clay eating) among negro women in the southern states of America in whom iron deficiency is also common. But according to Jenkins (1980) there is good evidence that geophagia is a widespread cultural practice. Pagophagia (ice eating) appears to be more specific for iron deficiency states, and responds rapidly to iron treatment before any haematological improvement (Reynolds et al., 1968).

Cognitive studies. There have been a number of studies on human cognitive function and behaviour in iron deficiency. Webb and Oski (1973) compared 92 anaemic high school students with non-anaemic controls. The anaemic students performed worse on "Iowa Tests of Basic Skills". Werkman et al., (1964) followed 28 infants with iron deficiency anaemia and 28 controls for one year. They found more abnormal behaviour such as crying, fearfulness and irritability in the anaemic group, but attributed these abnormalities to parental failure

rather than iron deficiency. Beller and Howell (1971) reported that anaemia (9.0-10.5 g/dl) had no effect on IQ but was associated with lack of attentiveness, a narrower attention span and more aimless manipulation. Sulzer et al., (1973) noted a decrease in IQ in anaemic 4-5 year-olds and also a defect in associative learning. All of these foregoing studies suffer from the disadvantage that they were cross-sectional and not controlled for social and environmental covariants.

Cantwell (1974) noted an increase in "soft neurological signs" in 32 six to seven year-olds who had iron deficiency anaemia in infancy when compared with 27 controls who received intramuscular iron dextran at six months.

Oski and Honig (1978) assessed 24 iron deficient infants 9-26 months of age using the Bayley Scales of Infant Development. Infants were randomly assigned to receive intramuscular iron or placebo and retested five to eight days after injection. Improvement was noted in the children who received iron, for reactivity, gross muscle movements and fine motor co-ordination but attention span showed no significant intergroup differences. More recently Deinard et al., (1981) divided 212 non-anaemic infants of 11 to 13 months of age into three groups according to serum ferritin levels. Thirty-four had SF less than 9 ng/ml; 21 had SF 10-19 ng/ml; 157 had SF of 20 ng/ml and over. Bayley scales were tested in all infants as in Oski and Honig's 1978 study, as well as other tests for psychological development. No significant differences were demonstrated in overall performances between the three groups. But, as the authors admit, even the group with SF <9 ng/ml were only in stage 1 iron deficiency and this study can make no predictions for Stage 2 and 3 iron deficiency.

In a careful study in Guatemala, Lozoff et al., (1982) psychologically tested 68 babies with and without iron deficiency

anaemia before and after one week of oral iron or placebo therapy. Bayley Scales showed developmental deficits in the anaemia group which showed no significant improvement after one week on iron. Multiple regression analysis was used to control for independent social and anthropometric variables and results indicated a probable real effect due to iron deficiency anaemia. Pollitt et al., (1982) compared anaemic 3-6 year-old children with matched controls using discrimination learning tasks. Iron deficient children showed relative performance deficits in simple tasks, but not in more complex ones. Differences disappeared after 11-12 weeks of iron repletion.

There have been a number of attempts to identify a biochemical lesion in the brain in iron deficiency. Interest has centred around the finding of depressed monoamine oxidase (MAO) activity (Youdim et al., 1975) and increased urinary norepinephrine excretion (Voorhess et al., 1975). An experimental study in the rat (Mackler et al., 1978) showed no alteration in brain MAO but depression of aldehyde oxidase with elevation of serotonin and 5-hydroxy-indole compounds in the brain. A more disturbing finding was that young rats fed iron deficient diets between 10 and 48 days had permanently depressed levels of brain iron in spite of subsequent dietary correction (Dallman et al., 1975).

Growth. The time honoured concept of the fat, flabby infant with iron deficiency was questioned by Judisch et al., (1966) in a retrospective study of 156 infants with hypochromic anaemia. They found the majority to be underweight for age and children with the lowest weight centiles had a higher incidence of low birth weight. There was a noticeable improvement in weight centiles on iron medication. Tonkin (1970) reported that Maori infants who received iron dextran at birth had a greater weight gain than controls but gave no statistical evaluation of the small differences observed. Conversely in Andelman

and Sered's (1966) massive, controlled, prospective study of iron fortification of cow's milk given to infants in Chicago, there were no anthropometric differences observed between treatment and control groups in spite of large haematological differences. James and Combes (1960) also found no nutritional differences between study and control groups in their prospective study of intramuscular iron to preterm infants.

IRON AND SUSCEPTIBILITY TO INFECTION

Different authors claim either that iron deficiency helps or hinders defences against infection. The controversy is compounded by confusion between different physiological states of iron balance both normal and abnormal, one-sided arguments, predominance of *in vitro* over *in vivo* work, and in particular the lack of adequately controlled prospective clinical studies.

Many reviews have been published or refer to the subject (Weinberg, 1971, 1974, 1978; Stockman 1981; Jacobs, 1969, 1982; Buckley, 1975; Committee on Nutrition, 1978; Anon, 1974, (Lancet); Lukens, 1975; Beard *et al.*, 1981; Anon, 1975; Kochan, 1973; Bullen *et al.*, 1978b; Oppenheimer and Hendrickse, 1983).

The most comprehensive review for the clinician remains that by Pearson and Robinson (1976).

To discuss previous work, it is necessary first of all to define terms of reference for "iron status", then to examine the effects of iron status on immune defence systems, and interactions with individual infecting agents, before looking at *in vivo* experimental and clinical results.

Iron status:

Without interference from the clinician the body may be in 3 broad states of iron equilibrium: depleted, normal, and chronically overloaded (as in haemochromatosis or thalassaemia major). The first

two states have been described earlier in the Chapter. Overload occurs in homozygous β thalassaemia (and older patients with homozygous sickle cell disease) and primary and secondary haemochromatosis, where transferrin saturation is very high. In the most severe cases transferrin may be fully saturated allowing low molecular weight iron to circulate loosely bound to albumen (Hershko et al., 1978; Batey et al., 1978; Anuwatanakulchai et al., 1984; Wang et al., 1986; Wagstaff et al., 1985; Gutteridge et al., 1985).

The fourth iron state is "under treatment" and is not an equilibrium. The effect of iron treatment on body iron compartments depends on the dose, the route of administration and is also dependent on the time elapsed after administration. In the assessment of the effects of iron status on susceptibility to infection, the equilibrium iron status should not be confused with the temporary effects of iron therapy (Stockman, 1981). There is a massive but temporary hyperferraemia following administration of iron parenterally, which lasts up to 3 weeks following intramuscular iron dextran (Will, 1968) or 2-3 days after intravenous iron dextran (Kanakakorn et al., 1973). The effect is not seen with oral iron supplementation in normal doses (Gross, 1968), although gut intraluminal iron may be high (Murray et al., 1980). Circulating iron dextran complex may be a source of iron for bacterial growth immediately following injection, and bacteriostasis is lost in this period (Becroft et al., 1977).

In vitro studies

Iron deficiency: The two component systems of active immunity, humoral and cell mediated immunity, have been extensively studied in relation to iron deficiency mainly in vitro. Little evidence exists for major humoral deficiencies in iron deficient humans (Strauss, 1978; Bagchi et al., 1980). Specific defects in cell mediated immunity described in iron deficiency include:

1) Altered polymorph neutrophil (PMN) function: Mackler et al., (1984) showed a 60% decrease in maximum respiratory rates in PMN's from iron deficient rats. The same authors showed decreased myeloperoxidase activity in PMN's while Baggs and Miller (1973; 1974) showed decreased numbers of myeloperoxidase containing PMN's in response to infection. Opsonisation and phagocytosis appear to be normal in iron deficient PMN's (Chandra, 1973; Chandra and Saraya, 1975; Kulapongs et al., 1974) while intracellular bacteriocidal activity has been reported as impaired (Chandra, 1973; Chandra and Saraya, 1975; MacDougall et al., 1975; Srikantia et al., 1976).

2) Depression of T lymphocytes (Srikantia et al., 1976; Bhaskaram and Reddy, 1975) with thymic atrophy (Smythe et al., 1971).

3) Defective lymphocyte transformation probably due to impaired DNA synthesis (Joynson et al., 1972; Chandra and Saraya, 1975; MacDougall et al., 1975; Phillips and Azari, 1975; Smythe et al., 1971). Recent work has suggested that the availability of transferrin bound iron is a critical determinant of lymphocyte transformation (Mainou-Fowler and Brock, 1985; Bomford et al., 1986; Brock and Mainou-Fowler, 1983). A possible mechanism for iron dependant reduced DNA synthesis in these cells is the activity of ribonucleotide reductase (an iron dependant enzyme) (Hoffbrand et al., 1976).

4) Reduced production of macrophage migration inhibition factor (Joynson et al., 1972).

Not all reports have shown these defects in cell mediated immunity (Kulapongs et al., 1974). The in vitro effects of iron deficiency and replacement are reviewed by Strauss (1978); Chandra et al., (1977); and Pearson and Robinson (1976).

Iron Overload: Iron overload appears to affect both PMN's and peripheral blood monocytes. In vitro added iron inhibits neutrophil chemotaxis and bactericidal activity (Gladstone and Walton, 1970;

Walton and Gladstone, 1976). Using neutrophils obtained from uraemic patients with transfusion iron overload, Waterlot et al., (1985) showed an impairment of phagocytosis and myeloperoxidase activity inversely correlated with serum ferritin levels and reversible by desferrioxamine therapy. Akbar et al., (1986) showed decreased natural killer activity in mononuclear cells from patients with thalassaemia major which was reversed by desferrioxamine. Ballart et al., (1986) showed decreased lytic activity of peripheral blood monocytes against C.pseudotropicalis in thalassaemia major which correlated with age and ferritin levels.

Iron binding proteins: The third set of defence systems which are uniquely associated with iron metabolism are cellular and extracellular iron binding proteins (transferrins and lactoferrin). Schade and Caroline (1946) first showed inhibition of in vitro bacterial growth by plasma transferrin reversable by iron. Milk and polymorph neutrophils were later also shown to contain an iron binding protein (lactoferrin) with bacteriostatic properties (Malmqvist et al., 1978; Bullen et al., 1972).

Lactoferrin: Bullen et al., (1972) showed that the bacteriostatic action of human milk was abolished by in vitro addition of iron. In this context the Murrays (1980) showed an increase in Entamoeba histolytica infection in cow's milk drinking nomads during supplementation with oral iron. This was not seen in the control group nor in those having parenteral iron. They related the effect to saturation of the cow's milk lactoferrin by oral iron. Bullen and Armstrong (1979) showed that the bacteriocidal power of neutrophils was reduced by introducing ferritin-antibody complex into the cells thereby presumably saturating intracellular lactoferrin. Cow's milk formulae contain no active lactoferrin and therefore iron fortification in theory should not compromise artificially fed infants

(Committee on Nutrition, 1978). However, there is considerable debate about the advisability of giving oral iron to breast fed infants (Fomon and Strauss, 1978; Bullen et al., 1978a; Fomon et al., 1979) which is largely unresolved due to lack of hard clinical evidence and disagreement as to whether normal breast fed infants become iron deficient (Saarinen, 1978; Garry et al., 1981). Lonnerdal et al., (1980) point out that since the bulk of the iron in breast milk is not attached to lactoferrin and yet is highly bioavailable for the baby, an alternative iron supplementation approach might be to supplement feeding mothers.

"Nutritional immunity": As stated above, the growth of a variety of bacteria and fungi is inhibited in vitro by transferrin (and lactoferrin) (Reviews: Weinberg, 1971, 1974, 1978; Bullen et al., 1978b; Kochan, 1973; Bezkorovainy, 1981). The thesis of "nutritional immunity" as stated by Kochan (1973) and elaborated by Weinberg and the Murrays is that a major host defence against certain bacterial infections is to make iron unavailable, for instance with transferrin, to prevent bacterial multiplication. The elaboration to this is that the lower the saturation of iron on transferrin (or lactoferrin) the greater the effect. The evidence for the main thesis is hard to challenge. It has been shown repeatedly that adding iron to serum abolishes its bacteriostatic effect (Bullen et al., 1967). Furthermore, the ability of various bacteria to acquire iron (directly from transferrin) using their own iron binding proteins (siderophores) has been equated with virulence (Kochan, 1973; Kochan et al., 1977; Miles et al., 1979; Crosa, 1984; Archibald and DeVoe, 1980; Simonson et al., 1982; Mickelsen et al., 1982; Holbein, 1980, 1981; Herrington and Sparling, 1985). The corollary of this is that raising transferrin iron saturation will have less enhancing effects on more iron avid bacteria. Put in another way, lowered transferrin saturation should not affect virulence of disseminating organisms with high iron

avidity. This was neatly demonstrated by Payne and Finkelstein (1978) using a variety of Neisseria and other bacterial strains in chick embryos.

Given that the level of plasma transferrin saturation may be less relevant to pathogenic organisms with powerful iron acquisition mechanisms, there remains the question as to how critical the actual degree of transferrin saturation is to organisms that do not have these systems. One of the arguments in the "nutritional immunity" hypothesis is that the hypoferraemia associated with infection (Hershko et al., 1974; Konijn and Hershko, 1977; Elin et al., 1977; Bullen et al., 1978b) represents an important enhancement of antimicrobial defences (Weinberg, 1974).

Most in vitro or animal in vivo studies of transferrin saturation and infection rely on either directly adding iron or injecting it parenterally. In a neat series of in vitro experiments Baltimore et al., (1982) cast some doubt on the validity of previous in vitro studies by showing that sera from thalassaemics, normal and iron deficient subjects representing a complete range of non-manipulated transferrin saturation had normal bacteriostatic action against E.coli throughout the range. In contrast when ionic iron was added to the sera bacteriostatic action was lost. This suggested that the exogenously added iron was not strongly bound to transferrin. The implications of this observation apart from raising a question over much of the previous experimental work are that oral iron therapy or supplementation in normal doses should not affect serum bacteriostasis and that lower transferrin saturations in iron deficiency or acute infection may be unlikely to protect further against bacterial infections.

Masawe & Nsanzumhire (1973) found slower growth of Staphylococcus albus in whole blood of iron deficients than in that of homozygous sicklers; however, small numbers were used (with no statistical analysis), the age range for the 2 groups was different, and the

effects of in vitro haemolysis could not be controlled for.

One group of micro organisms with an iron requirement in a special situation in relation to iron availability are the erythrocytic forms of the Plasmodia. Although inside cells with the highest iron content in the vertebrate body they apparently do not possess enzymes such as haem oxygenase capable of splitting iron from haem (Sherman and Tanigoshi, 1981; Fritsch et al., 1985). The mature red cell does not possess transferrin receptors (Enns et al., 1981). However, several groups have shown inhibition of in vitro parasite growth using desferrioxamine (Pollack and Fleming, 1984; Raventos-Suarez et al., 1982; Fritsch et al., 1985) and authors have taken this to mean that the parasite acquires iron normally from the surrounding medium (i.e. from transferrin). Peto and Thompson (1986) have shown on the other hand that if care is taken to ensure that all iron is appropriately bound to transferrin, no iron uptake occurs from the medium of in vitro cultures of Plasmodium falciparum and suggest that the desferrioxamine may act by another mechanism than binding transferrin iron. In contrast, Rodriguez and Jungery recently (1986) reported a protein on Plasmodium falciparum infected red cells which binds transferrin. This finding needs to be confirmed.

To summarise in vitro evidence, iron deficiency depresses certain aspects of CMI including neutrophil and macrophage function; humoral immunity is unaffected and the significance of hypoferraemia (as opposed to normal transferrin saturation) on growth of micro-organisms is arguable. Severe chronic iron overload may be associated with a fraction of serum iron only loosely bound to albumin; and there is a short period following parenteral iron administration when plasma bacteriostasis is lost. Given that there are conflicting effects of deficiency and treatment on defence systems, it becomes more important to study the situation in vivo.

Animal in vivo studies.

Iron deficiency: Studies on the effect of iron deficiency on susceptibility to infection in animals have produced some conflicting results. Hart et al., (1982) using Proteus mirabilis induced pyelonephritis in the rat, showed differential effects, with severe iron deficiency protecting less than mild deficiency. Baggs and Miller (1973) on the other hand claimed severe deficiency enhanced defences in rats while mild deficiency impaired them. Puschmann and Ganzoni (1977) showed increased resistance of iron deficient mice to Salmonella typhimurium infection, while Chu et al., (1976) showed an increased mortality of severely iron deficient rats infected with Streptococcus pneumoniae. Harvey et al., (1985) showed reduced parasitaemias and reduced mortalities in iron deficient mice infected with Plasmodium chabaudi.

Intervention: Pharmacological manipulation of iron balance in vivo has also produced conflicting results: Elin and Wolff (1974) showed that ferric ammonium citrate increased mouse mortality from Candida albicans and suggested that iron could reverse endotoxin induced non-specific resistance to infection. The evidence they gave for the latter, however, was from in vitro results not from their in vivo model. Bullen et al., (1967) showed that ferric ammonium citrate abolished the protective effect of antiserum in Clostridium Welchii infection of Guinea pigs. Al-Suttan (1984) increased lethality of Pasteurella haemolytica in mice with I.V. ferric ammonium citrate. Lalonde and Holbein (1984) showed an enhancing effect of iron dextran and a protective effect of desferrioxamine in mice infected with Trypanosoma cruzi.

Fritsch et al., (1985) have shown a rapidly acting protective effect of desferrioxamine in Plasmodium vinckei infected mice. As mentioned above, the effects of desferrioxamine may be complex (Singh

et al., 1985) and caution should be expressed in interpreting the physiological significance of such studies.

In contrast to the above studies, Smith et al., (1977) showed an enhancement of survival in fowl typhoid with the administration of iron dextran.

Clinical studies: In contrast to the plethora of in vitro and in vivo animal studies, clinical studies relating iron deficiency and iron supplementation to susceptibility to infections remain few and difficult to interpret.

Iron deficiency: some studies have reported an association of anaemia in patients admitted to hospital for various infections; Lovric, 1970; Oppenheimer, 1980 (see Chapter 2); Kaplan and Oski, 1980. Masawe et al., (1974) reported fewer bacterial infections in simple iron deficiency anaemia than in a "control" inpatient group with a variety of other causes of anaemia (megaloblastic and refractory) but they also reported more malaria in iron deficient patients in 8 cases (after initiation of therapy). Unfortunately they did not give details of which patients were on oral or parenteral therapy.

The Murrays (1978b) in their report of iron treatment in Somalia (vide infra) also noted that nomads entering the feeding camp had no infections if they were iron deficient (n = 26) in contrast to a high rate of infections in those with normal iron status (19/64). Higgs and Wells (1973) noted that iron deficient patients with chronic muco-cutaneous candidiasis improved on oral and parenteral iron therapy with a regression of oral lesions and development of delayed hypersensitivity to candida.

These anecdotal and post de facto studies, however, cannot replace prospective studies in establishing causal links.

Iron overload: as mentioned earlier, in severe chronic iron overload transferrin may be completely saturated with excess iron loosely bound

to albumen and thus potentially available for bacterial growth. Reports of death due to infectious disease in iron overload states have been quoted as evidence of increased susceptibility (Weinberg, 1978). Pearson and Robinson (1976) reviewed the clinical literature and found the evidence inadequate to incriminate iron overload specifically as the cause of the reported infections. The syndrome of shock in haemochromatosis has variously been related to cirrhosis (Macsween, 1966), massive toxic iron release (Taylor, 1951) and iron induced sepsis (Buchanan, 1971). Increased deathrate from infection in thalassaemia major has generally been related to splenectomy (Smith et al., 1962; Eraklis and Filler, 1972). In homozygous sickle cell disease haemosiderosis is generally a later complication (O'Brien, 1978; Vichinsky et al., 1981; Haddy and Castro, 1982), whereas the increased susceptibility to infections is a feature of early childhood (Powers, 1975) and may be more related to functional hyposplenism and disorders of opsonisation (Winkelstein and Drackmann, 1968).

Other situations where high transferrin saturation has been suggested as a causative factor in sepsis are leukaemia (Caroline et al., 1964) and kwashiorkor (Macfarlane et al., 1970). In the latter study low serum transferrins were a poor prognostic sign and death often occurred soon after starting therapy which included in some cases iron. This anecdotal study is difficult to interpret because of lack of control and the possibility that the low transferrins were an epiphenomenon of profound metabolic derangement preceding death.

Iron treatment: Several studies have incriminated iron therapy in acute exacerbations of pre-existing or latent infections. Briggs et al., (1963) noted an increase in urinary white cell excretion in patients with presumed urinary tract infections following intramuscular iron-sorbitol-citric acid complex. It is possible since iron is loosely bound in this complex that the white cell excretion

was secondary to a toxic effect of urinary iron excretion. Masawe's and Macfarlane's reports have already been mentioned, and in the Murrays' oral iron study in Somalia (1978b) which will be discussed further below infections were noted during therapy and most were likely to have been pre-existing. Byles and D'Sa (1970), in an uncontrolled study, reported 11 cases of clinical malaria in 917 pregnant women immediately following parenteral iron therapy.

In New Zealand serious E.coli sepsis was reported in 2% of neonates who received iron dextran at birth (Barry and Reeve, 1977; Farmer and Becroft, 1976). The increase in infections was confined to the 2-5 days following the last injection and was detected retrospectively with the attendant problems of control. Neonatal sepsis fell to 0.2% following discontinuation of the practice. Becroft et al., (1977) showed a marked reduction in the bacterostatic action of serum of these neonates on E.coli in vitro. Thus it appears that in spite of the observation that saturation of transferrin does not occur after iron dextran injection (Cox et al., 1968) iron may still be available from free circulating iron dextran complex for bacterial growth. In spite of the obvious methodologic problems of these retrospective studies, the evidence from New Zealand for increased E.coli sepsis in neonates is strong. However, in a controlled prospective study in the same area as Barry and Reeves, Cantwell (1972) found apparently the opposite effect, that is a reduction in hospitalisations for infections during the first two years of life. A possible reason for these apparent contradictions may be as follows: The absolute rate of neonatal sepsis in iron treated babies reported by Barry and Reeve (1977) was about 2% which might not have been detected by the other studies (including Cantwell's) mentioned because of small numbers. Cantwell (1972) whose follow-up spanned 2 years found 42% and 32% hospitalisations for infections in control and iron dextran treatment groups respectively.

The early neonatal period is likely to be a bad time to give injections of iron. The neonate has a high iron saturation (Saarinen and Siimes, 1977) and has a compromised immunity (Miller, 1969; Forman and Stiehm, 1969). Farmer and Becroft (1976) noted that the high incidence of neonatal E.coli meningitis with iron dextran injection was reduced when no premature infants received injections under one month of age and there was more selective administration. There is no evidence (excluding the present study) that administration of iron dextran to normal older infants has adverse effects on infection rates.

The apparent discrepancy between Barry and Reeves and Cantwells' reports underlines the importance of differentiating the immediate effects of treatment from the effects associated with steady state iron balance.

Although the majority of the above adverse effects were associated with parenteral therapy, the Murrays' important study in Somalia used oral iron. Murray et al., (1978b) reported more infections in nomads treated with oral iron than in a control group. Infections which were significantly more frequent in the iron group were malaria and schistosomiasis. The study was single blind and no attempt was made to control for other variables and no follow-up was made after finishing the 30 days treatment although the treatment group still had high reticulocyte counts and transferrin saturation at the end of this period. Documentation of infections was scanty. Murray et al., (1975) also reported attacks of malaria associated with hyperferraemia following refeeding of acutely starved patients without iron supplementation. The latter study is difficult to interpret due to lack of documentation and the potential confounding associated with other nutritional factors.

Prospective longitudinal studies are clearly needed to separate the effect of treatment from the effect of steady state iron balance:

The earliest longitudinal study of the effect of iron supplementation on infection rates was that of MacKay (1928) who reported that infants given dietary supplements of iron had 50% fewer respiratory and gastrointestinal infections than infants not supplemented. Unfortunately observations on study and control groups were not made in the same year. The largest study was that of Andelman and Sered in Chicago, when 1048 infants were randomly assigned to receive formula milk with or without iron fortification. Follow-up was for 18 months. Anaemia (Hb<10 g/dl) occurred in 76% of the control and 9% of the study group. These cases were then unfortunately removed from analysis. Significantly fewer respiratory infections occurred in the study group at each 3 month follow-up to 15 months. Unfortunately no details of methods of morbidity recording were given, and one suspects that mother's recall was used. Burman (1972) conducted a similar study in Bristol. Again methodology of morbidity recording was not elaborated. No differences in illnesses were noted between the control and study groups but the small difference in haemoglobins between the 2 groups only reached significance in the second year, suggesting a low rate of iron deficiency in the placebo group.

Cantwell's prospective controlled study of intramuscular iron dextran to neonates (1972) which showed less hospitalisation for infection in the study group has already been mentioned. In a prospective controlled trial of intramuscular iron to premature infants James and Combes (1960) found no significant differences in hospital admissions and outpatient attendances for any disease in a total of 171 infants followed-up for 1 year. Salmi et al., (1963) reported twice the incidence of infections in the control over the

study group in a prospective trial of iron medication to premature infants in Finland. No details were given in the letter.

1 other recent study published since this present one has looked specifically at the effect of oral iron supplementation to pre-pubescent school children in malarious areas. Harvey et al., (1987) gave oral iron for 16 weeks to half of a group of school children (with placebo to the other half) in Madang. Haematological status was significantly improved but no effects were seen associated with parasite rate or density, spleen size, or antimalarial antibody. They suggest that immunity may modify any disadvantage associated with iron.

In summary, in vitro evidence suggests specific defects of cell mediated immunity associated with iron deficiency and reversed by iron therapy; iron binding by transferrin and lactoferrin are important non-specific bacteriostatic defences which can be abolished by exogenous iron, but the relationship of the degree of in vivo transferrin saturation to the degree of antibacterial action is controversial; in vivo animal studies show protection by iron deficiency against some infections, in particular malaria. Clinical studies supporting these results are few; there is in vitro and in vivo evidence that the acute effects of parenteral and oral iron administration to normal and compromised hosts such as newborn and severely malnourished patients may be deleterious; on the other hand longitudinal clinical studies of iron suplementation to date have if anything given evidence of a protective effect of iron when the follow-up was beyond the immediate treatment period. The lack of control and defined morbidity reporting has made these studies difficult to interpret.

CONCLUSION

Iron deficiency is a global problem. Clinical effects may be divided into those resulting from anaemia, and a variety of tissue effects. Anaemia restricts maximal physical activity in direct proportion to its severity. Physiological compensatory mechanisms allow even severely anaemic people to continue activities at a lower level of efficiency until decompensation results in congestive heart failure. The pregnant woman and her foetus are at particular risk from anaemia.

Non-haematological effects of iron deficiency include: impairment of aerobic performance of skeletal muscle; a deleterious effect on most epithelial tissues especially in the gastrointestinal tract; an effect on the central nervous system which is poorly characterised in humans but may include a defect of attention; effects on overall growth which are as yet not clearly substantiated in humans; and effects on immune status and susceptibility to infection which have yet to be clearly characterised in vivo. Although in the main the effects of iron deficiency are harmful there is some evidence that iron deficiency could be antagonistic to certain infections. Supplementation and in particular parenteral iron therapy should not be given in the neonatal period. Parenteral iron also should be avoided in patients with compromised immune status and in kwashiorkor.

The clinician's reponsibility is to prevent harm due to iron deficiency and not to cause harm by iron administration. The former depends on accurate diagnosis of iron status at the individual and group level and knowledge of clinical consequences in relation to the degree of iron deficiency. The latter depends on a critical evaluation of iron requirements in relation to attendant risks of iron administration.

Iron supplementation to children and pregnant mothers is a world-wide practice; mainly by the oral route including milk fortification but also by parenteral administration. Certain developing countries with a high prevalence of iron deficiency have fortified other food-stuffs with iron. Common salt has been fortified in India (Nadiger et al., 1980) and trials in rural communities have been conducted (Datta et al., 1982). In Thailand, fortification of fish sauce with iron and iodine has been proposed (Suwanik et al., 1980). In Papua New Guinea oral and parenteral iron are prescribed as part of the standard presumptive treatment for anaemia, and at the time of this study, low-birthweight infants routinely received parenteral iron dextran at birth. Health policies in relation to these practices should be based on careful prospective evaluation of effects.

CHAPTER 2.

RETROSPECTIVE SURVEY OF HAEMOGLOBINS IN INFANTS WITH PENUMONIA AND MENINGITIS ADMITTED TO TWO PAPUA NEW GUINEA HOSPITALS - 1975-1977

(first published as a paper in Ann. Trop. Med. Para. 1980: 74, 69-72).

SUMMARY

A retrospective survey of admission haemoglobins of infants with meningitis and pneumonia in two Papua New Guinea hospitals revealed significant anaemia. The meningitis cases were more anaemic than the pneumonia cases. Those with Haemophilus influenzae meningitis were more anaemic than those with pneumococcal meningitis, but better nourished.

The patients in this study were seen early in the course of acute infections. It is argued that the marked anaemia observed is thus more likely to have been pre-existing than the result of the infection. These preliminary data suggest an association between anaemia and specific bacterial infection. In New Guinea infants a major cause of anaemia is iron deficiency.

INTRODUCTION

This study was carried out while the author was working as a government paediatrician in New Guinea (1976-1978) and initiated his interest in investigating the relationship between iron deficiency and infection.

In Goroka, a highland hospital in Papua New Guinea, meningitis and pneumonia accounted for 16% and 22% respectively of all paediatric hospital deaths reported in 1976 (Health Department Annual Report). This represented 59% of post-neonatal infant deaths. During 1976 it was noted that many infants with meningitis were also anaemic and several required transfusions. In order to see if there was a specific association of anaemia and bacterial infection in infancy, a

retrospective study of meningitis and pneumonia cases was carried out. A similar smaller study was also performed in Wewak, a coastal town with a different spectrum of disease, including hyperendemic malaria.

MATERIALS AND METHODS

The ward discharge book was used to find case notes of all patients with a primary diagnosis of meningitis or pneumonia where admission haemoglobin had been recorded (normally a routine). The period studied was 1975-1977 for the meningitis cases and 1976-1977 for the pneumonia cases in Goroka. In Wewak, the period studied was 1976-1977 for all cases. Only cases of proven purulent meningitis were used, and those secondary to skull fractures or otitis media were excluded. All cases with slide-positive malaria were also excluded. Data obtained from the notes included age, sex, weight, clinical diagnosis, admission haemoglobin, CSF cytology and bacteriology and outcome. The results of previous haematological and nutritional surveys of the highlands and coastal infants are used for comparison (Vines, 1970; Venkatachalam, 1962; Kariks, 1969; Ferro-Luzzi et al., 1978). Weight for age is expressed as a percentage of the Harvard Median (Jelliffe, 1966).

For the Goroka study, comparison is restricted to cases aged between two months and one year on admission in order to make the groups comparable with respect to age.

Two-tailed Students' 't' tests are used for comparison of means.

The haemoglobin values quoted for the highlands infants are not corrected for altitude (>1500 m). A suitable correction factor would be 0.94 (Vines, 1970).

RESULTS

Goroka Study (New Guinea Highlands)

Haemoglobin was estimated in 39 cases of meningitis and 69 cases of pneumonia admitted to Goroka base hospital. Of these, 32 and 43

respectively were between two months and one year of age. The sex ratio in the meningitis group was about three (male) to one (female), whereas there was an equal sex ratio in the pneumonia group.

The mean haemoglobin on admission was 8.3 g/dl (SE±0.41) for the meningitis cases and 9.5 g/dl (SE±0.26) for the 43 pneumonia cases, the difference being significant (see Table). When the meningitis cases were divided according to the infecting organism, the mean admission haemoglobin for the ten cases with pneumococcal meningitis was 9.2 g/dl (SE±0.70) and not dissimilar to that for the pneumonia cases, whereas the mean for the 19 cases with Haemophilus influenzae meningitis was 7.6 g/dl (SE±0.43) (there were three cases in whom no organism was isolated). The difference between the mean of the latter group and the pneumonia group was significant ($p<0.001$). Of four cases of meningococcal meningitis all were male and over one year of age. The mean haemoglobin was 8.4 g/dl.

TABLE

Comparison of weight for age and haemoglobin values in meningitis and pneumonia cases in Goroka - age group two months to one year

Cases	No.	Age (months) mean (SD)	Weight for Age (% Havard Median) mean (SE)	Admission Hb (g/dl) mean (SE)
Haemophilus influenzae meningitis	19	5.7(±2.3)	95(±3.0) ++	7.6(±0.43) +
Pneumococcal meningitis	10	6.2(±3.4)	80(±3.4) ++	9.2(±0.70) +
All meningitis	32*	5.7(±2.7)	91(±2.6)	8.3(±0.41) ++
Pneumonia	43	5.9(±2.8)	93(±2.6)	9.5(±0.26) ++

* Including three in whom no organism was isolated.
\+ Probability of obtaining observed difference by chance = 0.05
++ Probability of obtaining observed difference by chance <0.01

Seven cases of meningitis required blood transfusion, but this was not necessary for any of the pneumonia patients. Of the meningitis group, eight children died, six of these having H.influenzae isolated. The mean admission haemoglobin of these six was 7.1 g/dl (SE±0.41), which was lower than that for those who survived.

There appeared to be little difference between the nutritional status of the meningitis cases and the pneumonia cases as judged by weight for age (Jelliffe, 1966). The mean weight for age, however of the pneumococcal meningitis group was lower than that for the H.influenzae group, being 80% (SE±3.4) and 95% (SE±3.0) respectively (see Table).

Wewak Study (New Guinea Coast)

Twenty-one cases of meningitis and 83 cases of pneumonia were admitted in whom admission haemoglobin was estimated. The majority of these cases were aged between two months and one year. The mean admission haemoglobin of the meningitis cases was 8.4 g/dl (SE±0.35) compared with 9.2 g/dl (SE±0.15) for the pneumonia group. The difference between these means was just significant (P = 0.05). The mean admission haemoglobin for six cases of H.influenzae meningitis was 7.9 g/dl (SE±0.63) as compared with a mean of 8.5 g/dl (SE±0.7) in 13 cases of pneumococcal meningitis. Of two cases of meningococcal meningitis the mean haemoglobin was 9.2 g/dl.

Haemoglobin Values Recorded in Normal Infants in New Guinea

Previous surveys of haemoglobin values in normal infants have given the following results:

Vines (1970)	Highlands Infants	* Hb:11.8 g/dl (SE±0.29)
Venkatachalam (1962)	Highlands Infants	* Hb:11.4 g/dl
Kariks (1969)	Coastal Infants	Hb:10.01 g/dl

* These figures are uncorrected for altitude (>1500 m).

DISCUSSION

The mean haemoglobin levels in the children with meningitis and pneumonia were lower than those recorded in normal infants living in the same environment. The meningitis group as a whole had a significantly lower mean haemoglobin level than the pneumonia cases. But, when the meningitis cases were examined according to the infecting organism (where isolated) the haemoglobin values in H.influenzae meningitis were found to be significantly lower than in pneumococcal meningitis cases, whose values were similar to patients with pneumonia (see Table). The lowest haemoglobin values were found in infants who subsequently died, despite blood transfusions (see Table).

Using weight-for-age as a measure of nutritional status, infants in this survey are nutritionally similar to those recorded in highland infants in previous surveys except for the group with pneumococcal meningitis which contained more malnourished children.

The results described suggest an association between anaemia and infection which is independent of nutritional status judged by weight for age. This association was most apparent in cases of H.influenzae meningitis.

Many surveys have confirmed the prevalence of anaemia in both highlands and coastal New Guinea. Several of the larger studies remark on the especially marked hypochromic anaemia and low iron stores in New Guinea infants thus implicating iron deficiency as a cause (Bailey, 1966; Kariks, 1969).

An alternative explanation, that anaemia in these infants was secondary to infection, cannot be discounted but seems less likely in view of the fact that most patients were seen within 36 hours of the onset of symptoms. Inappropriate ADH secretion also cannot be excluded as a cause of a temporary fall in haemoglobins.

The effect of iron deficiency on immune mechanisms is reviewed in Chapter 1. Pneumonia and meningitis together accounted for 59% of reported post-neonatal infant mortality in a New Guinea highland hospital (Goroka) in 1976. If iron deficiency was the main cause of the anaemia observed in such cases and if iron deficiency was an aetiological factor in susceptibility to infections, a decrease in infant mortality and morbidity might be anticipated from a programme of iron prophylaxis in the post-neonatal period. These questions would be more appropriately answered by a controlled prospective trial of iron supplementation to infants in New Guinea.

CHAPTER 3.

INTRODUCTION AND AIMS OF STUDY

Clinical trials of iron supplementation, although showing haematological benefits, have given conflicting results in relation to the effect of iron on susceptibility to infection (see Chapter 1). Short- term effects have generally been deleterious, particularly when iron was given in the neonatal period (Barry & Reeve, 1977) or to severely malnourished individuals (Macfarlane et al., 1970). However, when iron dextran was given in the post neonatal period the sepsis risk was diminished (Farmer, 1976). Acute deleterious effects have also been noted with oral iron (Murray et al., 1978b). Long term follow-up studies, however, have tended to show a beneficial effect of iron supplementation (if any) on infectious susceptibility (Mackay, 1928; Andelman & Sered, 1966; Cantwell, 1972; Salmi et al., 1963). However, all of these studies have been criticised on grounds of design, control, and quality of morbidity recording.

Conflicting results, the lack of a definitive clinical study in the world literature (from developed or developing countries), the continuing empirical assumption in many countries that iron supplementation should be beneficial, the local practice in Papua New Guinea of prophylactic and presumptive use of iron dextran made a prospective controlled study of iron supplementation justifiable.

Such a study should be as capable of detecting no significant effect as of detecting a beneficial or deleterious effect.

The author's personal standpoint for initiating the study, starting from the initial observations described in Chapter 2, was that the balance of results from previous longitudinal studies in infancy indicated a probable beneficial outcome of iron supplementation if the neonatal period was avoided.

The main aims of the project were to study prospectively under controlled conditions the relationship in infancy between a) iron deficiency and b) iron supplementation and susceptibility to infection as measured by: (i) incidence and prevalence of infection; (ii) severity of infection; and (iii) mortality from infection. (It was accepted that mortality effects might not be detected in a study of this size).

Corollaries to these aims were 1) it should be established that iron deficiency existed in the study population; 2) the effect of iron should reverse deficiency without causing overload; and 3) other potential causes of anaemia and potential confounding effects (e.g. genetic disorders of the red cell) should as far as possible be detected and, if necessary, controlled for in analysis.

Supplementary aims were 1) to study the effect of total dose iron infusion given in antenatal clinics to mothers of the study newborns; 2) to investigate the relationship of maternal variables to newborn and postnatal iron status in the study group; and 3) to study the relationship of iron status and postnatal growth.

CHAPTER 4.

DEMOGRAPHY, GEOGRAPHY, ETHNOLOGY AND DESCRIPTION OF HEALTH SERVICES IN CHOSEN STUDY AREA

SUMMARY

Madang district was selected for a longitudinal study of the effects of iron prophylaxis on infectious morbidity in infancy. The topography, climate, domicile, ethnology, demography, disease patterns, nutrition and health services of the district are described. The area has a tropical, humid climate and a mixed economy. Pneumonia was the main killing disease at all ages, and malaria was endemic. A base hospital and well organized maternal and child health services ensured that morbidity surveillance would be optimal.

STUDY AREA: MADANG DISTRICT

The area used for the study includes Madang town and Madang district (Fig. 1). Madang Province lies on the north coast of Papua New Guinea (PNG) between 4° and 6° south of the equator. All villages in the study area (300 km^2) are within a 45 minute drive of Madang Hospital.

Topography and climate

The study area terrain may be divided into inland hills, with secondary tropical rainforest and extensive shifting cultivation (altitude <400 m) and a thin coastal strip (2 km) with alternating swamps and large copra plantations (altitude <15 m).

The climate is tropical and humid. Two seasons correspond with the Northwest and Northeast monsoons. A short relatively dry season lasts from June to September. Annual rainfall is 3.5 m with a monthly average ranging from 128 to 433 mm. Temperature varies little throughout the year with an average daily maximum of 30°C and minimum of 23°C. Relative humidity during the day ranges from 74% to 87% throughout the year.

Figure 1 Map showing Madang district and study area.

Domicile

Domicile in the study area may be divided into urban and rural. The urban area consists of accommodation designated as follows:

(1) "Urban residential": mainly government-built accommodation occupied by 45% of the population, with the majority of family wage earners in the public service. Allocation of urban residential accommodation depends on income and divides sharply into high and low cost development of standard designs with mosquito mesh on all windows.

(2) "Urban settlement": self-built housing where 55% of the population live, on income which is generally lower than in "urban residential". Accommodation varies from well built timber houses to houses of bush material; generally neither have mosquito mesh.

The rural population is divided by domicile into the following groups:

(3) "Village" (Indigenous): these groups live in villages or collections of hamlets on ancestral ground and, with the exception of coastal dwellers, have large food gardens and a stable, mainly subsistence, economy. The coastal indigenes, having little land for subsistence cultivation as a result of land alienation for plantations during the colonial period, have successfully moved towards a cash economy.

(4) "Rural Settlement" (Migrants): these generally come from inland areas of Madang province and live in small bush settlements, either around established villages or on plantations. Their access to land for cultivation is limited and, because of transport problems, their access to urban cash economy is also limited.

Rural houses, with few exceptions, are made of bush materials with no mosquito protection except sleeping nets.

(5) "Rural institution": a small additional group consists of people living in association with missions, hospitals and schools. They are usually wage-earners with accommodation varying in quality between "urban residential" and "urban settlement" standard.

In analysis in Chapters 5, 7, 9 and 10, "domicile" refers to the above five categories. Housing is divided for analysis into (A) high cost, and (B) low cost and (C) bush materials.

Ethnic types (Figure 2)

Papua New Guineans are often loosely described as Melanesians, but there are distinct major ethnic divisions based on linguistic differences and area of origin. Most mainland dwellers (>2,000,000) speak "non-Austronesian" (or Papuan) languages unique to Melanesia, of which there are over 700 (186 spoken in Madang province alone) (Z'graggen, 1975), and may be sharply separated, culturally and geographically, into highlanders, who inhabit the central cordillera (table >1600 m), and lowlanders, who inhabit the coastal lowland and hills (Z'graggen, 1975). An intermediate group, who are represented in Madang Province, inhabit the mountains and lowlands of the Huon peninsular but speak languages closely related to the East New Guinea highlands stock (Wurm, 1964).

The second major language phylum in Papua New Guinea is the "Austronesian language" group. Austronesian languages are spoken throughout South East Asia and may have been brought to New Guinea 3000 years ago by migrations which culminated subsequently in the Polynesian expansion into the Pacific (Wurm, 1976). Austronesian speakers are found on the coastline and islands of Papua New Guinea (see Fig. 2).

In the study area the rural population may be divided clearly into (a) Austronesians living on the shoreline and islands and (b)

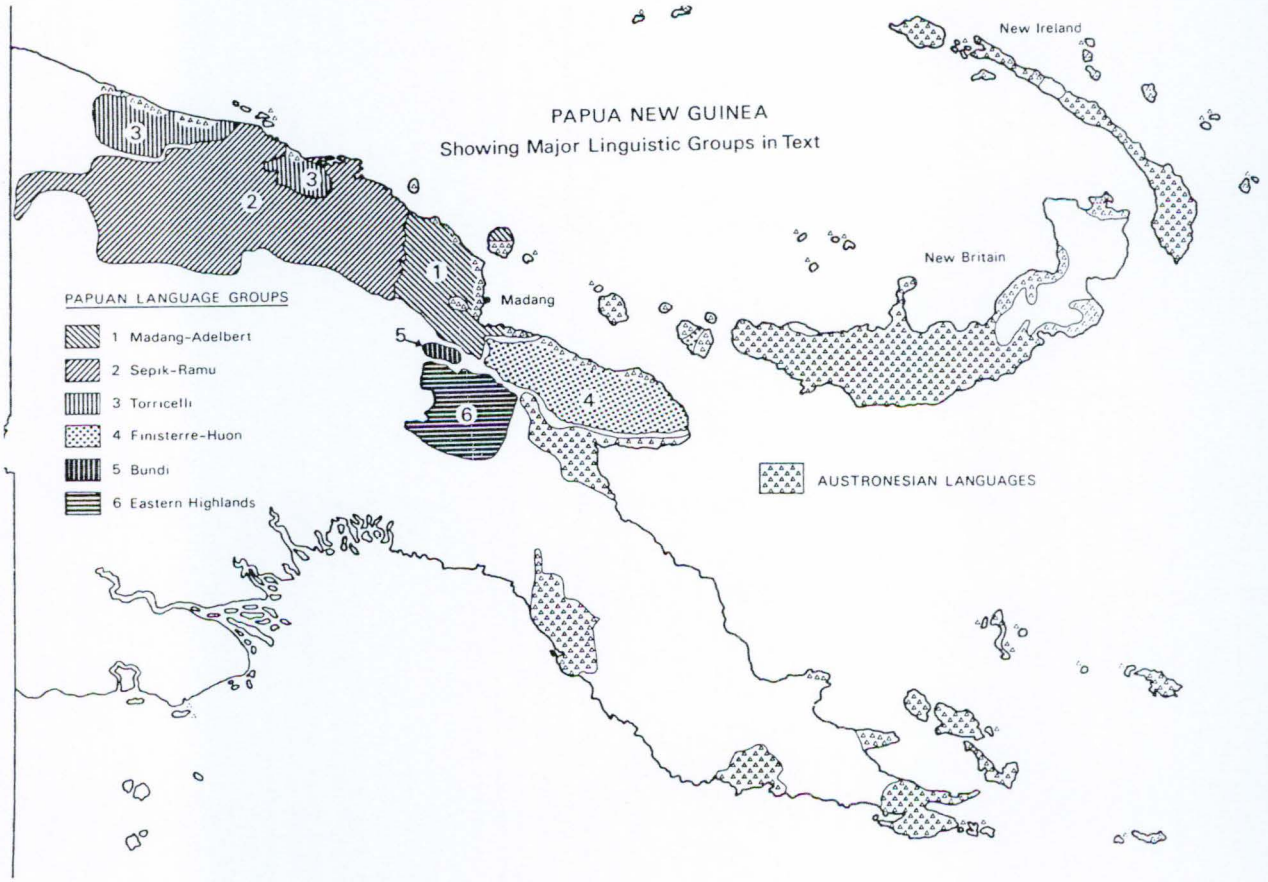

Figure 2 Map of Papua New Guinea showing distribution of major linguistic groups represented in the study. ("Papuan" and "non-Austronesian" are synonymous.

lowland non-Austronesian speakers living more than 1 km inland. The urban dwellers consist mainly of Madang indigenes, lowlanders (non-Austronesian and Austronesian speakers) from the neighbouring provinces of Morobe and Sepik, and a minority of highlanders. One thousand and seventy-eight urban dwellers are expatriate.

Demography

The Madang urban population is 20,335 (Papua New Guinea 1980 Census) with an annual increase of 5.5% influenced by inward migration. The population of the rural villages and settlements from which infants were recruited is approximately 9,000 (Papua New Guinea 1978 Census). The annual natural increase for the Madang district (rural) is 3.5% (Papua New Guinea 1978 Census).

Estimated crude birth rates for Madang Province vary from 38 to 51 per 1000 population (Winyard, 1979). Infant mortality in Madang has been estimated at 62 per 1000 livebirths (Bakker, 1983). Madang Hospital neonatal mortality in 1979 was 25 per 1000 livebirths (author's observation). In both the town and rural areas children under five years of age constitute 17% of the population.

Disease patterns

Causes of morbidity and mortality derived from hospital and health centre statistics give pneumonia as the main cause of death in children (30-40%) followed by meningitis (12%), gastroenteritis (6%) and malaria (5%) (Winyard, 1979). Tuberculosis is an infrequent cause of death in children, but a major cause of adult hospital mortality (12.5%) in Madang (Winyard, 1979).

In spite of the high malarial endemicity, mortality from cerebral malaria among hospital admissions in Madang appeared to be very low by world standards and affected mainly older children in the report of Stace et al. (1982). However, differences in diagnostic criteria do not allow strict comparison between studies.

Morbidity patterns differ between outpatients and inpatients. Malaria, diarrhoea and pneumonia account for 25%, 21% and 14% of paediatric admissions, respectively, while malaria, respiratory infections and skin conditions account for 24%, 24% and 20% of outpatients, respectively (Winyard, 1979).

Malaria

Malaria is endemic in lowland Papua New Guinea but classification of malarial endemicity is complicated. High adult spleen rates occur in areas where frequency of transmission and high levels of acquired immunity suggest holoendemicity (Metselaar, 1956). Detailed malarial epidemiology, undertaken by the PNG Institute of Medical Research (IMR) in the study area in 1980 and 1981, suggests that meso-, hyper- and holoendemicity exist side by side in adjacent parts of this small area (Cattani et al., 1983). Anti-mosquito spraying was stopped in these two census divisions in 1979. Chloroquine resistance has been reported at R1, R2 and R3 levels in proportions of isolates of 44%, 4% and 5%, respectively, in 1979 (Gibson, 1980) and 29%, 36% and 7% in 1980 (Darlow and Vrbova, 1981). It was mainly for this reason that an intervention programme involving amodiaquine prophylaxis in some villages in the study area was abandoned in 1981 (Papua New Guinea Institute of Medical Research, 1981). A further intervention programme involving presumptive treatment of fevers with chloroquine and amodiaquine was started by the IMR shortly after that time (Papua New Guinea Institute of Medical Research, 1981).

Nutritional surveys

A National Nutritional survey in 1978 (Papua New Guinea 1978 Census) showed that among children aged one to five years, 42% in Madang town and 46% in Madang sub-district weighed less than 80% of the Harvard median weight for age (Jelliffe, 1966). Only 28% of infants under one year of age fell into this weight category.

Health services

Madang town has a base Hospital of 250 beds staffed by 15 doctors including a specialist paediatrician, and two urban clinics staffed by Maternal and Child Health (MCH) nursing sisters. The urban clinics operate a mobile MCH service which runs monthly clinics in the nearby villages for mothers and well babies. Immunizations given include tetanus toxoid for pregnant mothers and triple antigen, oral polio-vaccine (Sabin) and BCG for babies. Sick children are also seen in the MCH clinics. "Standard" out-patient treatment for anaemia (Hb <10 g/dl) in mothers and babies includes antimalarials, anthelminthics and iron dextran injection (Biddulph, 1979). Low birthweight infants (<2.2 kg) also routinely received 2 ml iron dextran i.m. shortly after birth in PNG at the time of the study (Biddulph, 1979).

The rural areas in the study are also served by 40 aid posts, controlled from the Provincial health office, and one health centre (Yagaum) (Fig. 1). Each aid post is staffed by one "aid post orderly" who is trained to give a limited range of drugs including procaine penicillin and antimalarials. Yagaum health centre is staffed by State Registered and MCH nurses and has inpatient beds, outpatient services and facilities to deliver babies (180 per year). It also provides a mobile MCH service to the area between the Gum and Gogol rivers (see Fig. 1). The rest of the study area is served by mobile MCH clinics from the town. Because of the relatively easy road access to Madang the main hospital is much used as a primary health resource by surrounding villagers. There was easy communication by telephone links between the only two hospitals in the area and the MCH services, which afforded close monitoring of infant morbidity and mortality in the study.

Of the 1200 annual deliveries in Madang hospital 35% were urban, 41% were from Madang district and 24% were referrals from remote areas

(author's observation). This indicated potentially adequate numbers for study recruitment.

DISCUSSION

These studies of the geography, demography and pattern of child health in Madang indicated that it would be a suitable area for an intervention study to investigate the relationships between iron deficiency, iron supplementation and infection in infants for the following reasons:

(1) In previous longitudinal studies, respiratory infections were the main category of disease claimed to be reduced by iron prophylaxis and pneumonia was identified as the major disease associated with death in infancy in Madang.

(2) Births to mothers living within easy access of Madang hospitals were sufficient for recruiting numbers.

(3) Utilization of health services in the area was high and infants falling sick could only be admitted to one of two local hospitals (Madang and Yagaum) which enabled surveillance on a day to day basis.

(4) Iron dextran was already routinely administered by the health department to low birthweight neonates, and any patient with haemoglobin less than 10 g/dl. Assessment of this policy was therefore needed.

CHAPTER 5.

PILOT HAEMATOLOGICAL SURVEYS

SUMMARY

Two pilot haematological studies were performed in the study area to assess the degree and causes of anaemia in the infant population. Mean haemoglobin fell sharply in the first eight weeks of life before stabilising at less than 9 g/dl for the rest of the first year. Laboratory investigations showed a hypochromic microcytic picture with low serum iron and transferrin saturation. Factors associated with low haemoglobin levels were presence of splenic enlargement and domicile in new settlement areas.

INTRODUCTION

As a preliminary to the main longitudinal study, and on request of the PNG Medical Research Advisory Council prior to finalisation of the protocol, two pilot haematological surveys were performed. Their main purpose was to assess the degree and causes of anaemia in the infant population. The other aims were firstly, to assess the effects of environmental factors on anaemia, in order to determine which variables to use for matching of paired infants for different treatments in the main study and secondly, to test under field conditions the clinical and laboratory methods of study.

Pilot Study I

One hundred and fifty-eight infants aged 0-52 weeks (mean:23 weeks) were seen in a number of rural and urban well-baby clinics between February and March 1980. Infants who were ill were excluded. Apart from mother's consent no other selection procedure was used. All infants were examined and their social details, birthweight, birth date, sex, weight, temperature, pulse and spleen size recorded. Haemoglobin and packed cell volume were estimated on capillary samples

using the COMPUR mini photometer and minicentrifuge (both accurate portable methods) (Oppenheimer, 1979) (see Appendix I.i.).

Variables derived for analysis included (a) domicile: (1) urban residential, (2) urban settlement, (3) rural indigenous, (4) rural migrant settlers (see Chapter 4); (b) age in weeks; (c) sex (male/female); (d) nutritional status using weight expressed as a percentage of the Harvard 50th centile for age (Jelliffe, 1966); (e) splenomegaly - presence or absence of palpable spleen; (f) presence or absence of fever - (T>36.8°C axillary).

Results - Pilot Study I

Nutrition. Twenty-six per cent of infants weighed less than 80% of the Harvard 50th centile for age (Jelliffe, 1966). (See also Chapter 4).

Haematology. Mean haemoglobin values at all ages were over 3 g/dl lower than might be expected in an iron-sufficient population (Fig. 1) (Saarinen and Siimes, 1978, Matoth et al., 1971) (Table 1). Mean MCHC was 30.8 g/dl under nine weeks of age but fell to 28.6 g/dl in 9-52 week-olds (Table 1). This is around 4 g/dl lower than would be expected in an iron-sufficient European population (Saarinen and Siimes 1978).

TABLE 1

Pilot Study I Haemoglobin and MCHC of Madang infants

		Age	
		1-8 weeks	9-52 weeks
Haemoglobin (g/dl)	Mean (SD)	10.65 (0.50)	8.59 (1.66)
	n	29	129
MCHC (g/dl)	Mean (SD)	30.84 (3.02)	28.57 (3.90)
	n	19	90

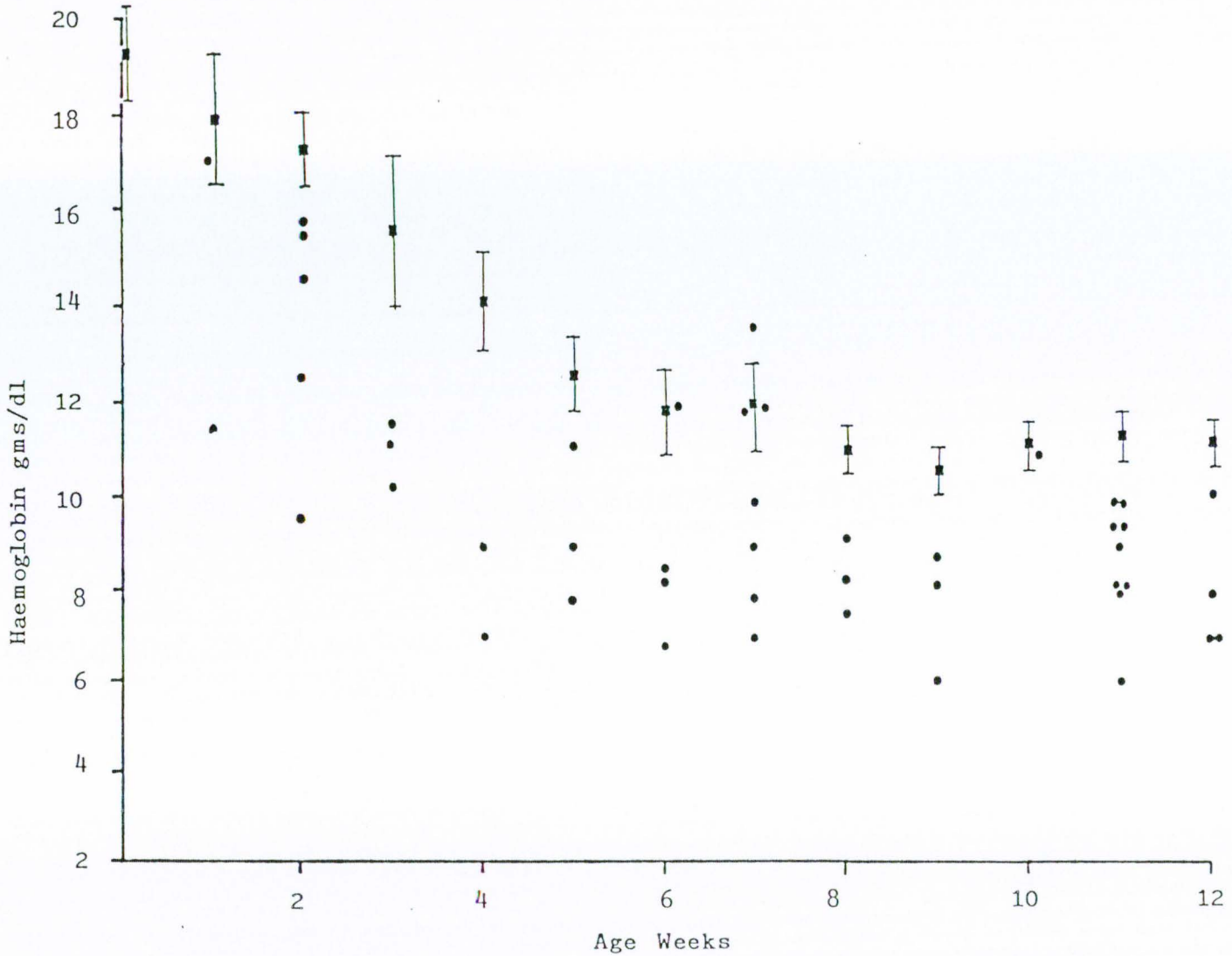

Figure 1 Capillary haemoglobin values in first 12 weeks in Pilot I compared with normal European values (means ± 2SD) - after Mattoth et al (1971)

Analysis of variables affecting haemoglobin in Pilot Study I

Between the ages nought and eight weeks haemoglobin fell sharply with age. Values below 10 g/dl occurred at two weeks and some below 7 g/dl at seven weeks of age (Fig. 1). A linear regression of haemoglobin against age for these 29 cases gave a significant negative correlation ($r = -0.52$; $p = 0.002$) with the regression equation:

Hb (g/dl) = 13.65-0.616 x age (weeks).

From nine weeks to one year there was no evidence of any further change with age, and mean haemoglobin stayed below 9 g/dl (Fig. 1). For this age group, nutritional status and physical and social variables were analysed in relation to haemoglobin. There was no evidence of a correlation between any of these variables and haemoglobin. Domicile and presence/absence of a palpable spleen did show some association with haemoglobin values (Table 2). The mean haemoglobin was 1.3 g/dl lower in infants with a palpable spleen ($p<0.01$), and settlement dwellers (urban and rural) had lower mean haemoglobin values than the other two groups ($p<0.01$). In a two-way Analysis of Variance, spleen and domicile account for 19% of variation in haemoglobin (Table 2). The effect of domicile was independent of, and greater than, the effect of spleen. Domicile was thus used as a matching criterion in the main study (see Chapters 6 and 7).

TABLE 2

Pilot Study I Mean Haemoglobin (g/dl) by domicile and splenomegaly for infants aged 9-52 weeks*

Spleen	Urban residential	Urban settlement	Rural indigenous	Rural settlement	All domiciles*
Not palpable					
Mean (SD)	9.07(1.47)	8.42(1.08)	9.73(1.74)	8.31(1.72)	8.76(1.64)
n	14	27	21	28	90
Palpable					
Mean (S.D.)		7.53(1.81)	8.40(1.78)	7.00(1.12)	7.50(1.47)
n	-	4	3	6	13

Analysis of variance

Source of variation	Sum of squares	DF	Mean square	F	P
Domicile	3316.6	3	1105.5	4.79	p<0.01
Splenomegaly	1151.7	1	1551.7	6.72	p<0.01
Interaction	43.3	2	21.6	0.09	NS
Residual	22,153.2	96	230.8		
Total	27,458.7	102	269.2		

* At least one variable not recorded in 26 cases

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Pilot Study II

Forty-nine infants aged from 0 to 52 weeks, seen in May 1980 in rural and urban well-baby clinics, were processed in a fashion similar to the first field study, but venous blood samples were obtained. EDTA specimens were obtained from 41 infants and clotted specimens from all. Haemoglobin was estimated by the cyanmethaemoglobin method, using a Corning Colorimeter 252, and packed cell volume using the COMPUR minicentrifuge (Oppenheimer, 1979). Red cell morphology was assessed on fresh blood films. Hypochromasia and/or microcytosis were assessed in particular and scored according to severity. Blood was also examined for malarial parasites, total and differential white cell count. Frozen serum specimens were transported to the Department of Tropical Paediatrics, Liverpool School of Tropical Medicine, to estimate the following:

(1) Serum ferritin, using an enzyme-linked immunosorbent assay (see Appendix I.iii.).

(2) Serum iron and total iron-binding capacity using SIGMA Kit No. 565 adapted for small samples (see Appendix I.ii.).

(3) Serum folate (see Chapter 6).

(4) Serum B_{12} level (see Chapter 6).

Washed red cells were lysed in 0.2% ascorbic acid and transported frozen to Liverpool for estimation of red cell folate. The folate and the B_{12} assays were performed in the combined Department of Haematology, Liverpool University.

Results - Pilot Study II

The haemoglobin and MCHC values were similar to those in Pilot Study I. There was no evidence of Folate or B_{12} deficiency at any age (Table 3). In the 9-52 week age group mean serum iron and transferrin saturation values were low (Figs. 2 & 3) (Table 3) even by standards given for infants (Koerper and Dallman 1977). Hypochromasia microcytosis were present in all 35 films examined from infants over eight weeks old.

TABLE 3

Pilot Study II Haematological indices

	Age (weeks)			
	0-8		9-52	
Index	Mean (SD)	n	Mean (SD)	n
Haemoglobin (g/dl)	10.5(1.40)	11	8.1(1.17)	30
Serum iron (µmol/l)	12.5(3.39)	8	5.9(2.97)	21
Transferrin saturation(%)	23.2(5.50)	8	11.7(5.56)	21
Serum ferritin (ng/ml)*	176.3(2.64)*	12	35.7(3.00)*	25
Serum folate (µg/l)	8.1(2.51)	9	13.4(4.16)	23
Red cell folate (µg/l)*	651.6(1.26)*	13	662.8(1.41)*	36
Serum B_{12} (ng/l)*	422.7(1.50)*	10	518.0(1.60)*	23

* Geometric mean (antilog of SD of $\log_e$ transform).

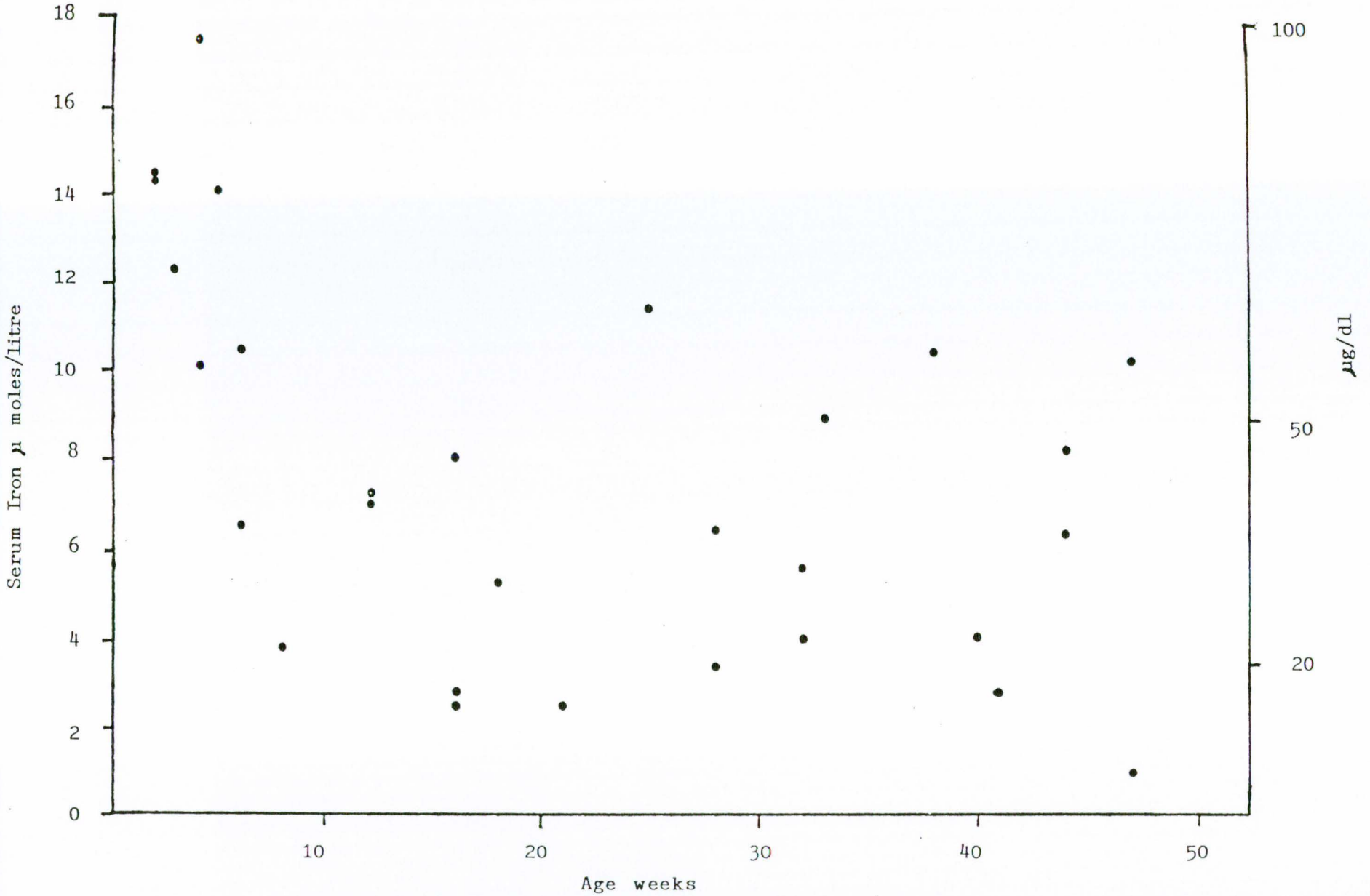

Figure 2 Serum iron levels plotted by age in Pilot II

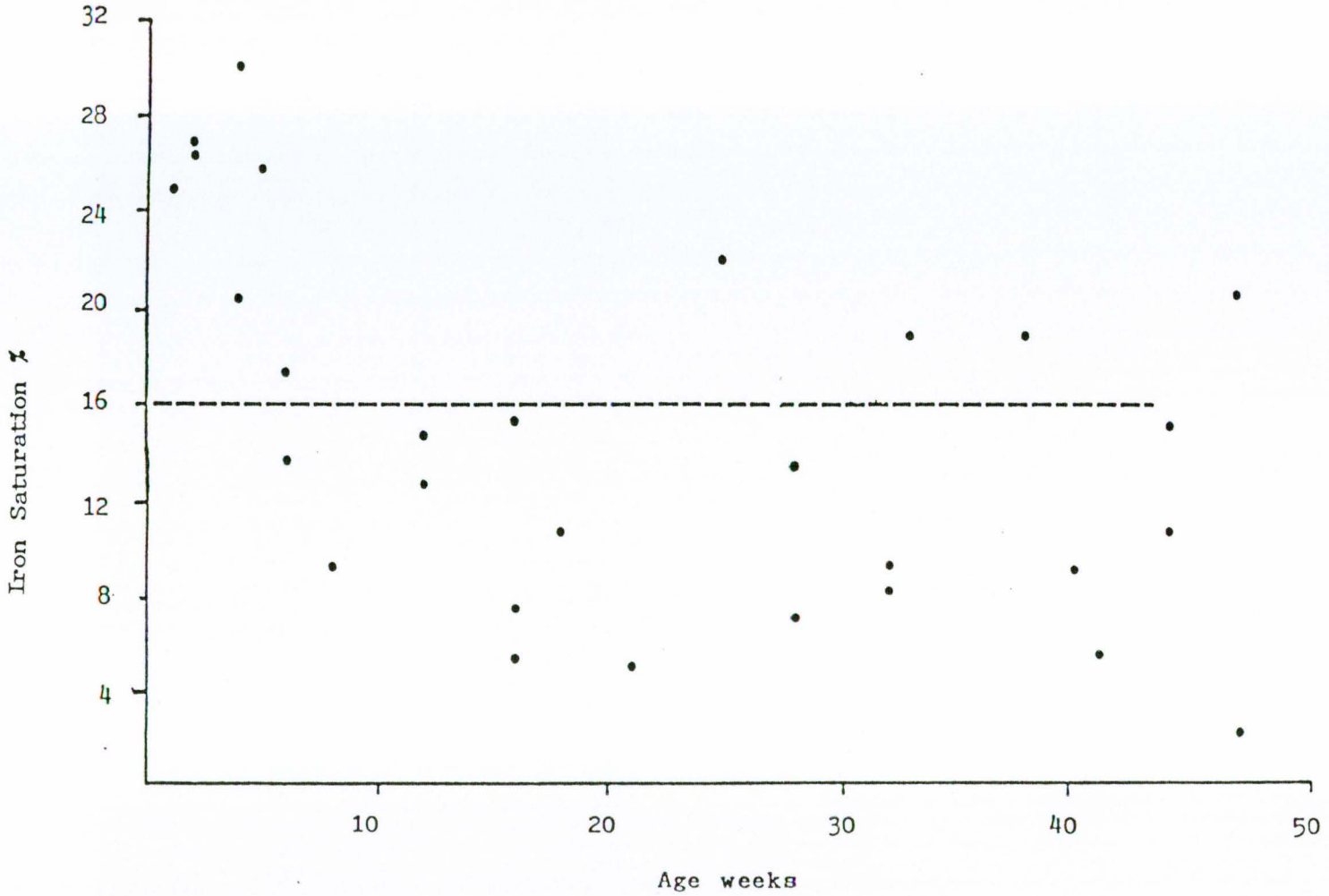

Figure 3 Transferrin values plotted by age in Pilot II

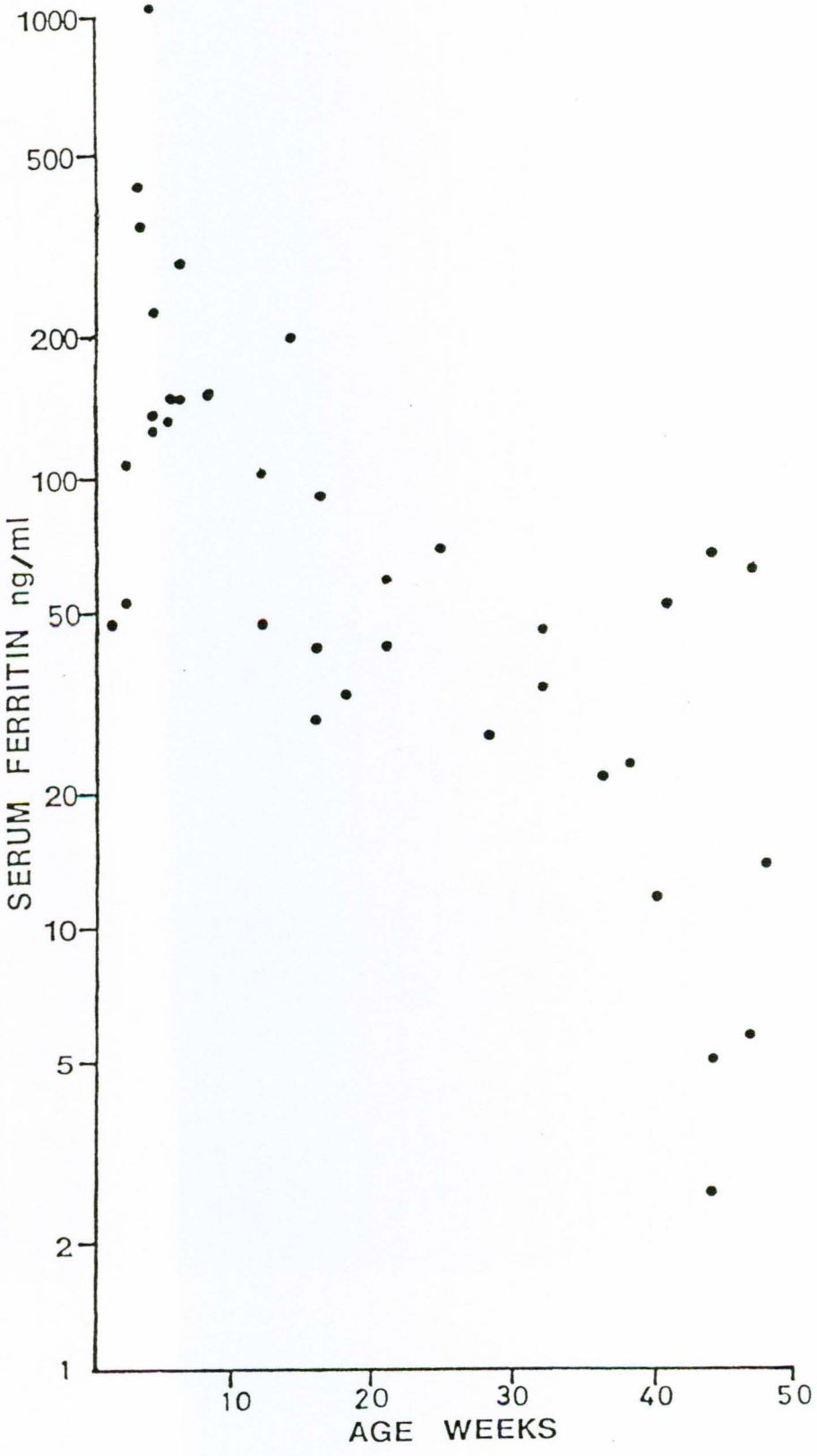

Figure 4 Serum ferritin values plotted by age in Pilot II. Log scale.

Serum ferritin fell significantly with age; however values of serum ferritin less than 10-15 ng/ml (a lower limit of normal serum ferritin for adults (Worwood, 1979)) were only seen in infants over 40 weeks of age (Fig. 4). A linear regression of log serum ferritin with age for 37 cases gave the equation

$\log_{10}$ serum ferritin (ng/ml) = 2.308-0.0246 x age (weeks);

($r = -0.71$; $P<10^{-5}$)

Two of 49 thick blood films examined showed Plasmodium vivax. No other parasites were seen.

DISCUSSION

The two pilot surveys in Madang district revealed a population of infants with marked anaemia and hypochromia, and results are in agreement with previous surveys in infants in Papua New Guinea (Vines, 1970; Kariks, 1969). Suggested causes for anaemia in coastal PNG have included folic acid deficiency (Powell and Booth, 1969), iron deficiency (Kariks, 1969), malaria (Vines, 1970), and genetic disorders (Amato, 1977). In these pilot studies there was no evidence of folic acid or B_{12} deficiency, but there was evidence that both malaria and iron deficiency affect this population.

The presence of a palpable spleen was associated with lower haemoglobins in infants over eight weeks of age in the first pilot study. This is good presumptive evidence of the role of malaria in some of the anaemia. In the second pilot study, infants over eight weeks of age showed low haemoglobins, low serum iron values, and universal hypochromia and microcytosis in blood films, evidence suggestive of a high prevalence of iron deficiency. Serum ferritin, however, only fell to sub-normal levels after 40 weeks of age, whereas all the other indices suggestive of iron deficiency were already abnormal by two months of age. These findings are not necessarily incompatible: iron-deficient erythropoesis in the presence of

apparently adequate serum ferritin levels has previously been noted in infants in other studies (Saarinen and Siimes, 1978; Lundstrom, 1980).

The pilot surveys indicated that it would be important to match for domicile in a prospective study of anaemia.

The haematological surveys revealed a high prevalence of hypochromic microcytosis apparently mainly an iron deficiency effect. At the time of these surveys and the commencement of the trial the uniquely high prevalence of alpha thalassaemia (81%) in this population (Oppenheimer, 1984) (see Chapter 7.2) was not known. In retrospect some of the hypochromia and microcytosis noted in the survey could have been attributable to this factor.

CHAPTER 6.

PROTOCOL AND METHODS (Clinical/Laboratory/analysis).

Chapter 6.1 PROTOCOL

SUMMARY

The protocol for a prospective, randomized, double-blind, placebo controlled trial of iron prophylaxis in infants is described. Specific design points discussed include (i) control and "blind", (ii) dose, preparation and age of administration of iron, (iii) standardization of morbidity recording, (iv) data analysis and (v) ethical review.

INTRODUCTION

Pilot studies in Madang, Papua New Guinea (see Chapter 5) confirmed the suitability of the venue, the population and the methods proposed for study (Oppenheimer et al, 1984). The cohort study was undertaken to investigate the relationship between a) iron deficiency and b) iron supplementation and susceptibility to infection as measured by: (i) incidence and prevalence of infection; (ii) severity of infection; (iii) mortality from infection.

Analysis of results was 2-tailed and based on the null hypothesis of no effects.

When planning this study special attention was directed toward overcoming a number of factors which have detracted from the validity of previous studies: (i) controls; (ii) standardised morbidity recording; (iii) route of administration and dosage of iron; (iv) age This Chapter details the protocol and laboratory methods agreed with the Papua New Guinean Ethical Board following the pilot studies, which was observed very closely during the project.

METHODS OF STUDY

Cohort study and clinical trial of intramuscular iron dextran

(a) Method of recruitment. All infants born in the base hospital to women living in the study area were eligible for inclusion. The following were excluded: babies with major congenital anomalies, or extremely low birth weight (<1.4 kg); and those who had exchange transfusion or serious illness in the neonatal period, or a mother unwilling to participate in the study. Infants were recruited at birth, the nature and details of the study were explained to their mothers and their consent obtained.

(b) Treatment: trial cohort allocation. Prior to two months of age the infants were matched and paired by sex, domicile and birth weight and then allocated paired code numbers. These numbers corresponded with batched pairs of masked, coded syringes containing either 3 ml of iron dextran or a sterile pyrogen free saline placebo. The order of the two treatments within a pair was randomized by the supplier (Fisons). A sealed key to the treatments given was kept by a scientist not directly involved in the study, in case it proved necessary to break the code in the interests of a particular baby. These injections were given at two months of age after obtaining a full history, physical examination and confirmation of consent. The code number was then inserted in the babies' records. If at the time of the two month visit the baby was sick, or had a fever, the injection was deferred by one week or until there was full recovery. Babies were fully re-examined one week after the injection or earlier in the case of sickness. The trial was thus planned to be double blind, using matched randomised pairs (see Chapter 7).

(c) Field follow-up. All subjects were then followed in an identical manner with clinical and haematological observations recorded at birth, two months, six months and one year. Maternal and Child health

records independently kept by the MCH services were also analysed. Information recorded at each visit by a field worker (state registered nurse or doctor) included:

(1) Birth. Mother: marital status, village, occupation, antenatal care, education, obstetric history, haemoglobin and weight. Infant: sex, birth weight (beam balance), length, head circumference and details of any jaundice and/or infection in the neonatal period.

(2) Two months, six months and 12 months. Details were obtained of any infections present or in the previous two weeks. The infant was fully examined and weight (by Salter hanging scale), length, and head circumference recorded. Any illness requiring attention was managed by the field worker in co-operation with the MCH sister present. Details of all these visits were recorded on standard proforma (Appendix IV). Diagnoses, with standardized accuracy rating, were made on the basis of the field worker's questionnaire, examination and the MCH record book. No attempt was made to obtain a comprehensive record of all illnesses since the previous visit as the inaccuracies of recall for longer than two weeks would have made the information suspect. Thus, in effect, point prevalence only was measured in the field part of the study.

(3) Infants were also visited one week after the injections in order to detect any possible ill effects. This follow-up was recorded on an extra form with identical details to those recorded on other field visits (Appendix IV).

(d) Admissions. All hospital admissions of infants from the cohort were recorded on a standard proforma (Appendix IV). Diagnoses were coded using the 9th WHO International Classification of Diseases (WHO,

1975). Details of history, examination and investigations were also individually coded (Appendix IV).

(e) Informed consent. Informed consent was obtained on two occasions, at birth and again at the two month visit, before injection. This was deemed necessary in order that mothers should fully appreciate the voluntary nature of their participation and understand the study. The father's consent was obtained where possible.

(Explanation of the potential effects of iron dextran injection was considerably helped by the fact that it was already a commonly used standard treatment. Thirty-four percent of the mothers had received iron dextran during pregnancy and a great many had either seen it given to other mothers or to their children in MCH clinics).

(f) Independent ethical review. The protocol was reviewed and approved by the Papua New Guinea Medical Research Advisory Council (Official Ethical Board) in April 1980. It was also endorsed by the Public Health Department, the Senior Specialist paediatrician in the University of Papua New Guinea, and the Madang provincial paediatrician.

DISCUSSION OF PROTOCOL

There have been several studies to date of the effects on morbidity of iron supplementation in infancy. These have been criticised on the basis of design and methods of recording (Pearson and Robinson, 1976). This study aimed to overcome these problems as far as possible. A number of factors which needed special attention are listed below:

Route of iron administration

Iron may be given parenterally or as a regular oral supplement. In New Guinea there is nearly universal breast feeding. For this

reason an iron-fortified milk formula was out of the question, and regular oral medication had major problems. Firstly, it would not have been possible to control for the theoretical disadvantage of oral iron interfering with lacoferrin bacteriostasis in the gut and, secondly, it would not have been possible to supervise or ensure long-term therapy by the mothers. Iron dextran injection was already in routine use in New Guinea both for the presumptive treatment of anaemia (Biddulph, 1979), and for prophylaxis in low birth weight neonates (Biddulph, 1979). Therefore, from the community point of view, this practice was acceptable and from a medical stand point due for assessment.

Dosage of iron

The dose used was 150 mg elemental iron (3 ml of iron dextran injection). This was calculated as a mean requirement for the Madang infant population by two independent approaches: (a) expected iron requirements in the first year based on total body iron at birth and postnatal growth and (b) total body iron deficit observed at one year. These calculations were made using data obtained in the pilot surveys (Chapter 5) which included birth weights, post-natal growth patterns, and haemoglobin and serum ferritins observed at different ages in the first year. Methods and assumptions used for calculating total body iron using these variables have been described (Saarinen and Siimes, 1979).

It should be noted that 150 mg elemental iron is considerably less than that used previously in Maori neonates in New Zealand (250 mg) (Barry and Reeve, 1977) and less than that used routinely in Papua New Guinea for anaemic infants (250 mg Fe for infants weighing 3-5 kg; 350 mg Fe for infants weighing 6-9 kg) (Biddulph, 1979).

Age of administration

Iron dextran given to neonates has been reported in retrospective studies to have increased the rate of sepsis due to E.coli (Barry and Reeve, 1977). This increase in sepsis was restricted to the week following injections and was markedly reduced when injections were delayed until after one month of age (Farmer, 1976). Since in the neonatal period serum iron saturation and iron stores are high (Scott et al, 1975) and there is susceptibility to opportunistic infections such as E.coli (Miller, 1978), it was decided to delay the administration of iron dextran until two months of age. The iron dextran was administered as a single intramuscular treatment in order to minimize the period of hyperferraemia; and the field check at one week after injection was instituted to pick up and control any possible effects associated with this.

Design of prospective longitudinal trials

(a) Controls. Previous studies have been criticized for inadequate controls. In Mackay's study of iron supplementation in 1928 (Mackay, 1928), control and treatment observations were made in different years. In Barry and Reeves' retrospective longitudinal survey of iron dextran administration in Maori infants, control and treatment observations were also made in different populations at different times (Barry and Reeve, 1977). In other prospective studies of iron supplementation to infants, though randomization for the treatment and control groups was performed it is not clear whether follow-up morbidity observations were double or even single blind (Cantwell 1972), (Andelman and Sered, 1966). In the study by Andelman and Sered, removal of anaemic controls (75% of that group) from the analysis was a potential source of considerable bias in their results (Andelman and Sered, 1966).

The project design followed recent standard recommendations for longitudinal clinical studies (Peto et al, 1976). Following matching, randomization and treatment, we had a trial cohort consisting of roughly equal numbers in treatment and control groups. In order to categorize the study cohort as fully as possible for factors that might influence morbidity and iron balance, a number of social, anthropometric, clinical and laboratory variables were recorded on mothers and babies at the time of delivery. There were no significant differences between the iron dextran and placebo groups for any variable (see Chapter 7). Being a hospital based study, this was inevitably a biased sample of the population, but comparisons were made between randomized groups of the same sample, thus neutralizing that bias. The logistic advantages of hospital recruitment were considerable. For accurate recording of perinatal details, hospital recruitment was essential.

The infants in the trial cohort were followed "blind" in an identical manner over an average period of ten months commencing with the injection at two months. Withdrawals prior to injection were excluded from the analysis but follow-up was performed on most of these cases. Subsequent to the injection at two months no further cases were regarded as withdrawals from final morbidity analysis even for reasons such as non-attendance at field follow-up visits. The only exclusions from analysis allowed after randomization and injection were permanent migrations which occurred immediately after injection, when no follow-up was possible (Peto et al, 1976).

Events which might, at first glance, be thought to justify withdrawal after injection were the administration of iron dextran and/or blood transfusion to either the treatment or placebo group during the surveillance period. However, withdrawal of such cases from analysis would be likely to introduce bias in the morbidity

rates. Since the trial was testing the real effect of a policy of iron dextran prophylaxis at two months of age, within the context of the health policy and services available, morbidity analysis had to include children in either group who subsequently received iron. Numerical breakdown and description of the cohort are given in Chapter 7.

(b) Standardization of morbidity recording is a crucial factor in studies such as this, but receives little mention by most authors. In the detailed study by Andelman and Sered (1966), which is the largest reported to date, it is not clear whether morbidity recorded is based on mother's recall or on physical examination.

Standarized pre-coded morbidity forms which recorded and coded hospital admissions were used in this study. The only differences between 'field' and hospital forms were that:

(i) the hospital forms included standard special investigations e.g. chest X-ray and microbiology and

(ii) the final diagnostic coding in hospital followed the four digit ICD (WHO, 1975) while the field visit forms used a modified three digit tabulation (WHO, 1975).

Advantages of this system were:

(a) sign and symptom complexes could, if necessary, be analysed independently of the clinician's diagnosis and be correlated with WHO acute respiratory morbidity classifications, which were already in use in the Highlands of Papua New Guinea (WHO, 1981).

(b) the results of hospital special investigations e.g. chest X-ray, could be used to perform discriminant analysis on the coded clinical findings and thus validate the field diagnoses which would be based on the same clinical observations as hospital diagnoses minus special investigations (see Appendix I.iv.).

Chapter 6.2 SUMMARY OF LABORATORY METHODS* AND INVESTIGATIONS

INTRODUCTION

In planning laboratory methods to be used for a study of this nature in a third world country, decisions needed to be made as to which investigations were desirable, essential, or practicable. The main constraints in Madang are inaccessibility for 1) servicing of specialised equipment; and 2) transport of perishable specimens. Other considerations were that a study on infants requiring at least 4 separate blood taking episodes necessitated use of accurate micromethods for analysis.

Tests which had to be performed in Madang

Basic red cell indices (Hb, PCV, MCHC), blood film spreading, staining, white cell counting, separation of serum for freezing, preparation of RBC lysates, reticulocyte preps and packed red cells all had to be performed within hours of blood collection. Ideally a Coulter counter would have produced rapid and accurate RBC indices and WBC counts, and would have added the major advantage for this study of accurate determinations of MCV and MCH. This was originally budgeted for, but became impractical because of lack of servicing facilities, disposables, and quality control supplies in Papua New Guinea. More rugged traditional manual methods thus had to be used.

Venous blood could not always be obtained in the field; accurate portable methods* of estimation of Hb and PCV on very small volumes therefore needed to be evaluated for occasional use in the field (see Appendix I.i.).

* for details of development and evaluation of new methods see Appendix I.

Red cell enzymes, particularly G6PD are not stable enough for prolonged storage and needed to be performed soon after blood collection using standard screening kits.

Tests performed in England

1) Transport:

All blood and serum specimens sent to England had to be transported frozen. For the 4000 specimens sent over a 3 year period dry ice and liquid nitrogen, even if easily available, would have been impractical. Given the necessity for 3 airline changes between Madang and Liverpool, the minimum transit time for booked cargo was 36 hours. A system of dry frozen blocks in expanded polystyrene containers (TRANSTEMP) was used. This guaranteed 3 days at -11°C and only required a -21°C deep freeze to freeze the blocks. In practice no specimen ever defrosted in transit. Storage in PNG and UK was at -45°C and -30°C respectively.

2) Microtests for serum iron/TIBC and ferritin

It was important to monitor iron status in individuals. Serum ferritin and transferrin saturation were desirable measures. At the start of the study (1979) serum iron and transferrin saturation could only easily be estimated from larger serum samples (>0.5 ml); serum ferritin could only be estimated in Liverpool using immunoradiometric assays requiring serum volumes in excess of 200 µl. Given the small volumes of serum available and the number of other tests performed on serum, it was necessary to develop accurate microassays for both these two tests (see Appendix I).

SUMMARY OF CLINICAL AND LABORATORY INVESTIGATIONS

Blood Tests

Venous blood, 0.5 ml in potassium EDTA and a 1 ml clotted specimen, was obtained at birth, at each field visit and on each hospital admission. Cord blood was also obtained where possible at birth. Routine tests included the following:

(1) Haemoglobin by the cyanmethaemoglobin method (Dacie and Lewis, 1975) using a Corning colorimeter 252.

(2) Packed cell volume (PCV) using the COMPUR minicentrifuge (Oppenheimer, 1979) (see Appendix I.i.).

(3) Reticulocyte count (Dacie and Lewis, 1975).

(4) White cell count (Dacie and Lewis, 1975).

(5) Thin blood film (HAEMOFAST-Modified Wright's stain): red cell morphology (assessed by a standarised scoring system for hypochromasia, microcytosis, poikilocytosis, anisocytosis and polychromasia (Vines, 1970)); white cell differential; presence of toxic granulation in neutrophils; presence of malarial pigment in monocytes; presence and identity of malarial parasites.

(6) Thick blood film for presence, identity and density of malarial parasites. All blood films were read by two independant microscopists. In addition, all positives and 10% of negatives were confirmed by David Gibson, of the Papua New Guinea Institute of Medical Research, (formerly WHO malariologist).

(7) Red cell folate, using an automated radioimmune assay (Becton Dickinson SIMULTRAC) 15 µl washed red cells were lysed in 0.2% ascorbic acid and frozen for transport to Liverpool University Department of Haematology, where the assay was performed by Mr. P Cashin.

(8) Serum folate using an automated radioimmune assay (Becton Dickinson SIMULTRAC).

(9) Serum iron and total iron-binding capacity using a micro modification of Sigma Kit No. 565 (see Appendix I.ii.).

(10) Serum ferritin, using an enzyme-linked immunosorbent assay (see Appendix I.iii.). Reagents: Dako anti-human liver ferritin and peroxidase conjugated anti-human liver ferritin (Mercia Brocades); Standards and Controls RIA (U.K.) Ltd.

(11) Serum B_{12} level assayed in Liverpool University Department of Haematology using an automated radioimmune assay (Becton Dickinson SIMULTRAC).

(12) Red cell pyruvate kinase (Sigma Kit No. 205) and glucose-6-phosphate dehydrogenase (Sigma Kit No. 202). These tests were both performed in Papua New Guinea in the project laboratory.

(13) Haemoglobin electrophoresis and HbF/HbA_2 quantitation at one year:

a) Preparation of haemolysate:

1. Whole blood in potassium EDTA in Madang - washed 3 times with isotonic saline, packed, frozen and transported to England.

2. Packed red cells in Liverpool - cells were lysed by dilution 3 times with distilled water, and thoroughly mixed. Approximately 2 cm^3 carbon tetrachloride and a few drops of 1% KCN were added, thoroughly mixed, and spun 20 minutes at 3000 rpm. Supernatent was pipetted off, and stored at 4°C while in use, then at -20°C or below.

b) Detection of abnormal haemoglobins by starch gel electrophoresis at pH 8.6 in Tris EDTA Borate buffer (Weatherall, 1983).

c) Cellulose Acetate Electrophoresis for quantitation of HbF (alkali denaturation; Weatherall, 1983), and HbA_2 (elution from cellulose acetate; Weatherall, 1983).

d) For Barts estimation in cord bloods see Chapter 7.2).

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Haemoglobin and PCV were estimated from capillary samples if the mothers preferred this to venepuncture (Oppenheimer, 1979) (see Appendix I.i.). Capillary blood was also used to obtain thick blood

films for malaria, parasites, and thin films for differential and total white blood counts when necessary.

Additional investigations performed on children admitted to hospital were as follows:

(1) Routine: chest radiography - Madang Hospital; nose and throat swabs for bacteriological culture; stool parasitology - Madang Hospital Pathology Department.

(2) Where clinically indicated: lumbar puncture, blood culture and bone marrow examination - Madang Hospital Pathology Department.

Chapter 6.3 CODING, DATA ENTRY AND METHODS OF ANALYSIS

Coding, checking, entry and datavetting

Clinical data were entered directly on pre-coded forms (see Appendix IV) while laboratory investigations were transferred from laboratory day books to pre-coded forms (see Appendix IV) and coding sheets, thus reducing the likelihood of transcription errors. The forms were checked twice in PNG and again in Liverpool before being punched directly into the Liverpool University computer system, using double-entry and verification.

The final database contained over 650,000 characters, necessitating systematic datavetting using standard range- and logic-checks.

Notes on analysis and use of logistic regression and analysis of variance

The bulk of analysis was performed using the programme Statistical Package for Social Sciences (SPSS). Analyses used only cases where complete data were known for that analysis. Denominators are given.

Where appropriate, X^2 and 2-tailed Student's 't' tests were used as tests of significance of observed differences. Odds ratios were used to quantify and compare risks.

Where distributions showed marked skew (for example periods of hospitalisation of infants in the cohort), non-parametric tests were used to compare differences (e.g. the Kruskall-Wallis one-way analysis of variance).

Interactions between iron dextran treatment and uncontrolled factors such as malaria were a potential source of confounding effects in the trial, and needed to be tested for, using multivariate analysis (see below).

Logistic regression

In analysis of the multiple factors potentially affecting risk of admission in the period 2 - 12 months of age (Chapter 10), it was necessary to use logistic regression since the dependant variable was discrete and multiple linear regression could not be used. A Generalised Linear Interactive Modelling (GLIM) package was therefore used in this analysis.

In the above analysis "interactive terms" were tested for. By this is meant that the risk (e.g. of admission) associated with one "explanatory" factor (e.g. low weight-for-height at the start of the trial) may be affected by the presence of another "explanatory" factor (e.g. iron dextran treatment). The possibility of such an interaction is tested for after controlling for the independant effects of the respective explanatory factors.

A note on the use of analysis of variance (ANOVA)

In Chapters 8, 9, 11 and 13, 2 and 3 way analysis of variance is used to separate effects of various factors on haematological measurements. The need for this somewhat complex analysis in a balanced intervention study is related to confounding and interactive effects mainly associated with malaria:

1) Separation of main effects. Implicit in the aims of the study is the need to measure and explain the haematological effects of iron treatment in this population.

The relative increase in haemoglobin levels in the iron group when compared with the placebo group should be direct evidence of the degree of anaemia due to iron deficiency in the infant population. Levels of serum ferritin and transferrin saturation in the iron treatment group should likewise give an indication of the degree of increase in storage and circulating iron. However, as will become apparent (see also Chapter 5) malaria was associated with lower

haemoglobins and (in this study) higher serum ferritin levels and transferrin saturation (see Chapter 9). There was, moreover, more malaria slide positivity in the iron treatment group thus tending to confounding effects on these measures. There was therefore a need to separate the main independent effects associated with iron treatment from those associated with malaria positivity using 2-way analysis of variance.

2) Interactive effects. Higher malaria rates were noted in the iron treatment group (see Chapter 9). This was one of the main detectable outcomes of the study. Measurement of the severity of effects associated with malaria was thus the next step.

Haematological effects (particularly low Hb levels) were the only objective measures of malarial severity observed in this study. Not only was it necessary to separate and measure malaria effects while controlling for iron effects - see above, but it was also important to test for "interactive effects" between malaria and iron on the haematological variables in particular haemoglobin, since this should give information on the relative severity of malaria in the respective treatment groups.

Malarial slide positivity was clearly unevenly distributed between cells which affects the assumptions of "balanced design" ANOVA. However, the ANOVA programme used in this study adjusts for imbalanced design.

Tabulation of ANOVA results

Analysis of variance attempts to explain the variation in the dependant variable by adjusting for explanatory factors. Total variation in the dependant variable is the sum of squares of differences of each value from the mean. The sum of squares left after adjusting for explanatory factors is the "residual variation".

Full results of the ANOVA are generally expressed in the first place as "sum of squares" or "variation" explained separately by each factor followed by "sum of squares" explained by "interactions".

The total sum of squares explained by the explanatory terms (e.g. treatment group and malaria), expressed as a percentage of the total sum of squares of the dependant variable (e.g. Haemoglobin) gives a measure of total variation explained by these terms and is the equivalent of the "r^2" value in multiple regression.

An F statistic is derived for each term by dividing the mean sum of squares explained by the term by the residual mean square for the whole model. The F statistic is used to test for significance; however, since it gives a measure of the relative power of each term in explaining variation it is also used for this purpose in summary tables of ANOVA in Chapter 9. It should be noted that ANOVA is used only to test prior hypotheses and the directions of effects are illustrated in the figures and breakdown tables.

Exact p values are generally shown in tables. Significant values ($p<0.05$) imply that a factor/covariate/interactive term explains a significant proportion of variation in the dependent variable (e.g. Hb) while non-significant values ($p>0.05$) fail to disprove the null hypothesis.

CHAPTER 7.

DESCRIPTION OF COHORT

SUMMARY

The study cohort at birth is described, and rationale for exclusions and reasons for withdrawals are discussed. An initial descriptive comparison is made of treatment and control groups entering the trial at two months of age.

Chapter 7.1.1 EXCLUSIONS, WITHDRAWALS AND RANDOMISATION

Five hundred and sixty-five infants were initially recruited at birth in Madang hospital between June 1980 and December 1981. Nine were excluded in the neonatal period mainly for protocol reasons: (a) neonatal death (two); (b) major cardiac anomaly (two including one death); (c) congenital hypothyroidism (one); (d) extreme low-birth-weight (one); (e) mother changed mind immediately after initial consent (two); and (f) exchange transfusion (one) (Figure 1).

After the neonatal period the remaining 556 recruits were randomly allocated to receive iron dextran (273) or placebo (283) (Figs. 1 and 2). As previously stated (Protocol: Chapter 6), the trial commenced at the two months visit when infants were to be given either iron dextran or placebo. Of the 556 cases randomized, 70 were withdrawn before trial entry at two months. Reasons for withdrawal are shown in Table 1. The reasons for withdrawal were distributed evenly between treatment and placebo allocations.

TABLE 1

Reasons for withdrawal after allocation before two month entry

Allocated	Not found at 2 months	Refused at 2 months	Migrated before 2 months	Chronic sickness	Died before 2 months
Iron dextran	11	11	11	1	3
Placebo	11	10	12	-	-

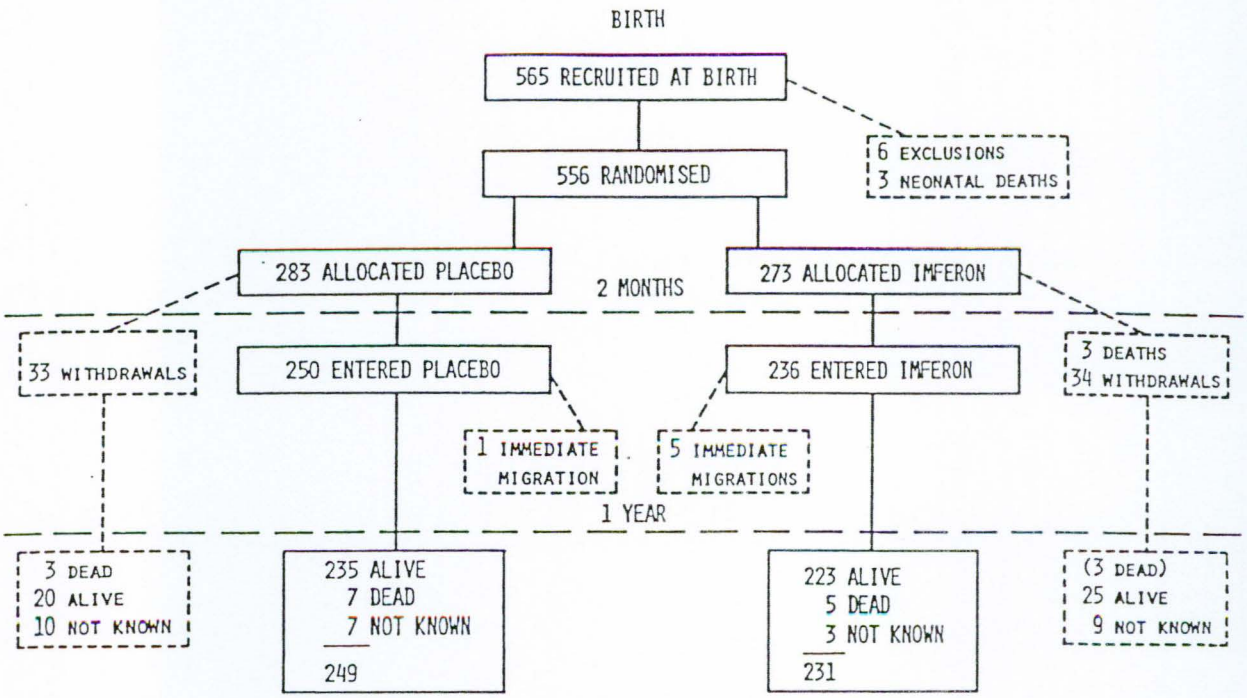

Figure 1 Flow chart of cohort study to show exclusions, allocations, withdrawals and follow-up numbers.

Batched trial supplies were not available at the commencement of the trial and the first 135 of 556 babies were matched and allocated iron dextran injection or no injection randomly. These infants, whose mothers therefore knew whether or not they had been injected were therefore effectively single-blind. During the later double-blind period, using trial supplies, both treatment and placebo infants received a masked, coded injection. There was a theoretical possibility of bias in withdrawals at the time of injection in the single blind period. Figure 2 shows the distribution of withdrawals during both periods and between groups. No significant bias is apparent in withdrawals between iron dextran and placebo groups in either period. The overall withdrawal rate was less in the double-blind period; this was due to improved follow-up procedures. It should be stressed that following the two months visit all babies were "observer blind" since records of allocations were coded.

Infants from single and double-blind periods are combined in subsequent analysis.

Among 486 infants entered into the trial there were 6 immediate, permanent migrations to other provinces, with no possibility of systematic follow-up or of ascertaining admissions to hospital in the first year (Fig. 1). One of these (in the iron dextran group) was known to be alive at one year. There were two further immediate migrations to remote inland parts of Madang province. One year survivorship was known in 470 (98%) of the remaining 478 and in 471 (97%) of the 486 infants originally entering the trial.

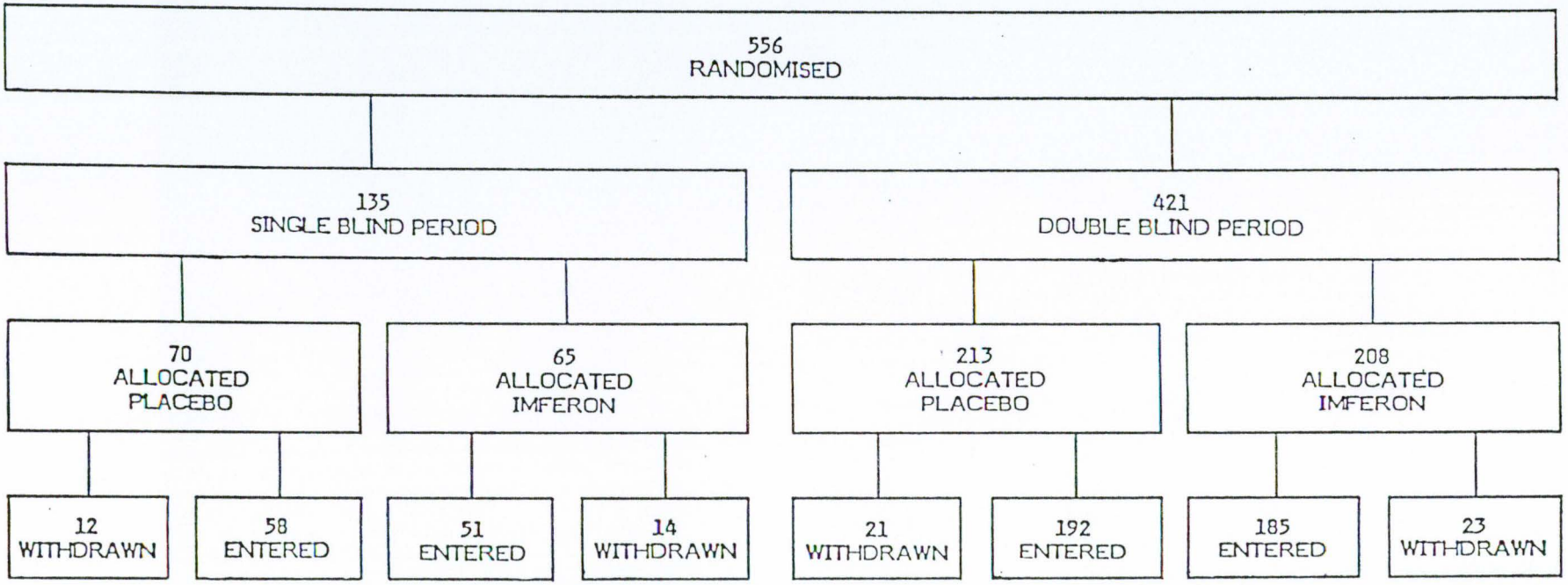

Figure 2 Flow chart of allocations, withdrawals and entries in single and double blind periods to show balance of cells.

Chapter 7.1.2 DESCRIPTION OF INITIAL BIRTH COHORT (556 INFANTS INITIALLY RANDOMISED

Mothers. Two-thirds of the mothers were from urban areas, the majority from periurban settlements. Chapter 3 describes the domicile and housing classification. Most mothers had some antenatal care (average four clinic visits), and 34% received total dose iron infusion (35 ml iron dextran) for anaemia during pregnancy. (This was given in the antenatal clinic and was not related to the study intervention). Full details are given in Chapter 8. Forty mothers (9%) had perinatal slide positive malaria. Mean (SD) antenatal haemoglobin was low: 9.2 g/dl (1.48) n = 362; mean postnatal parity was three, while modal parity was one. Mean maternal postnatal weight was 52 kg. Mothers' ethnic origin (for details of classification see Chapter 4) was known in 96% of cases and showed: 36% indigenous Madang non-Austronesian speakers; 23% Sepik-Ramu non-Austronesian speakers from the neighbouring East Sepik province; 27% Austronesian speakers; 6% from the Finisterre Huon mountain range (non-Austronesian speakers); 3% from Eastern highlands; 3% Bundi (Gende) and 1% from the Toricelli ranges in the West Sepik province (non-Austronesian speakers). The rest were non-Austronesian speakers from other parts of New Guinea* (see Map Fig. 3). The distribution of fathers' ethnic origins showed 78% concordance with mothers'.

Birth cohort (Table 2)

Fifty-two per cent of babies were boys. Mean (SD) birthweight was low: 2.79 kg (0.43). Gestational age was assessed by the Dubowitz scale (Dubowitz and Dubowitz, 1977), which was validated on the 20% of mothers with known dates for last menstrual period. Mean (SD) gestational age was 38.8 weeks (1.34); with a minimum of 33 weeks.

* In this context "non-Austronesian" and "Papuan" are synonymous.

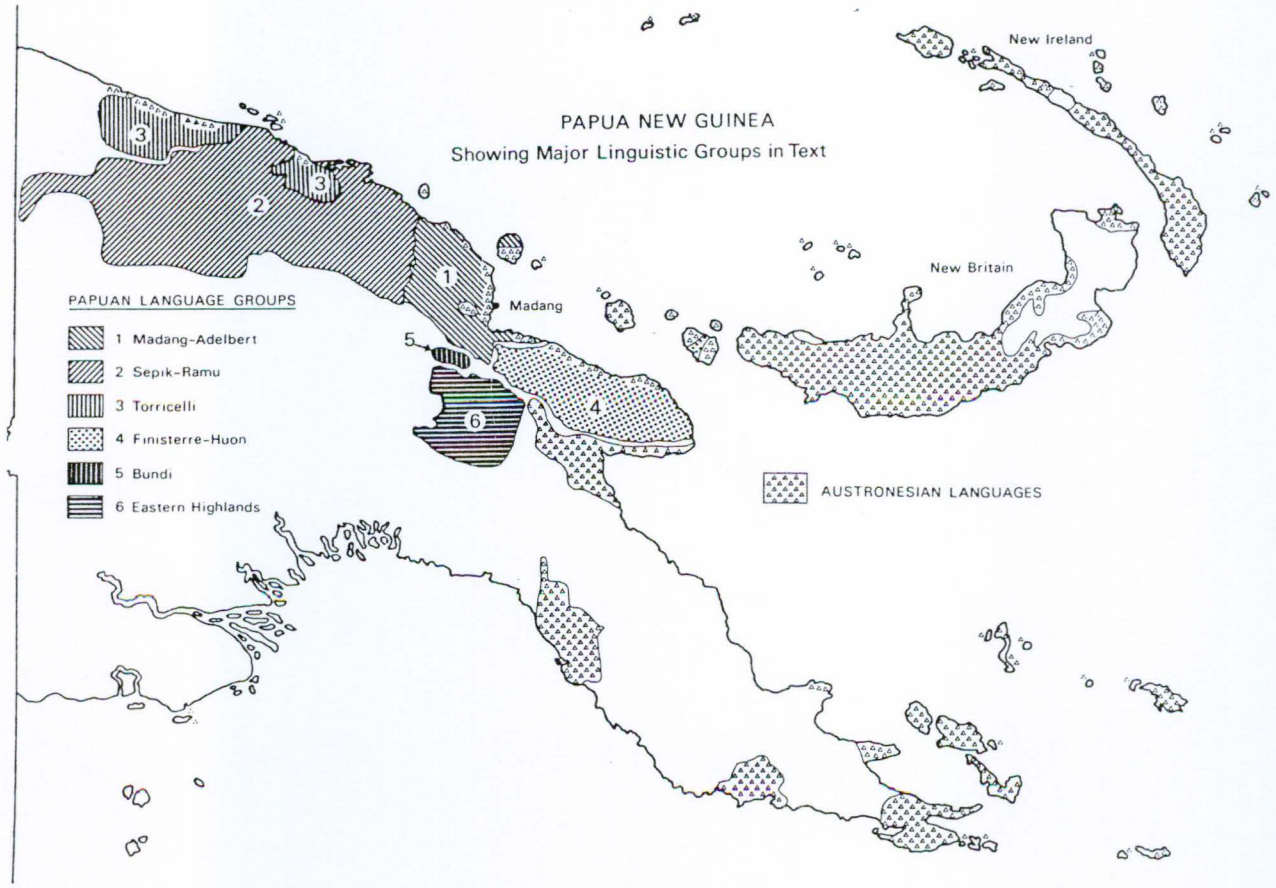

Figure 3 Map of Papua New Guinea showing distribution of major linguistic groups represented in the study. ("Papuan" and "non-Austronesian" are synonymous).

Twenty-five per cent of newborns developed jaundice and 10% had a neonatal infection. Mean (SD) cord haemoglobin, estimated on a small sample of 35 infants, was 12.3 g/dl (1.61). Neonatal haemoglobin was estimated on 553 venous samples, taken between three and 100 hours after birth. Mean (SD) neonatal Hb was 14.7 g/dl (2.13) which is around 4 g/dl lower than reported in western neonates (Guest and Brown, 1957; Gross, 1968). No significant relationship with time after birth was noted in the Hb values. No B_{12} or folate deficiency was detected in birth bloods. The mean (SD) serum iron was 12.3 µmol/l (6.6), and mean (SD) transferrin saturation was 22.8% (8.6). These values are 40-50% lower than those quoted for American three-day-olds (Gross, 1968). The geometric mean for serum ferritin was 139.3 ng/ml. Garry et al, (1981) give geometric mean values of 260-290 ng/ml for one- to two-day-old Mexican infants.

TABLE 2

Birth recruits: descriptive variables

Variable	Mean	SD	n.
Birthweight (kg)	2.79	0.42	556
Birthlength (cm)	48.4	2.64	530
Head circumference (cm)	33.2	1.60	533
Gestational age (weeks) (Dubowitz)	38.8	1.34	527
Cord haemoglobin (g/dl)	12.3	1.61	35
Postnatal haemoglobin (g/dl)	14.7	2.13	554
Serum B_{12} (ng/l)	696.3*	1.62+	238
Serum folate (µg/l)	11.6	4.02	237
Serum iron (µmol/l)	12.3	6.56	508
Transferrin saturation (%)	22.8	8.6	499
Serum ferritin (µg/l)	139.3*	2.04+	513

* Geometric mean
\+ Antilog of SD of $\log_{10}$ transform.

Chapter 7.1.3. GENETIC ANOMALIES

A number of genetically determined abnormalities were noted in the blood of the birth cohort: 160 out of 200 tested (80%) had haemoglobin Barts on electrophoresis (Oppenheimer et al, 1984) (see 7.2 below and Chapter 11). Ten out of 491 (2%) were fully deficient for RBC pyruvate kinase while 20 out of 491 (4%) were partially deficient. Seventeen out of 431 (4%) showed marked G6PD deficiency (one of these was a girl), and five out of 431 showed partial deficiency. Some degree of ovalocytosis was seen in 47 out of 557, 25 of whom showed more than 50% ovalocytes with knizocytes or stomatocytes. There were no significant differences in rates of genetic traits between iron and placebo treatment groups.

Effects on haematological variables associated with α^+ thalassaemia.

With the uniquely high prevalence of α^+ thalassaemia found in this cohort, it was important to check for any associated effects on haematological variables which might confound other results, particularly those relating to iron status. For the purpose of comparison, infants who had birth Hb Barts estimated and entered the trial are divided into 3 groups: 1) "Normals" (no Hb Barts); 2) "Low Barts" (Hb Barts up to 3.5%); 3) "High Barts" (Hb Barts >3.5%). These groups in this population correspond approximately with the 3 genotypes: Normals; Heterozygotes; Homozygotes. The rationale for these divisions is given in Chapter 11.

Mean venous haemoglobin at birth was 14.7 g/dl, and was 0.41 g/dl higher in the "Normals" than in those with Hb Barts detected at birth. The difference was not significant, and there was no significant correlation between birth Hb and % Hb Barts. Serum iron transferrin saturation at birth was significantly higher in the "Normals" than the "thalassaemics" ($t = 6.22$; $p<0.001$). Serum ferritin (logged) at birth showed no significant correlation with Hb Barts %. Venous MCHC was

negatively correlated with Hb Barts % ($r = -0.371$; $n = 173$; $p<0.0001$). A comparison of these variables in the 3 Barts categories is shown in Table 3.

TABLE 3

Birth and 2 month haematological variables by birth Hb Barts status

mean (SD)

	Haemoglobin g/dl	MCHC g/dl	Transferrin saturation %	Serum ferritin µg/l
Birth				
Normals	15.0(2.44) n=35	31.93(1.56) n=35	25.3(9.86) n=31	133(13.6%)* n=32
Low Barts	14.5(2.07) n=40	31.06(0.97) n=40	21.1(7.64) n=38	134(13.7%)* n=39
High Barts	14.6(2.18) n=98	30.71(1.48) n=98	21.2(8.08) n=91	145(13.1%)* n=92
2 months				
Negative Barts	9.0(1.07) n=34	31.6(1.69) n=34	25.8(9.04) n=30	121(18.6%)* n=30
Low Barts	9.0(1.14) n=41	31.6(1.91) n=41	29.1(10.12) n=29	132(15.3%)* n=29
High Barts	8.9(1.00) n=96	31.0(1.83) n=94	27.4(9.67) n=67	157(15.3%)* n=71

* Geometric mean (coefficient of variation in log transform).

At the 2 month visit (pre injection) there was no significant correlation between Hb Barts % at birth and Hb, MCHC, serum iron, transferrin saturation or serum ferritin (logged). No microcytosis was seen in any of the infants with negative Hb Barts at birth or with low Barts at birth. 32% of infants with high birth Hb Barts had microcytosis seen on thin film at this visit.

In subsequent visits (6 and 12 months) there were no significant main effects associated with birth Hb Barts status on haemoglobin, MCHC, transferrin saturation or serum ferritin. Interactive effects with iron and malaria are described in Chapter 11.

Comparison of electrophoresis at birth and one year:

There were 241 estimations of HbF % at one year and 189 estimations of HbA_2 at one year in the full cohort. The mean HbF (SD) was 2.34% (1.56). 5% of HbF results were over 5%, and 8% of results over 4%. Mean (SD) HbA_2 at one year was 2.5% (0.40). Two values were over 3.8%. The presence or absence of Hb Barts at birth did not affect mean HbA_2% at one year.

There was no significant correlation between (1) 1 year HbF and 1 year HbA_2, (2) 1 year HbF% and birth Hb Barts % (when present), (3) 1 year HbA_2% and birth Hb Barts % (when present). However, infants who had no Hb Barts at birth had a significantly higher mean HbF% at one year than infants with Hb Barts detected at birth: (3.16%: 2.22%; $t = 2.44$; $p<0.017$).

Chapter 7.1.4. TRIAL COHORT: COMPARISON OF TREATMENT AND PLACEBO GROUPS (n = 486). (Tables 4 and 5).

Of the 556 infants recruited at birth, 70 were withdrawn before the trial injection at two months, leaving 486 infants entering the trial. Differences between the two treatment groups could have resulted in confounding errors in the trial. Therefore comparisons were made between treatment groups for a number of potentially confounding variables. Students 't' tests, analysis of variance, and X^2 tests were used as appropriate to test hypotheses of no difference between the groups. Maternal variables tested included: domicile, housing type, antenatal care (+/-), total dose iron infusion during pregnancy (+/-), pregnancy booking haemoglobin, mean antenatal haemoglobin, post-natal haemoglobin, perinatal malaria (+/-), post-natal weight, parity, education and ethnicity. No significant differences were detected between treatment groups for any of these variables. A selection of the more important maternal variables are shown in Table 4. Descriptive variables compared in trial infants included sex, gestational age, the presence of individual genetic anomalies (Hb Barts at birth, G6PD deficiency, ovalocytosis, Pyruvate Kinase deficiency), haematological variables in cord, 24 hour and 2 month bloods (Hb, PCV, serum ferritin, serum iron, transferrin saturation, serum and red cell folate, serum B_{12}) anthropometric measures at birth and 2 months (weight, length, head circumference). No significant differences between groups were detected for any of these variables. Table 5 shows comparisons of the main potential confounders in trial infants.

DISCUSSION

Following matching, randomisation and treatment, the trial cohort consisted of roughly equal numbers in treatment and control groups. In order to categorise the study cohort as fully as possible for factors that might influence and confound morbidity and iron balance, a number of social, anthropometric, clinical and laboratory variables were recorded on mothers and babies at the time of delivery. There were no significant differences between the iron dextran and placebo groups for any variables tested. Being a hospital based study, this was inevitably a biased sample of the population, but comparisons were made between randomized groups of the same sample, thus in theory neutralizing bias. The logistic advantages of hospital recruitment were also an important consideration.

TABLE 4

Comparison of mothers' social, antenatal and postnatal variables between infants' treatment group in trial cohort (n = 486)

Infants' treatment	Domicile					Housing			Received antenatal care	Received total dose iron infusion in pregnancy	Had maternal perinatal malaria	Maternal postnatal		
	Urban resident	Urban settlement	Rural indigene	Rural migrant	Rural institute	High cost	Low cost	Bush material				Weight (kg)	Hb (d/dl)	Parity
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	Mean (SD)	Mean (SD)	Mean (SD)
Iron dextran (n = 236)	65 (27)	96 (41)	55 (23)	14 (6)	6 (3)	8 (3)	87 (37)	141 (60)	192 (85)	80 (38)	18 (9)	52.7 (7.50) n = 225	9.83 (2.11) n = 236	3.00 (1.91) n = 236
Placebo (n = 250)	76 (30)	93 (37)	53 (21)	14 (6)	14 (6)	8 (3)	89 (36)	150 (61)	220 (90)	73 (32)	14 (7)	52.5 (7.67) n = 236	10.18 (2.02) n = 247	3.0 (2.02) n = 249

TABLE 5

Comparison of birth anthropometric and laboratory variables between infants' treatment groups in trial cohort (n = 486)

Treatment	Sex (% male)	Birth weight (Kg) Mean (SD)	Gestational (weeks) Mean (SD)	Post-natal haemoglobin (g/dl venous) Mean (SD)	Post-natal serum iron (μmol/l) Mean (SD)	Transferrin saturation (%) Mean (SD)	Serum ferritin (ng/ml)
Iron dextran	55.1	2.79 (0.43)	38.9 (1.32)	14.7 (2.09)	12.4 (6.68)	23.1 (0.09)	131.5 (0.13)*
n = 236		n = 236	n = 226	n = 235	n = 215	n = 211	n = 217
Placebo	49.6	2.80 (0.42)	38.8 (1.37)	14.7 (2.19)	12.1 (6.50)	22.3 (0.08)	142.5 (0.15)*
n = 250		n = 250	n = 237	n = 250	n = 232	n = 229	n = 232

* Geometric mean (antilog of SD of $\log_{10}$ transform).

Chapter 7.2. ALPHA-THALASSAEMIA IN PAPUA NEW GUINEA

(first published in the Lancet - Feb 25, 1984).

SUMMARY

Haemoglobin Bart's was detected in cord blood samples from 81% of 217 infants born in Madang on the north coast of Papua New Guinea. Analysis of the α globin genes of 30 infants and adults from the same region showed that all but 3 were heterozygous or homozygous for the deletion form of α^+ thalassaemia. None of 18 cord blood samples from infants born in Goroka in the Eastern Highlands Province had haemoglobin Bart's, and in each case the α globin genes were normal. Preliminary geographical and linguistic analyses of both groups suggest that the prevalence of α thalassaemia may be related to altitude rather than to linguistic grouping and hence that resistance to malaria may be at least one reason why α thalassaemia is so common in some populations.

INTRODUCTION

Several surveys have suggested that inherited red-cell disorders are common in Melanesia (Curtain et al, 1965; Beaven et al, 1974; Booth, 1981). As part of a study of anaemia in Madang on the north coast of Papua New Guinea, the haemoglobin constitution of 217* newborn infants was analysed.

PATIENTS AND METHODS

Cord blood samples were collected from 217* infants born at Madang Provincial Hospital and from 18 born at Goroka Base Hospital in the Eastern Highlands Province (see Map 7.1.2.). The red cells were washed three times and stored at -45°C. Haemoglobin analysis by starch-gel electrophoresis, pH 8.6, and estimation of the relative

* 200 of these belonged to the birth cohort

amount of haemoglobin Bart's by quantitative cellulose-acetate electrophoresis, pH 8.9, were caried out by standard methods (Weatherall, 1983).

The ∝globin genes were studied in the cord blood samples of the 18 infants born in Goroka, cord blood samples of 21 infants born in Madang, and blood samples from 9 adults in Madang. Preparation of DNA from buffy coats and analysis of the ∝ globin genes after digestion with Bam Hl and hybridisation with an ∝-globin-specific probe were carried out as previously described (Weatherall and Clegg, 1981; Old and Higgs, 1980).

RESULTS

Of the 217 infants born in Madang Hospital, 175 (81%) had detectable haemoglobin Bart's, with levels ranging from 0.8% to 11.0%. None of the 18 infants born in Goroka Hospital had detectable haemoglobin Bart's. Two Highlands infants born in Madang had no haemoglobin Barts. A subset of 182 results based on the language spoken (Wurm and Hattori, 1981) and area of origin of the parents is shown in the Table. In the three lowland Papuan linguistic groups (Table groups 1-3; see also Map 7.1.2.) the combined frequency of detectable haemoglobin Bart's was 93% (103/111) and in the foothill ethnic groups (Table groups 4 and 5; see Map 7.1.2.) the frequency was 43% (6/14), whereas none of the 20 ethnic Highlands infants had detectable haemoglobin Bart's.

TABLE

FREQUENCY OF HAEMOGLOBIN (Hb) BART'S AT BIRTH IN THE DIFFERENT LINGUISTIC GROUPS STUDIED

Language*	Altitude m	Number (%) infants with: No Hb Bart's	Hb Bart's detected (%)
Austronesian	0	13	24(65)
Papuan:			
1 Madang	0-500	3	57(95)
2 Sepik-Ramu	0-500	5	44(90)
3 Torricelli	0-500	0	2(100)
4 Finisterre-Huon	0-1500	4	5(44)
5 Bundi (Gende)	600-1500	3	2(40)
6 Eastern Highlands	>1500	20	0(0)

* see Map 7.1.2.

To determine whether a high level of haemoglobin Bart's at birth reflects the presence of α thalassaemia in this population and to provide further information about the prevalence of α thalassaemia in these groups, α gene mapping was carried out on 48 samples. All the 18 samples from Highlands infants showed a normal αglobin genotype ($\alpha\alpha/\alpha\alpha$). Of the 30 samples from infants and adults from the Madang province, 3 showed a normal αgenotype, but the remainder were either heterozygous ($-\alpha/\alpha\alpha$) or homozygous ($-\alpha/-\alpha$) for the deletion form of α^+ thalassaemia. Of the 39 α^+ thalassaemia chromosomes, 27 were of the $-\alpha^{4.2}$ type, 10 were of the $-\alpha^{3.7}$ type, and 2 were unclassified $-\alpha$. When the αglobin genotype was compared with the level of haemoglobin Bart's at birth in 31 infants; 20 infants with normal αglobin genes ($\alpha\alpha/\alpha\alpha$) had no haemoglobin Bart's and 1 had 1.4% haemoglobin Bart's; 4 α^+ thalassaemia heterozygotes ($-\alpha/\alpha\alpha$) had 0%, 2.1%, 2.3%, and 2.4% haemoglobin Bart's; and 6 α^+ thalassaemia homozygotes had haemoglobin Bart's levels ranging from 4.7% to 8.2%.

DISCUSSION

This pilot study has shown that an extremely high proportion of newborn infants from the north New Guinea coastal lowland region have haemoglobin Bart's. Globin gene mapping analysis has confirmed that detectable haemoglobin Bart's at birth is caused by the deletion form of α^+ thalassaemia in this population. The apparent rarity of the more severe clinical phenotypes of α thalassaemia, the haemoglobin Bart's hydrops syndrome and haemoglobin H disease, presumably reflects the rarity of α^0 thalassaemia determinants (ie, conditions in which both α globin genes are deleted on the same chromosome (Weatherall and Clegg, 1981) in this region.

The deletion forms of α^+ thalassaemia are distributed widely throughout Africa, the Mediterranean, the Middle East, the Indian subcontinent, and South-East Asia (Weatherall and Clegg, 1981). The prevalence varies from 1-2% to 80% (Weatherall and Clegg, 1981; Brittenham et al, 1980). The prevalence of 95% in the Madang/Adelbert linguistic group in this study is the highest yet reported. It seems likely that the deletion forms of α^+ thalassaemia result from unequal crossing-over between homologous pairs of chromosomes 16, leaving one α gene on one of the pair and three on the other. However, in every population examined to date the single α globin gene chromosome is much commoner than the triplicated α gene arrangement (Higgs and Weatherall, 1983). Hence, it seems likely that under certain circumstances the single α gene chromosome has come under strong selection.

Although the selective factors that have maintained the high prevalence of the deletion forms of α^+ thalassaemia are not known, their geographical distribution suggests that resistance to Plasmodium falciparum malaria may have had a role. Although the numbers of cases studied are small, α thalassaemia is unevenly distributed among the

population and the prevalence appears to be related to the altitude of residence of the various groups examined. Lowlands New Guinea has endemic malaria, ranging from hyperendemic to holoendemic, which was present before European contact (Vines, 1970). Highlands New Guinea, in contrast, had no malaria transmission over 2000 m and only seasonal Plasmodium vivax transmission over 1300 m before European contact (Brittenham et al, 1980); in the Eastern Highlands no malaria was recorded in early surveys (Peters et al, 1958).

It is possible, of course, that gene drift and isolation may have a role in the apparently varying prevalence of α thalassaemia in the areas studied. There are, however, several reasons why this is unlikely. The migratory and centrifugal spread of the trans-New-Guinea language phylum throughout mainland New Guinea about 10,000 years ago not only gave rise to the closely related upland languages of groups 4-6 (see Table) but also to the lowland languages of Madang Province (group 1) (Wurm, 1975). Thus, the groups with the highest and lowest levels of α thalassaemia are linguistically more closely related than the other lowland groups. Furthermore, with triaxial mapping of ABO and MNS blood group frequency, the New Guinea populations fall into four slightly overlapping groups (Booth and Simmons, 1972). On two-dimensional representation, the Madang and Sepik populations are separated from the Austronesians by the Highlanders. Thus, it also appears from blood group data that the groups with high prevalence of α thalassaemia are more closely related to the group with the lowest prevalence than they are to each other.

A possible relation has been reported between altitude and the prevalence of β thalassaemia and glucose-6-phosphate dehydrogenase deficiency, two other red-cell disorders thought to afford heterozygous protection against P.falciparum malaria, in Papua New Guinea (Curtain et al, 1965; Gorman and Kidson, 1962). If more

extensive population studies of the type reported here demonstrate a similar relation for α thalassaemia, it may be possible to explain why this single-gene disease is so common throughout the world.

Postscript

Since this preliminary communication in the Lancet in 1984, the author went back to Papua New Guinea to collect 550 blood samples mainly from the Highlands which contributed to a survey of alpha thalassaemia frequencies in Melanesia confirming the correlation of frequency of this haemoglobinopathy with intensity of malaria transmission in the region (see Appendix V).

CHAPTER 8.

RELATIONSHIP OF MATERNAL AND NEONATAL IRON STATUS; EFFECTS OF TOTAL DOSE IRON INFUSION IN PREGNANCY ON MOTHER AND BABY

SUMMARY

An analysis is presented of haematological results from the 544 mothers and their 556 newborns in the birth cohort. 34% of mothers received total dose intraveous iron infusion (TDI) before delivery in the antenatal clinic as treatment for anaemia. A range of haematological tests were carried out on newborns and mothers in addition to anthropometry. 84% of mothers had had antenatal care and data were also collected retrospectively from antenatal records. TDI was associated with more slide positive perinatal malaria in primipara (odds ratio: 5.5) but not in multipara. When all relevant factors were considered TDI was not associated with an overall improvement in haemoglobin status from the first antenatal level recorded to the postnatal check. Postnatal malaria was associated with lower antenatal and postnatal haemoglobin levels. There was no evidence of any effect of TDI in pregnancy or of maternal malaria on foetal maturity or birth weight. Gestational age, maternal weight, parity and maternal postnatal haemoglobin were all significantly correlated with birth weight. TDI to the mother was associated with higher neonatal serum ferritins and lower neo-natal haemoglobins. Maternal postnatal malaria was associated with significantly lower iron in serum in newborns. It is suggested that routine total dose iron infusion to anaemic pregnant mothers in malaria endemic areas may be contraindicated.

INTRODUCTION

Malaria has been reported to have more deleterious effects and to be more common in pregnant than in non-pregnant females in endemic

areas (Brabin, 1983). Specific effects which may occur in areas of stable as well as unstable malaria endemicity include (i) severe acute maternal malaria (Wickramasuriya, 1937), (ii) chronic maternal anaemia (Fleming et al, 1969); (iii) an increase in foetal mortality (Hung Le Van, 1951); (iv) decrease in birth weight (Bruce-Chwatt, 1952); and (v) pre-term delivery (Menon, 1972). These effects were particularly pronounced in primipara. Malaria is also more common in primipara than in multipara, the increase in prevalence being more marked in early pregnancy (Brabin, 1983). The secondary effects of malaria on the mother have an interdependency which it is important to control for when analysing the effects of maternal malaria on the baby since birth weight, for instance, may be affected by prematurity, parity and maternal anaemia quite apart from any direct effect of maternal or placental malaria on foetal growth.

This section analyses the relationship between maternal and newborn iron status, and the effects of iron therapy and malaria on the mother and the baby, and reports an apparent increase in maternal malaria in primipara who received parenteral iron antenatally.

SUBJECTS AND METHODS

556 babies born to 544 mothers in the initial birth cohort (see Chapter 7) were used as case studies. There were ten sets of twins and two sets of triplets. Only two of each set of triplets were included for matching purposes connected with the main study. This group represented approximately 40% of deliveries in Madang hospital between June 1980 and December 1981. The results reported here represent an opportunistic analysis of perinatal and retrospective antenatal data.

The mothers came from urban, peri-urban and rural areas within easy access of the hospital; this fact, and initial consent to enter the study, formed the main factors in selection (see Chapter 6).

Madang has intense transmission of malaria with a high incidence of chloroquine resistance (Darlow & Vrbova, 1981). Maternal anaemia is common and drugs routinely supplied at clinics to all mothers include ferrous sulphate and chloroquine weekly prophylaxis. In spite of this, most mothers maintain haemoglobins less than 10 g/dl during pregnancy. Standard policy in the PNG health department recommends parenteral iron treatment for all pregnant women with unresponsive anaemia, but as this is impracticable a lower threshold of haemoglobin (around 8 g/dl) tends to be used as the indication for treatment. 34% of mothers nonetheless received total dose iron infusion (TDI = 35 cc iron dextran = 1750 mg elemental iron) at some time in the antenatal period at Madang hospital. This provided the opportunity to observe the effects of parenteral iron in pregnancy in a highly endemic malarious area.

Information recorded on mothers included:

(i) Antenatal details (retrospectively from antenatal records): presence or absence of antenatal care; dates of visits; haemoglobin measurements at each visit (cyanmethaemoglobin method; EEL colorimeter); haemoglobin at the time of the decision to give TDI and date of administration if different. "Booking haemoglobin" refers to first haemoglobin measurement in pregnancy.

(ii) Postnatal details used in this analysis include: postnatal weight; postnatal haemoglobin; and results of postnatal thick and thin blood films examined for presence and species identity of maternal parasites.

Variables recorded on newborns included:

(i) Anthropometric: birth weight, birth length, head circumference.

(ii) Gestational age by the Dubowitz method (Dubowitz & Dubowitz, 1977).

(iii) Haemoglobin, serum iron, total iron binding capacity, serum ferritin, serum and red cell folate and serum B_{12} on venous and cord blood. Methods are described elsewhere (Oppenheimer et al, 1984a, b, Chapter 6).

Methods of analysis include where appropriate X^2 and 't' tests. Analysis of variance and covariance were performed using SPSS programmes which allow for unequal cell sizes and non-manipulated experimental attributes, e.g. malaria. In all tables and analyses, cases with missing variables for that analysis are excluded. The denominator (n) is stated where appropriate.

RESULTS

Maternal cohort description

The study cohort is described in detail in Chapter 7; relevant facts only are given here. 84% of the 544 mothers had some antenatal care recorded with an average of 3.3 visits. Median and mode number of visits was three with a range of one to nine. 167 (31%) of mothers had total dose iron infusion (TDI) antenatally. Mean postnatal parity was three but the most frequent reported state was primiparous (28%). In mothers receiving TDI, mean (SD) haemoglobin at the time the decision was taken to give TDI was 7.0 g/dl (1.13). However mean (SD) booking haemoglobin in these mothers was 9.8 g/dl (1.70). Mean (SD) postnatal haemoglobin was 10.0 g/dl (2.08). 40 mothers had slide-positive malaria recorded at delivery (9% of 440). 79% were Plasmodium falciparum, 18% P.vivax and 3% P.malariae.

Preliminary analysis of maternal variables by parity showed that primipara differed from multipara on a number of features and that grades of multiparity showed little further between group variation. Further analysis therefore uses "parity" as a dichotomous factor (primipara vs multipara).

TABLE 1

Maternal variables broken down by parity

	Ante-natal attendance % (n)	Total dose iron infusion in pregnancy % (n)	Slide positive post-natal malaria % (n)	Booking haemoglobin mean (SD) n	Post-natal haemoglobin mean (SD) n
Primipara	95% (148)	43% (140)	12% (133)	9.73 g/dl (2.09) 113	9.64 g/dl (2.18) 152
Multipara	85% (376)	30% (352)	8% (305)	9.79 g/dl (1.70) 249	10.14 g/dl (2.03) 387
All	87% (524)	34% (492)	9% (438)	9.77 g/dl (1.83) 362	10.00 g/dl (2.08) 539
Comparison between the two groups	p<0.01	p<0.01	NS	NS	p<0.05

Table 1 shows maternal variables broken down into primipara versus multipara. Primipara received significantly more antenatal care, significantly more total dose iron infusions and had slightly more malaria. Primipara also had significantly lower postnatal haemoglobins although booking haemoglobins were similar to multipara. The haemoglobin difference between primipara and multipara was of the order of 0.5 g at the postnatal check (Table 1).

Effect of TDI on maternal perinatal malaria

When the effect of iron dextran on infant malaria rates was detected during analysis of the trial, maternal perinatal slide positivity was analysed retrospectively in relation to TDI during pregnancy to see if there was any similar risk.

Although there was only slightly more slide-positive postnatal malaria in primipara than multipara this difference was significantly increased when mothers who received total dose iron infusion (TDI) were considered. Table 2 shows that the odds ratio (TDI vs no TDI) of having postnatal malaria in primipara was 5.46 whereas the rate of malaria in multipara was unaffected by TDI.

Table 3 shows as expected that the mean postnatal haemoglobin was significantly lower in mothers with postnatal malaria than in those without. Mean antenatal booking haemoglobin was also lower in these mothers. The mean weight of mothers with malaria was also 3 kg lighter than those without.

In order to determine any net beneficial or deleterious effect of TDI on maternal haemoglobins, a three-way analysis of variance was performed with postnatal haemoglobin as the dependent variable, booking haemoglobin entered as a covariate to avoid selective confounding errors, and parity (primipara/multipara), TDI (given/not given), and malaria (positive/negative) as the three explanatory factors (Table 4). The results show that the main determinant of post-natal

TABLE 2

Odds ratio of post-natal malaria broken down by parity and total dose-iron infusion(TDI)

		No. with slide examination	% Positive for malaria	Odds ratio TDI vs no TDI (95% limits)
Primipara	TDI	54	20.4%	5.46 (2.20-13.53)
	No TDI	67	4.5%	p<0.01
Multipara	TDI	95	8.4%	1.12 (0.73-1.70)
	No TDI	184	7.6%	NS

TABLE 3

Maternal variables broken down by post-natal malaria positivity and by total dose iron infusion (TDI)

	Booking haemoglobin	Post-natal haemoglobin	Mother's Post-natal weight
Maternal Post-natal malaria slides	Mean (SD)	Mean (SD)	Mean (SD)
	n	n	n
Malaria positive	8.91g/dl (1.90)	9.31g/dl (2.13)	49.7kg (5.63)
	28	40	39
Malaria negative	9.78g/dl (1.79)	10.01g/dl (2.01)	52.7kg (7.49)
	269	397	380
Comparison between the two means	p<0.05	p<0.05	p<0.01
Maternal total dose iron infusion			
TDI given to mother	8.96g/dl (1.70)	9.70g/dl (2.04)	51.6kg (6.49)
	139	166	160
TDI not given to mother	10.32g/dl (1.70)	10.21g/dl (2.06)	53.0kg (7.50)
	212	325	307
Comparison between the two means	p<0.0001	p<0.01	p<0.05

TABLE 4

3-way analysis of variance of post-natal haemoglobin (dependent) by: maternal parity, TDI and post-natal malaria controlling for maternal booking haemoglobin

Explanatory Variables	Mean Square	DF	F	P
Covariate				
Maternal booking haemoglobin	275.98	1	88.08	<0.00005
Factors				
Parity (1/1+)	35.44	1	11.31	0.001
TDI given (+/-)	2.85	1	0.91	0.341
Post-natal maternal malaria (+/-	0.03	1	0.01	0.927
Residual	3.06	280		
Total	4.19	284		
Total variation explained: 28%				

haemoglobin was the initial booking value. Parity also had a strong effect on postnatal haemoglobin (primipara having lower values - see Table 1). TDI in contrast had a small non-significant (negative) effect and malaria had no effect.

In summary, (i) TDI was associated with more malaria in primipara, (ii) when all relevant factors and covariates are considered TDI was not associated with a net improvement in haemoglobin status from the booking antenatal visit to the postnatal period. Postnatal malaria was associated with lower ante- and postnatal haemoglobin levels.

Newborns

(i) Effects of maternal variables on birth-weight

Maternal malaria has been reported to affect maturity and birth-weight in newborns in endemic areas, particularly in primipara (Wickramasuriya, 1937; McGregor et al, 1983). The mean (SD) birth-weight in the infants in this study was low at 2.79 kg (0.43); the mean (SD) gestational age was 38.8 weeks (1.34). When birth-weight was broken down by parity, primipara clearly had lighter babies than multipara although gestational age was unaffected by parity (Table 5). Maternal malaria was however not associated with any difference in gestational age (Table 6).

In order to determine if there was an effect of malaria on birth-weight it was important to control for independent confounding maternal features which might also have significantly influenced birth-weight. A three-way analysis of variance was therefore performed with birth-weight as dependent variable and prior entry of gestation, maternal weight, and postnatal haemoglobin as covariates. The three explanatory factors used were malaria (slide positive/negative), TDI (given/not given), and parity (primipara/multipara). The results (Table 7) show that, when the other factors were

TABLE 5

Infants, birth-weight, maturity and 24-hour haematological variables broken down by parity

	Birth weight (kg) mean (SD) n	Gestational age (weeks) mean (SD) n	Venous Hb (g/dl) mean (SD) n	Serum iron µmol/l mean (SD) n	Serum ferritin ng/ml geometric mean n
Primipara	2.64 (0.40) 153	38.7 (1.46) 147	14.5 (2.06) 153	12.8 (7.33) 141	164.9 (103%)* 143
Multipara	2.84 (0.43) 401	38.9 (1.30) 379	14.8 (2.15) 399	12.19 (6.43) 366	131.2 (106%)* 369
All	2.79 (0.431) 554	38.8 (1.34) 526	14.6 (2.12) 552	12.37 (6.69) 507	140.0 (107%)* 512
Comparison between the two means	p<0.0001	NS	NS	NS	p<0.01

* co-efficient of variation in log transform

TABLE 6

Infants' birth weight, maturity and 24-hour haematological variables broken down by maternal post-natal malarial positivity and total dose iron infusion (TDI)

	Birth weight (kg) mean (SD) n	Gestational age (weeks) mean (SD) n	Venous Hb (g/dl) mean (SD) n	Serum iron µmol/l mean (SD) n	Serum ferritin ng/ml geometric mean n
Maternal post-natal malaria slide					
Malaria positive	2.69 (0.42) 40	38.6 (1.55) 38	14.6 (2.13) 40	9.7 (3.3) 40	162.2 (106%) * 40
Malaria negative	2.80 (0.44) 410	38.8 (1.37) 393	14.6 (2.12) 409	12.7 (7.09) 374	141.3 (107%) * 378
Comparison between the two means	NS	NS	NS	p<0.001	NS
Maternal total dose iron infusion					
TDI given to mother	2.77 (0.44) 171	39.0 (1.43) 160	14.2 (2.15) 171	12.2 (6.15) 157	164.9 (87%) * 157
Mothers received no TDI	2.80 (0.43) 335	38.8 (1.31) 318	14.8 (2.04) 334	12.6 (7.21) 305	127.5 (109%) * 310
Comparison between the two means	NS	NS	p<0.01	NS	p<0.001

* co-efficient of variation in log transform

TABLE 7

Three-way analysis of variance of birth weight by maternal parity, TDI, and post-natalmalaria controlling for gestation, maternal post-natal weight and post-natal haemoglobin

Explanatory Variables	Mean Square	DF	F	P
Covariates				
Maternal post-natal haemoglobin	0.714	1	4.80	0.029
Mother's post-natal weight	4.949	1	33.31	<0.0001
Gestation	9.267	1	62.37	<0.0001
Factors				
Post-natal malaria (+/-)	0.002	1	0.013	0.908
TDI given (+/-)	0.018	1	0.122	0.727
Parity (1/1 +)	2.480	1	16.70	<0.0001
Residual	0.149	365		
Total	0.202	371		

Total variation explained: 27%

controlled for, parity was still associated with a strong effect on birth-weight whilst there was no evidence of an effect of malaria or TDI on birth-weight.

Gestation assessed at delivery, maternal weight, parity and postnatal haemoglobin accounted together for 27% of variation in birth-weight (Table 7).

(ii) Effects of maternal variables on birth haematology

It has been suggested that the placenta may acquire iron directly when mothers receive TDI in pregnancy (Bauminger et al, 1982). Table VI shows comparative breakdowns of infants' haematological variables by malaria and TDI. TDI was associated with significantly higher neonatal ferritins whereas maternal malaria was not (Table 6). A three-way analysis of variance with $\log_{10}$ serum ferritin (dependent) by parity, maternal malaria, and TDI confirmed that the above effects of TDI were independent of parity.

Serum iron was significantly lower in babies of malarious mothers. There was no evidence of an effect of TDI on neo-natal serum iron (Table 6). Paradoxically neo-natal haemoglobin was significantly lower in babies of mothers who had had TDI (Table 6); this was in a reverse direction to the serum ferritin levels in these babies (Table 6).

In babies of mothers who had not received TDI, 24 hour venous haemoglobin and transferrin saturation correlated with mean maternal antenatal haemoglobin (r^2 = 1.3%; p=0.047; n = 212; and r^2 = 3%; p=0.01; n = 188 respectively). The effects were small and were not seen with postnatal haemoglobin. Apart from the above effects no other correlation was detected between maternal antenatal and post-natal haemoglobins and cord and 24 hour neonatal haematological variables.

DISCUSSION

Pregnant mothers, in particular primipara, have been widely regarded as peculiarly susceptible to malaria in endemic areas (Brabin, 1983; McGregor et al, 1983). Furthermore maternal malaria is thought to affect birth-weight (Bruce-Chwatt, 1952) especially in primipara (McGregor et al, 1983). Suggested reasons for the effect of malaria on birth-weight are: (i) prematurity (Lawson, 1967), (ii) maternal anaemia (Lawson, 1967), and (iii) a specific effect of the malarious placenta on foetal growth (McGregor et al, 1983). Theories as to the relative susceptibility on primipara vary from a concept of immune suppression or "naivete" of the primiparous mother (Brabin, 1983) to a concept of failure of alternative parasite killing mechanisms in the placental bed in early pregnancies (McGregor et al, 1983).

In this study in an area of endemic malaria, the relative risk of slide-positive postnatal malaria was over five times higher in primipara, who received total dose iron infusion during pregnancy, than in those who did not. Primipara who did not receive TDI had slide positivity rates similar to multipara. Slide positivity rates in multipara were unaffected by TDI. It should be noted that 95% of primipara attended antenatal clinic at some stage in the third trimester and therefore were given intermittent malaria prophylaxis and oral iron supplements during this time. Since the mothers who received TDI also belong to this group and were also the ones with more malaria, it is unlikely that oral treatment influenced the findings on which the above conclusions are based. In addition, since the emergence of high rates of chloroquine resistance in this area, amodiaquine prophylaxis has been shown not to affect parasite rates (Papua New Guinea Institute of Medical Research, 1981).

Postnatal maternal haemoglobins correlated with, but were higher than, antenatal (booking) haemoglobins. Primiparity was associated with lower postnatal haemoglobins. TDI and postnatal malaria however were not associated with any effects on postnatal haemoglobin when booking haemoglobin and parity had been controlled for. This is a somewhat surprising result since the decision to give TDI was based on finding a very low haemoglobin value at some stage in pregnancy (mean 7.0 g/dl). The average rise in mean Hb from this low value to the postnatal check was 2.8 g/dl (which to the obstetrician might at first sight be attributed to the TDI). However, the mean first antenatal booking haemoglobin for the TDI group was 9.0 g/dl, so the low haemoglobin values which prompted iron therapy were likely to be acute temporary falls. The most likely cause of acute mid-trimester anaemia in this area is malaria. Blood slides were not taken in antenatal clinic so this hypothesis cannot be checked. Nonetheless there is no convincing evidence that TDI was associated with any net beneficial effect on overall maternal haematological status from the booking visit to the postnatal check.

The analysis of materno-foetal influences showed:

(i) Total dose iron infusion and maternal malaria did not apparently affect foetal maturity or birth-weight, while antenatal haemoglobin levels, maternal postnatal weight, gestation and parity together accounted for 27% of variation in birth-weight.

(ii) Total dose iron infusion to the mother was associated with slightly higher neo-natal serum ferritins and lower neo-natal haemoglobins. This result supports Bauminger's suggestion (1982) and observations by MacPhail et al. (1980) and Fenton et al. (1977). However, information and control is inadequate to draw conclusions in this study.

(iii) Maternal malaria was associated with lower serum iron in neonates.

(iv) There was little evidence for a strong correlation between maternal anaemia and neonatal haematological indices. This is in contrast to the finding of Singla et al. (1978) who found strong correlations.

Maternal influences on neonatal iron stores are thus more likely to be related to birth-weight since this also correlates with total body iron (Widdowson & Spray, 1951). The implications of these findings for the management of anaemia in pregnancy in this malarious area argue against the routine use of total dose iron infusion which increases the risk of malaria in primipara without any apparent compensating benefit to either primipara or multipara, or any clear beneficial effects on their infants. Treatment of maternal gestational malaria would seem to be more appropriate first line therapy. Since the complications of malaria in pregnancy have to be more severe in areas of unstable malaria transmission than in a stable endemic area, TDI may be definitely contraindicated in the former situation in other parts of the world.

The problems of interpretation of an unbalanced observational study such as this suggest the need for controlled balanced prospective studies in malaria endemic countries where TDI is still widely used in pregnancy.

CHAPTER 9.

IRON SUPPLEMENTATION: EFFECTS ON MALARIA AND HAEMATOLOGICAL INDICES

SUMMARY

A placebo-controlled trial of intramuscular iron dextran prophylaxis for two-month-old infants was carried out on the north coast of Papua New Guinea where there is high transmission of malaria. The results indicate that the placebo group became relatively iron deficient whereas the iron dextran group had adequate iron stores and, in the absence of malaria, a higher mean haemoglobin. However in the iron dextran group there was (i) a higher prevalence of malaria, as judged by parasite and spleen rates at 6- and 12-month follow-up; (ii) a lower haemoglobin associated with malaria when compared with the placebo group. Within the placebo group it was noticed that the malaria rates were lower at follow-up in those infants who had had a low birth haemoglobin. In neither group was there apparent suppression of effective marrow activity, as judged by reticulocyte count, in the presence of malaria. Malaria infection in both groups was associated with significantly raised serum ferritin levels and transferrin saturations. Over-all these data give evidence for a protective role of iron deficiency against malaria and would argue against the injudicious use of iron replacement in areas where malaria is endemic.

INTRODUCTION

This section deals specifically with the results of the intervention study in relation to malaria prevalence and malarial anaemia.

MATERIALS AND METHODS

The study area, disease patterns, the study protocol and the study cohort are described in detail in Chapters 4, 6 and 7, and relevant points only will be given here. Malaria is endemic in the

region and accounts for 24% of out-patient attendances but has not been observed as an important cause of paediatric deaths at Madang hospital.

486 infants entered the trial cohort at two months of age. Nearly half (236) of these received an intramuscular injection of 3 ml iron dextran (50 mg/ml elemental iron) at this point.

Of the 486 infants in the original trial cohort six migrated to another province and two to remote parts of Madang province immediately after the injection, 422 were seen and examined for malaria at six months and 372 at 12 months. However, one year survivorship was known for 471 (97%) of the original trial cohort (see Chapter 7). One week following the injection all but seven infants were followed up and history and examination again performed. This check was carried out for 439 infants (211 in the treatment group). A further 34 infants (17 treatment/17 placebo) when visited were not available, but were reported to be well by the family. Procedure at all field visits (two months, six months and one year) was identical. History was taken, the infant was fully examined, and blood taken for various haematological tests. These included: haemoglobin, PCV MCHC, reticulocyte count (per 500 RBC), serum iron, total iron binding capacity, serum ferritin, serum B_{12}, serum folate, and red cell folate (see Chapter 6).

If mother preferred, capillary samples were used (see Chapter 6). This method was used in 29% of measurements. Comparisons of all direct haematological indices (Hb, PCV, WBC and parasite rates) obtained at all visits by these two methods showed no significant differences except that at six months the mean haemoglobin was 0.28 g/dl higher in venous specimens ($t = 2.62$, $p<0.01$). Mean MCHC was 0.5-0.6 g/dl lower at all field visits when measured by the capillary method ($p<0.01$). There was however no significant difference in the

frequency of capillary estimations between iron treatment and placebo groups, and no confounding effects were seen due to blood method when it was entered as a factor in analysis, so in the presentation of results, measurements by both methods are grouped together. Hypochomasia and microcytosis were assessed on an ordinate scale from 0 to 4 according to the frequency of the characteristic in the red cell population (Vines, 1970).

Malaria species were identified on thin films. Malaria asexual counts were performed on the majority of thick films per 200 white blood cells and then density/µl estimated using the white blood count. If less than 5 parasites were detected in this count, counting continued to 100 high power fields. Very high densities were estimated per high power field. Results thus obtained were then ranked for the purpose of analysis which used non-parametric tests for comparison. The majority of slides were read independently by two microscopists and in addition a supervisor (F.D. Gibson, previously WHO malariologist for PNG) reviewed all positives and 10% of negatives.

In the examination, spleen enlargement was measured in centimetres along a line perpendicular to the (L) lower costal margin and through the umbilicus. If the spleen was palpable but not proud of the costal margin, it was scored as 1 cm. Spleen rates were based on any palpable spleen.

Serum ferritin and reticulocyte counts have log normal distributions; analysis uses these variables in the $\log_{10}$ transform.

Not all the above variables were measured at all visits, and in the results the sample size (n) is given for each analysis. Each analysis includes only infants for whom all variables were recorded for that analysis. Analysis of variance was performed using Statistical Package for Social Sciences (SPSS), taking into account

unequal cell sizes due to non-manipulated experimental variables such as malaria (see Chapter 6.3).

Cases where iron dextran and/or transfusion were received after the two month visit from other health workers are indicated on the main tables; these were not removed from the analysis (see Chapter 6.1).

RESULTS

I. Factors influencing malaria prevalence at scheduled field visits

Malaria prevalence in this study is best assessed by studying results of the field visits at 2, 6 and 12 months since these occasions were clearly age matched and randomly related in time with respect to any episode of illness in the infants. The 2 month visit immediately preceded the injection and commencement of the trial, therefore the field check and the 6 and 12 month visits were the only scheduled events during the trial. Blood slide positivity and spleen enlargement are the only observations specifically indicative of malaria recorded in this study.

Domicile, age and season

Three independent factors were found to influence malarial positivity (and spleen rates): Domicile, age and season. Housing type also affected malarial positivity rates but this effect was closely related to domicile.

Domicile is described in detail in Chapter 4. Five categories were used: (i) urban residential (government and rented housing), (ii) urban settlement, (iii) rural village (ancestral landowners), (iv) rural settlement (migrants) and (v) rural institutions (missions). Fig. 1 shows over-all parasite rates at the three field visits separated by domicile. There was a progressive rise in prevalence of malaria with age in all domiciles, but there was also a clear, consistent difference in prevalence between the groups. The rural

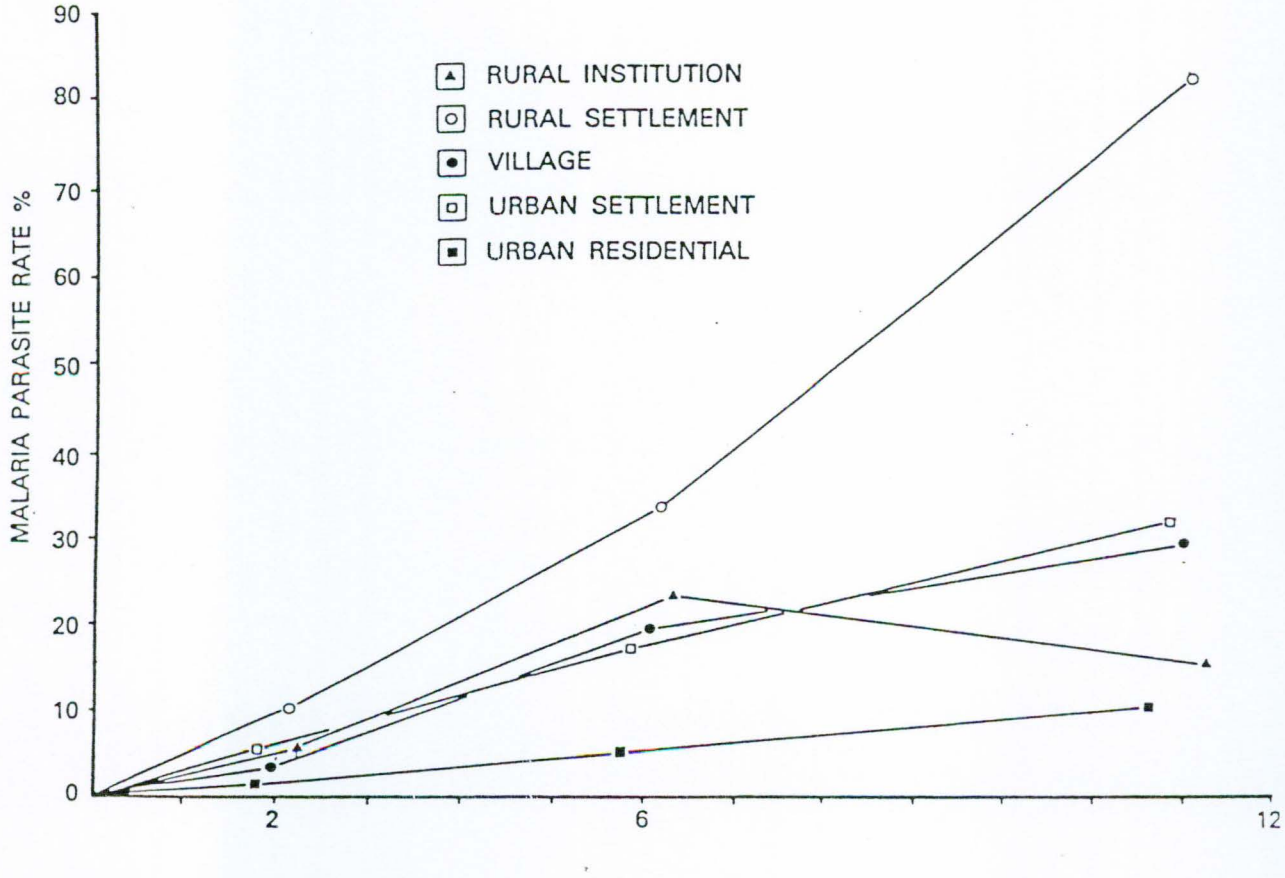

Figure 1 Malarial parasite rates at the 2, 6 and 12 month field visits broken down by domicile

settlement group had a high prevalence, whereas the urban residential (who generally have mosquito mesh on windows) had a low prevalence and the rural village, rural institutions and urban settlement groups had similar intermediate prevalence. When rural villages were divided into coastal and inland, no consistent differences in parasite rate were seen.

As mentioned in Chapter 7, the distribution of domicile between the treatment and placebo groups showed no significant differences, and this factor is ignored when analysing the effect of iron treatment.

The seasonal fluctuation in the prevalence of malaria is shown in Fig. 2. Although malaria is more prevalent in the wetter season (October-May), transmission occurs throughout the year. Indirectly evidence for this was seen at the 2 month visit where there were positive malaria cases throughout the year, even in the dryer season (June - September). Randomisation of treatment groups was performed using pairs proximate in date of birth. Seasonal variation did not affect slide positivity rates differentially between treatment groups.

The ratio P.falciparum:P.vivax was 3.9 at 6 months and 3.6 at one year. Two P.malariae infections were identified in the 1 year age group (Table 1).

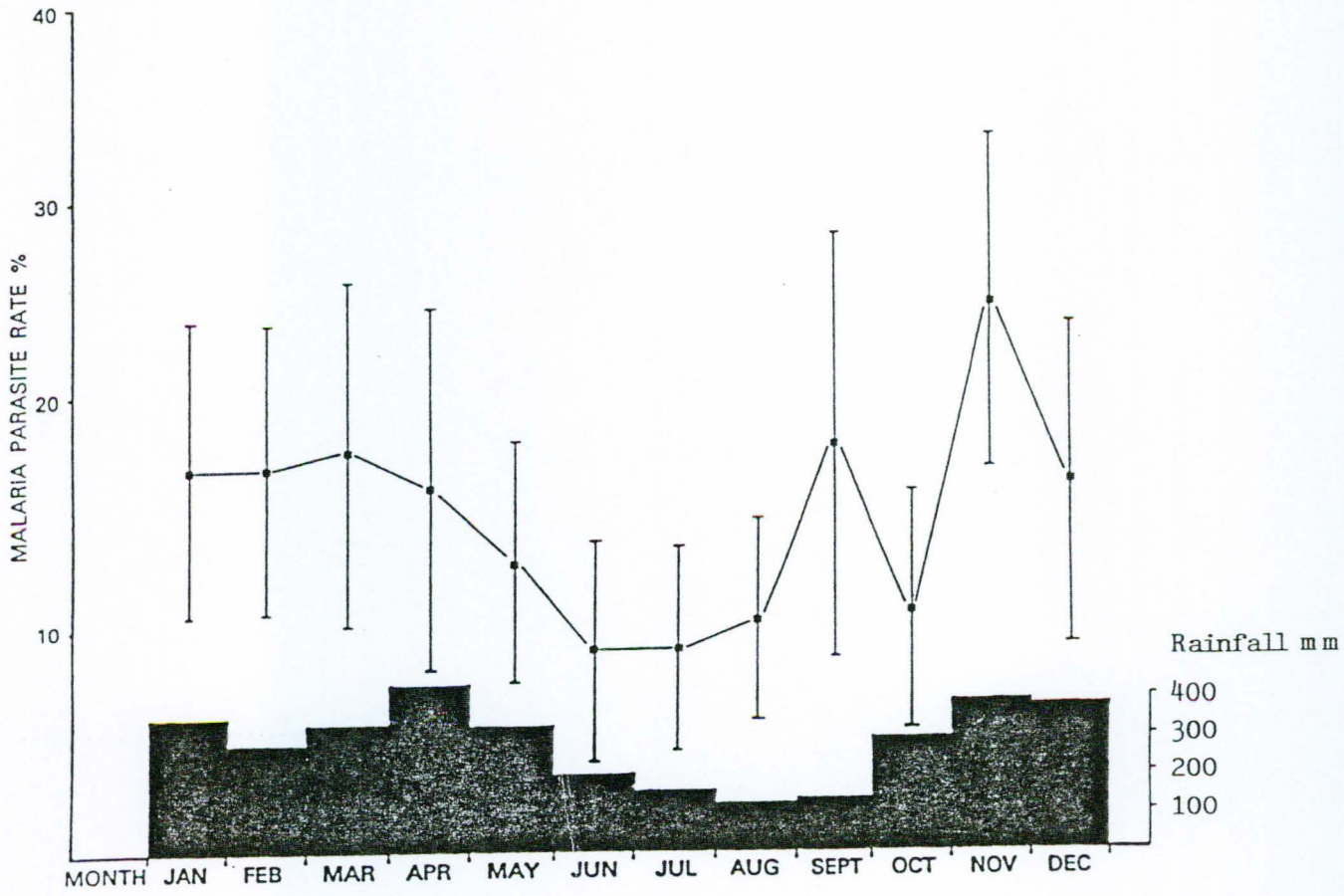

Figure 2 Malarial parasite rate (±2SE) (all visits combined) and average rainfall broken down by month of the year.

TABLE 1

Malaria species positivity, tabulated by treatment group at 6 and 12 month visits

6 months

Species	Iron dextran	Placebo
P.falciparum	29	19
P.vivax	5	5
P.falciparum + P.vivax	3	0
Negative	163	188
TOTAL	200	212

Approx relative risk (odds ratio): P.falciparum: 1.93; $X^2 = 4.70$ ($p < 0.05$)
P.vivax: 1.73; $X^2 = 0.91$ (N.S.)

12 months

Species	Iron dextran	Placebo
P.falciparum	39	33
P.vivax	11	4
P.malariae	1	0
P.falciparum + P.vivax	6	1
P.falciparum + P.malariae	1	0
Negative	119	152
TOTAL	177	190

Approx relative risk (odds ratio): P.falciparum: 1.61; $X^2 = 3.52$ (N.S.)
P.vivax: 3.93; $X^2 = 7.91$ ($p < 0.01$)

Iron treatment and malaria prevalence at field visits

The prevalences of malaria and splenomegaly were higher in the iron treatment group than in the placebo group both at 6 and 12 months; for slide positivity the differences were significant at both visits; for splenomegaly only at 12 months. (Figs. 3 and 4). (Table 2). All three species of malaria found in this study (Plasmodium falciparum, P.vivax and P.malariae) were more common in the iron treatment group (see Table 1).

The risk (odds ratio) of malaria slide positivity (any species) associated with iron treatment was 1.8 ($p<0.05$) at six months and 2.0 ($p<0.01$) at 12 months (see Table 2). For spleen enlargement the risk (odds ratio) associated with iron dextran treatment was 1.5 ($p>0.05$) at six months and 1.6 ($p<0.01$) at one year (see Table 2).

A major potential confounding factor for malaria effects in this cohort was the high prevalence of α^+ thalassaemia (80% - see Chapter 7). When infants with Hb Barts detected in birth blood were excluded from analysis, the risk of malaria associated with iron treatment increased: for malaria slide positivity, the risk (odds ratio) (6 and 12 months visits combined) increased from 2.0 to 14.6 (see Table 2, Chapter 11). For spleen enlargement the equivalent odds ratio for non-thalassaemics was 4.6 at six months (not significant) and 8.0 at 12 months ($p<0.05$). Presence of Hb Barts was not associated with any overall difference in slide positivity rate (Chapter 11) thus the observed depressive effect of Hb Barts presence at birth on the iron treatment risk appeared to be interactive. The effects associated with αthalassaemia are discussed in more detail in Chapter 11.

In contrast to the later field visits, no significant effects of iron treatment on malaria were noted at the field check one week after the injection. Eleven cases of malaria were diagnosed at this visit; six were in the iron dextran group and five in the placebo group. In

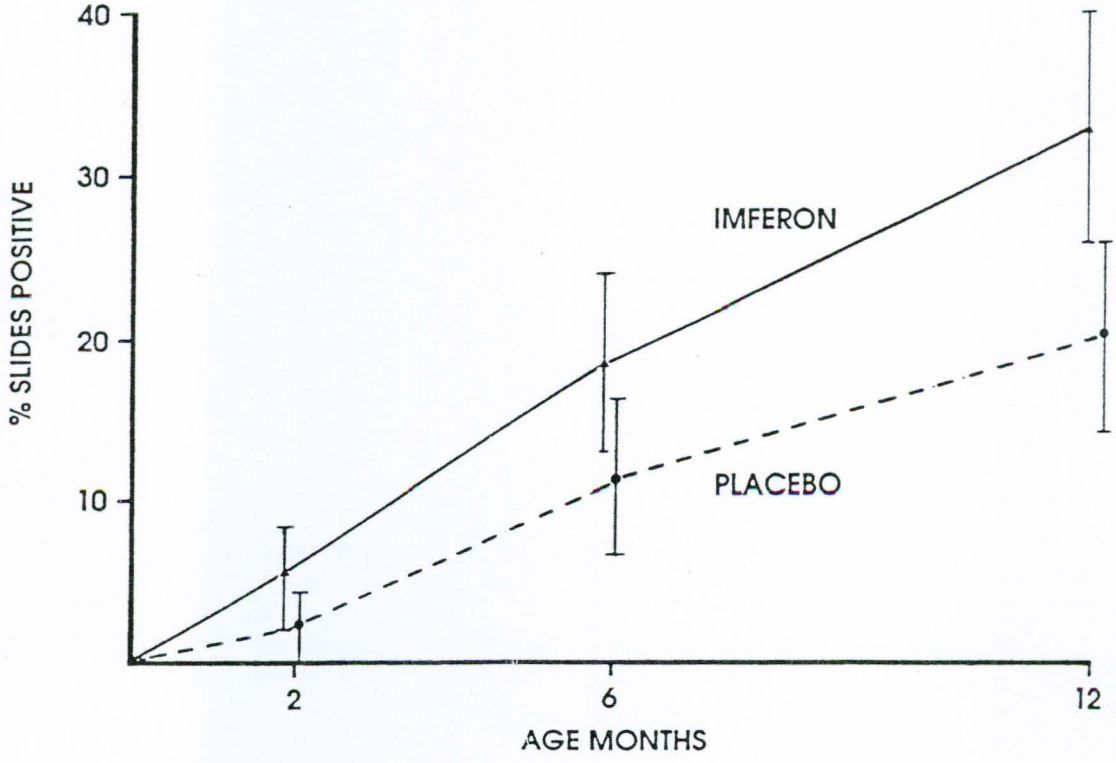

Figure 3 Malaria slide positivity rates % at 2, 6 and 12 months broken down by treatment group (± 2SE)

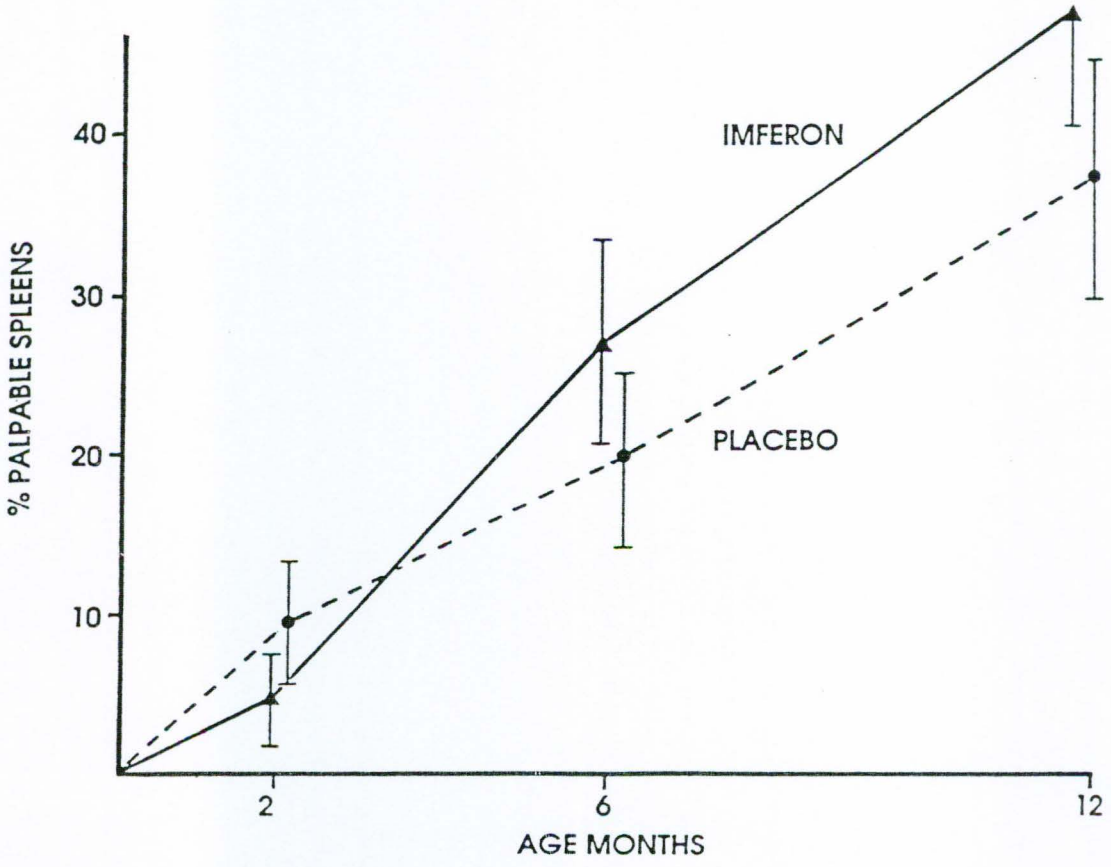

Figure 4 Spleen rates at 2, 6 and 12 months broken down by treatment group (± 2SE)

TABLE 2

Malaria slide positivity and splenomegaly at 6 and 12 months tabulated by treatment group

6 months Treatment	Malaria slide Positive No.	(%)	Total	Spleens Positive No.	(%)	Total
Iron Dextran	37	(18.5)	200(1)	53(1)	(26.9)	197(1)
Placebo	24(2)	(11.3)	212(2)	43(2)	(19.6)	219(2)
Odds ratio Iron Dextran v. Placebo	1.78*			1.51		
(95% confidence limits)	(1.02-3.10)			(0.95-2.33)		
12 months Treatment	Malaria slide Positive No.	(%)	Total	Spleens Positive No.	(%)	Total
Iron Dextran	58(3)	(32.8)	177(4)	81(2)	(47.6)	170(4)
Placebo	38(3)	(20.0)	190(9)	66(3)	(36.9)	179(7)
Odds ratio Iron Dextran vs Placebo	1.95**			1.56**		
(95% confidence limits)	(1.21-3.13)			(1.02-2.39)		

Superscript numbers in parentheses indicate infants who received iron dextran or blood transfusion from another source before this visit.

* significantly different from unity at 5% level.

** significantly different from unity at 1% level.

two cases malaria had been detected on blood film at the injection visit (one in each group) but in no case had there been splenomegaly or fever at the injection visit. All but two of these malaria cases had splenomegaly at the check, and all but one had malaria confirmed by blood slide. P.falciparum and P.vivax were present in equal proportions between treatment groups at this check.

Birth haemoglobin and prevalence of malaria in placebo group

In order to determine whether iron status before the start of the trial, as opposed to iron treatment, also affected risk of malaria at follow- up field visits, measures of iron status (Hb, serum ferritin, serum iron and transferrin saturation) at birth and 2 months in the placebo group were analysed in relation to slide positivity at follow-up field visits. The only variable associated with significant effects was birth haemoglobin:

Infants were ranked by haemoglobin at birth and the cohort divided into three equal parts by the 33.3rd and 66.7th centile points (13.7 g/dl and 15.7 g/dl). Malarial parasite rate in the resultant three cohorts in the placebo group at 6 and 12 month visits is shown in Figure 5, and Table 3. There was a trend of increasing slide positivity rates at field visits from low birth haemoglobin to high birth haemoglobin groups. This trend was significant at below the 1% level at 12 months.

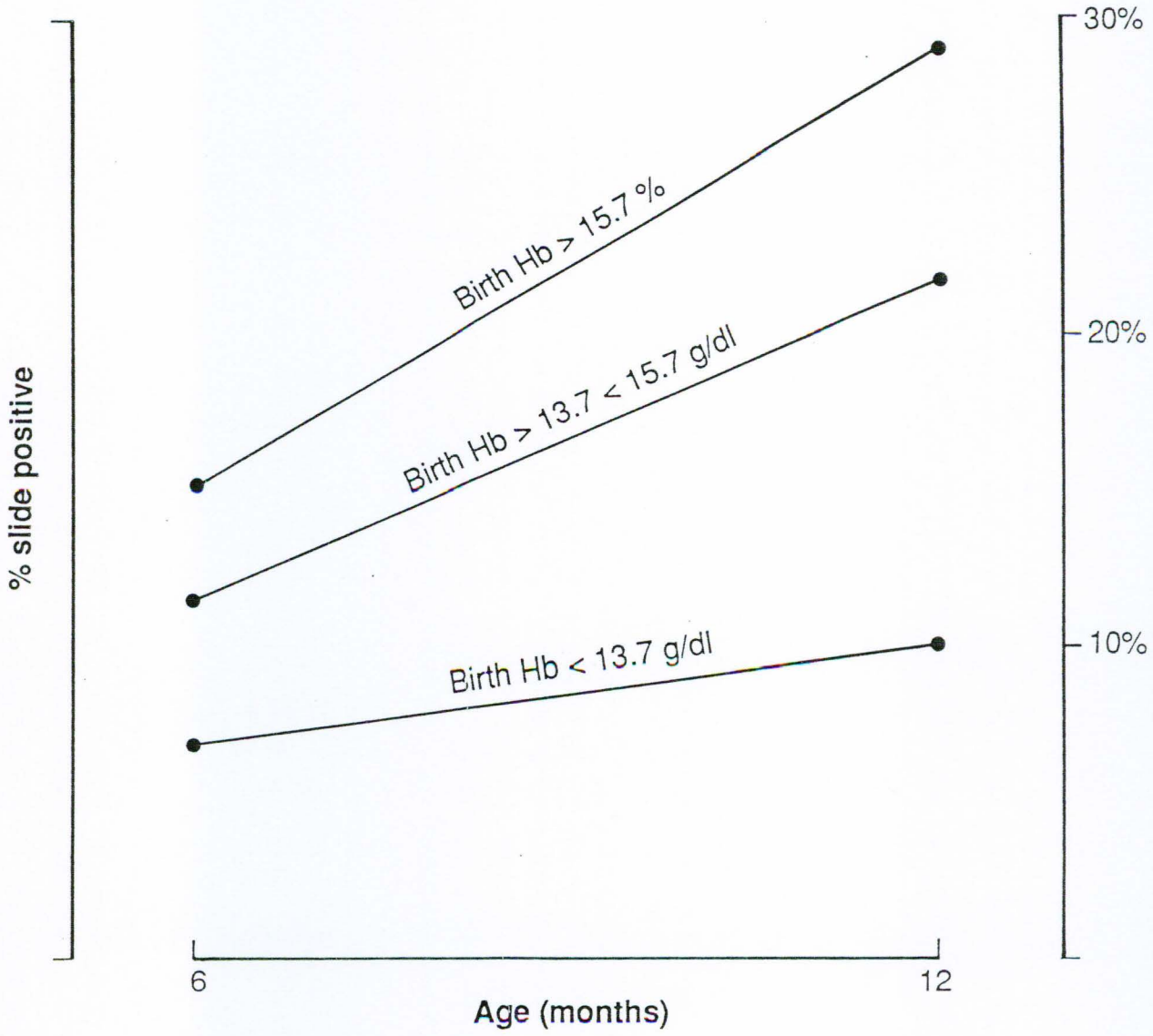

Figure 5 Malaria slide positivity rates at 6 and 12 months in placebo group broken down by birth haemoglobin.

TABLE 3

Malarial slide positivity in placebo group at 6 and 12 month visits categorised by birth venous haemoglobin

Malaria slide	Birth Haemoglobin <13.7 g/dl No.	Rate	13.7 to 15.7 g/dl No.	Rate	>15.7 g/dl No.	Rate
6 months						
Positive	5	(6.9%)	7(1)	(11.5%)	12(1)	(15.1%)
Total	72		61(1)		79(1)	

(Test for trend X^2 0 2.55, 1 d.f., p - 0.1)

12 months						
Positive	7(1)	(10.0%)	11(1)	(21.6%)	20(1)	(29.0%)
Total	70(3)		51(2)		69(4)	

(Test for trend X^2 = 7.84, 1 d.f., p<0.01)

Superscript numbers in parentheses indicate infants who received iron dextran or blood transfusion from another source before this visit.

II. Effect of iron treatment on severity of malaria

Criteria have been laid down for diagnosis of severe or complicated malaria (WHO, 1986). Diagnostic features include 1) hyperparasitaemia (>250,000/µl); 2) coma; 3) severe anaemia; 4) jaundice; 5) electrolyte imbalance; 6) renal failure; 7) hyperthermia (T>39°C); 8) complicating or associated infections; 9) pulmonary oedema; 10) hypoglycaemia; 11) shock; 12) bleeding disorders; 13) haemoglobinuria. Many of these features are more often seen in adult non-immunes with malaria.

Objective assessment of the above features in individuals is necessary if severity is to be compared.

1) Parasite Densities: There were no cases with hyperparasitaemia at any visit or on admission so this could not be used as a discrete criterion. Harvey et al. (1985) found quantitatively higher malarial parasitaemias in normal as opposed to iron deficient mice. To test this finding in the present study, parasite densities in positive slides were ranked, and compared between the iron treatment and placebo groups at 2, 6 and 12 month visits separately using the Kruskall-Wallis rank sum test. No significant differences were detected between density ranks of the two treatment groups at any visit or at first admission.

2) Coma: There were no cases of cerebral malaria from the cohort. Of the 12 febrile convulsions occurring on admission to hospital, five had positive blood slides, two of these had acute otitis media, one had measles, one had gastroenteritis with severe electrolyte imbalance, and in only one case (in the placebo group) was malaria clearly the most likely precipitating cause of convulsion. Thus disturbance of consciousness could not be used in this study to compare severity.

3) Severe Anaemia: Eight cases of severe anaemia (Hb<5 g/dl), clinically attributable to malaria, were admitted (see Chapter 10). Five of these were in the iron dextran group, thus, again giving too few cases for statistical analysis.

TABLE 4

Spleen enlargement (in centimetres) at 6 and 12 month visits tabulated by treatment group

6 months visit

	0	1cm	2cm	3cm
Iron dextran	144	32	17(1)*	4
Placebo	176(1)*	30	11(1)*	2

12 months visit

	0	1cm	2cm	3cm	4cm	5cm
Iron dextran	89(2)*	47	22(1)*	9(1)*	2	1
Placebo	113(4)*	38(2)*	24(1)*	2	2	0

* Numbers in parentheses indicate infants who received iron dextran or blood transfusion from another source before this visit.

X^2 for trend

6 months $X^2 = 4.04$ (1 d.f.; $p < 0.05$)

12 months $X^2 = 4.50$ (1 d.f.; $p < 0.05$)

X^2 for heterogenicity

6 months $X^2 = 3.3$ (2 d.f.; $p > 0.05$)

12 months $X^2 = 7.7$ (3 d.f.; $p < 0.05$)

Objective attribution of any associated "complication" (e.g. hyperthermia) to malaria is weakened in a stable endemic area by the high rate of asymptomatic parasitaemia in the general population and features 4), 5), 6), 9), 10), 11), 12), 13) clearly attributable to malaria were not seen in admissions. Feature 8) (associated infections) is discussed in Chapter 10.

Spleen size: There was evidence of larger spleen sizes in the iron treatment group at six months ($p<0.05$) and at 12 months ($p<0.05$) using a test for trend (Table 4).

Mortality: Of the 12 deaths which occurred in the trial cohort, seven were in the placebo group and five in the iron group. All but one of these occurred in hospital. Malaria could not be incriminated as the major contributing factor to death in any case in hospital. In the case of the one infant that died at home the history was strongly suggestive of measles complicated by pneumonia (see Chapter 10).

With the relative scarcity of the more severe forms of malaria in this cohort, it becomes more difficult to assess and compare severity. Another objective approach is comparative measurement of the associated effects of malaria on haematological indices which is discussed below.

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III. Haematological effects of iron treatment and malaria

Methods of analysis to separate the potentially opposite and therefore confounding effects of malaria and iron treatment on haematological indices were discussed in Chapter 6.3. Both of these factors affected haematological indices, and as is shown above, malaria was more prevalent in the iron treatment group.

Before discussing the detailed breakdown and analysis it is useful first to tabulate the net effects associated with iron treatment in the study.

Overall effects of iron associated with haematological indices

Comparison of the two treatment groups showed significantly higher levels in the iron dextran group of Hb, PCV, MCHC, serum iron, transferrin saturation and serum ferritin at both 6 and 12 month visits but not at the 2 month pre-injection visit (Tables 5 and 6). Total iron binding capacity was not significantly different in the two groups at any age. Mean haemoglobin levels were 0.5 grams higher in the iron group at six months and 0.7 grams higher at one year (Table 5). Mean MCHC was 0.6 g/dl higher in the iron group at six months and 0.4 g/dl higher at 12 months (Table 5). Mean transferrin saturation % was only slightly higher in the iron group (3% at six months and 4% at one year), however, the difference was significant (Table 6). Log mean serum ferritin was 50% higher in the iron group at six months and 38% higher at one year (Table 6).

Comparison of the degree of microcytosis (see Methods) between treatment groups showed no difference at 2 months but significantly less in the treatment group at 6 months ($p<0.01$) and at 12 months ($p<0.01$). Similarly for the degree of hypochromasia, no difference was noted at 2 months, but less was seen in the iron dextran group at 6 months ($p<0.001$) and at 12 months ($p<0.001$). It should be noted that both of these morphological abnormalities were reduced but not absent in the iron dextran group. A marked degree of anisocytosis was prevalent in both treatment groups at all field visits.

TABLE 5

Haemoglobin and MCHC levels at 2, 6 and 12 month visits broken down by treatment group: Means g/dl (SD)

Haemoglobin

	Iron dextran	n	Placebo	n	t	p
2 months	8.93 (1.00)	235	8.95 (1.16)	249	0.21	N.S.
6 months	9.82 (1.39)	201	9.14 (1.09)	220	5.54	<0.001
12 months	9.78 (1.36)	179	9.32 (1.34)	191	3.23	<0.002

MCHC

	Iron dextran	n	Placebo	n	t	p
2 months	31.3 (1.75)	233	31.3 (1.98)	247	0.1	N.S.
6 months	31.3 (1.68)	199	30.7 (1.77)	218	3.85	<0.001
12 months	30.8 (1.58)	177	30.4 (1.68)	190	2.21	<0.05

TABLE 6

Transferrin saturation % broken down by treatment group at 2, 6 and 12 month visits. Means (SD)

	Iron dextran	n	Placebo	n	t	p
2 months	28.0 (8.97)	170	27.3 (8.68)	168	0.72	N.S.
6 months	26.9 (3.32)	102	24.3 (9.19)	120	2.15	<0.05
12 months	27.5 (8.39)	120	23.1 (8.69)	144	4.19	<0.001

Serum ferritin levels and treatment group at 2, 6 and 12 months: Geometric means ng/ml (coeff var %) *

	Iron dextran	n	Placebo	n	t	p
2 months	132 (15.7%)	176	135 (17.8%)	179	0.3	N.S.
6 months	134 (16.1%)	107	26.4 (34.9%)	120	12.6	$\ll 0.001$
12 months	61.6 (21.1%)	120	19.9 (40.2%)	142	8.8	$\ll 0.001$

* calculated in log transform

Analysis of effects of iron treatment on the anaemia of malaria

When infant's haemoglobin levels are broken down by treatment group and malaria slide positivity at birth, 2, 6 and 12 month visits (Fig. 6) several observations can be made. Firstly if one looks at the malaria negative cases only, the difference in haemoglobin between iron and placebo increases to about 1 gram at the 6 months visit. Secondly, as expected infants with slide positive malaria have lower haemoglobins at all three visits. Thirdly, if the differences between slide positive and slide negative haemoglobins are compared separately for the two treatment groups, the effect appears to be greater in the iron dextran group, particularly at 6 months implying more active or more severe malaria in this group (see Discussion).

Iron treatment is thus associated with higher haemoglobins, malaria with lower, and there is an interaction between iron treatment and the effect of malaria. The contribution of these 2 main effects and their interaction on haemoglobin levels at 6 and 12 months was assessed using 2-way analysis of variance (see Chapter 6.3, Methods). The 2 main effects of iron and malaria were strong at both visits. The 2-way interactive effect was strong at six months but weaker at 12 months although still showing a trend (Table 7).

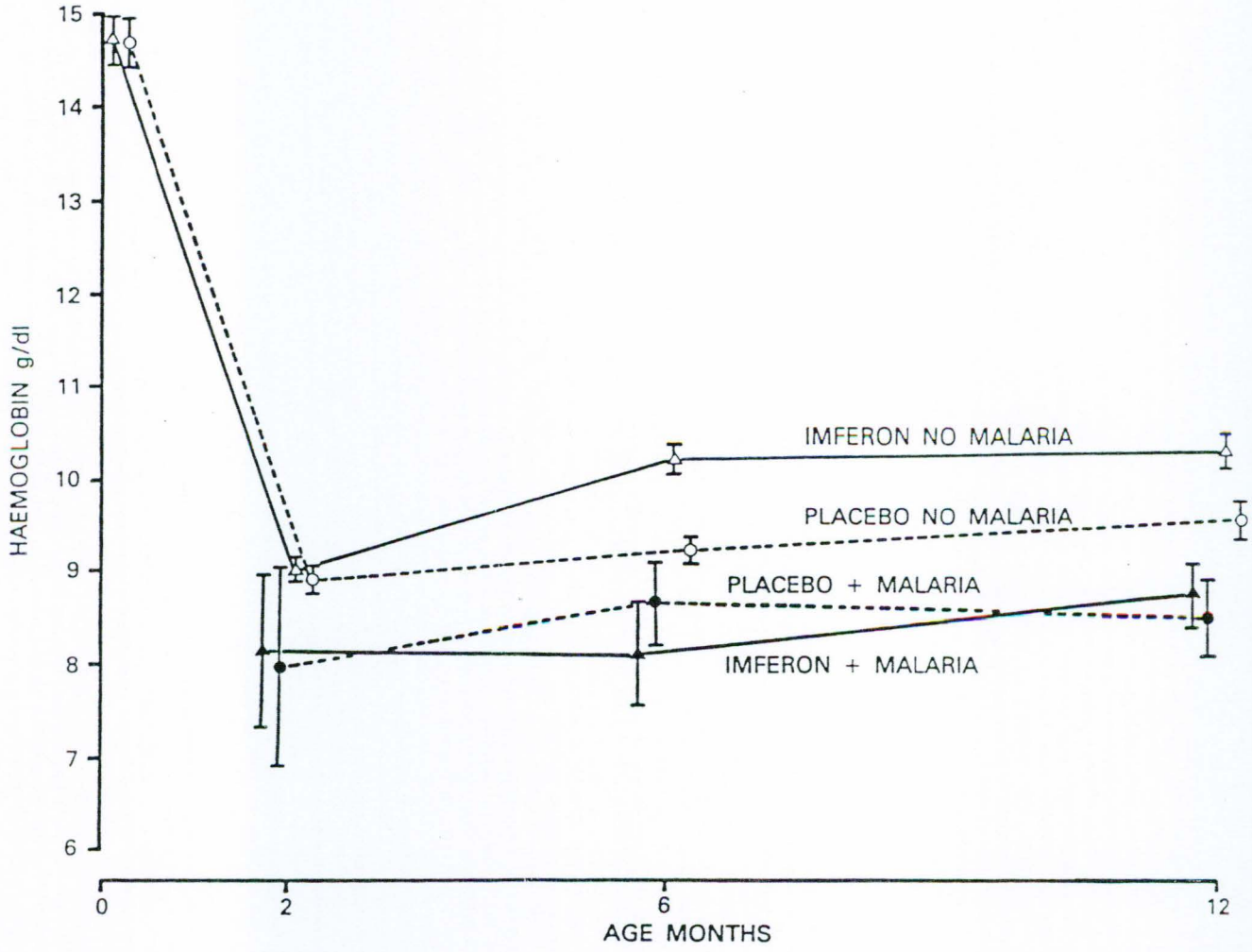

Figure 6 Mean haemoglobin levels (95% confidence interval) at birth, 2, 6 and 12 months broken down by treatment group and malaria slide result at each visit.

TABLE 7

Summary results of analyses of variance of the effect of iron treatment and malaria slide result (factors) on haemoglobin, transferrin saturation and serum ferritin (dependent variables) at 6 and 12 months visits

Source of variation (factors)	Dependent Variables					
	See Fig. 6 Haemoglobin		See Fig. 9 Transferrin Saturation		See Fig. 8 Serum ferritin (log)	
	F	p	F	p	F	p
6 months visit						
Main Effects						
Iron dextran +/-	53.6	<<0.0005	4.13	0.043	149.8	<<0.0005
Malaria slide +/-	93.6	<<0.0005	3.72	0.055	13.9	<0.0005
2-way interactions	25.0	<<0.0005	0.02	0.943	3.44	0.065
	n=410; d.f.=1;407		n=220; d.f.=1;217		n=224; d.f.=1;221	
Total variation explained	28%		4%		45%	
12 months visit						
Main Effects						
Iron dextran +/-	24.9	<<0.0005	13.3	<<0.0005	13.2	<0.0005
Malaria slide +/-	86.6	<<0.0005	7.29	0.007	64.9	<0.0005
2-way interactions	3.2	0.077	1.70	0.193	5.2	0.024
	n=363; d.f.=1;362		n=261; d.f.=1;258		n=259; d.f.=1;256	
Total variation explained	22%		9%		27%	

Analysis of the effects of iron on the reticulocytosis associated with malaria

Reticulocyte counts may be a useful indirect guide to the rate of red cell destruction and also to effective erythropoesis in response to a low haemoglobin. When geometric mean reticulocyte counts seen at 2, 6 and 12 months are broken down (as for haemoglobin) by treatment group and malaria slide positivity, effects nearly the inverse of those seen for haemoglobin may be observed (Fig. 7). Firstly, as expected, malaria is associated with higher counts. Secondly, an interactive effect is seen at 6 months with the effect of malaria on reticulocyte counts being greater in the iron group (implying a brisker response in this group). This is not seen at one year. Apart from the interactive effect, iron treatment is not, however, clearly associated with higher counts. Interpretation of these breakdown results is complicated and confounded by the inverse relationship of reticulocyte counts to haemoglobin levels.

There was a strong inverse correlation between reticulocyte counts and haemoglobin at both the 6 and 12 month visits (Table 8). In order to assess bone marrow responsiveness to iron and malaria independently it is therefore necessary to correct for haemoglobin level. The results of a 2-way Analysis of Variance of reticulocyte count (logged to the base 10) (dependent) by treatment group and malaria slide positivity (explanatory factors), controlling concurrently for haemoglobin (covariate) at 6 and 12 months are shown in Table 8. As stated above, this was designed to test the effect of iron treatment on reticulocytes independantly of haemoglobin levels and malaria, and showed that whereas malaria had a positive effect independantly of haemoglobin, iron treatment had no such effect. In this analysis when controlling for haemoglobin there were also no significant interactions between malaria and iron on reticulocyte count at either visit.

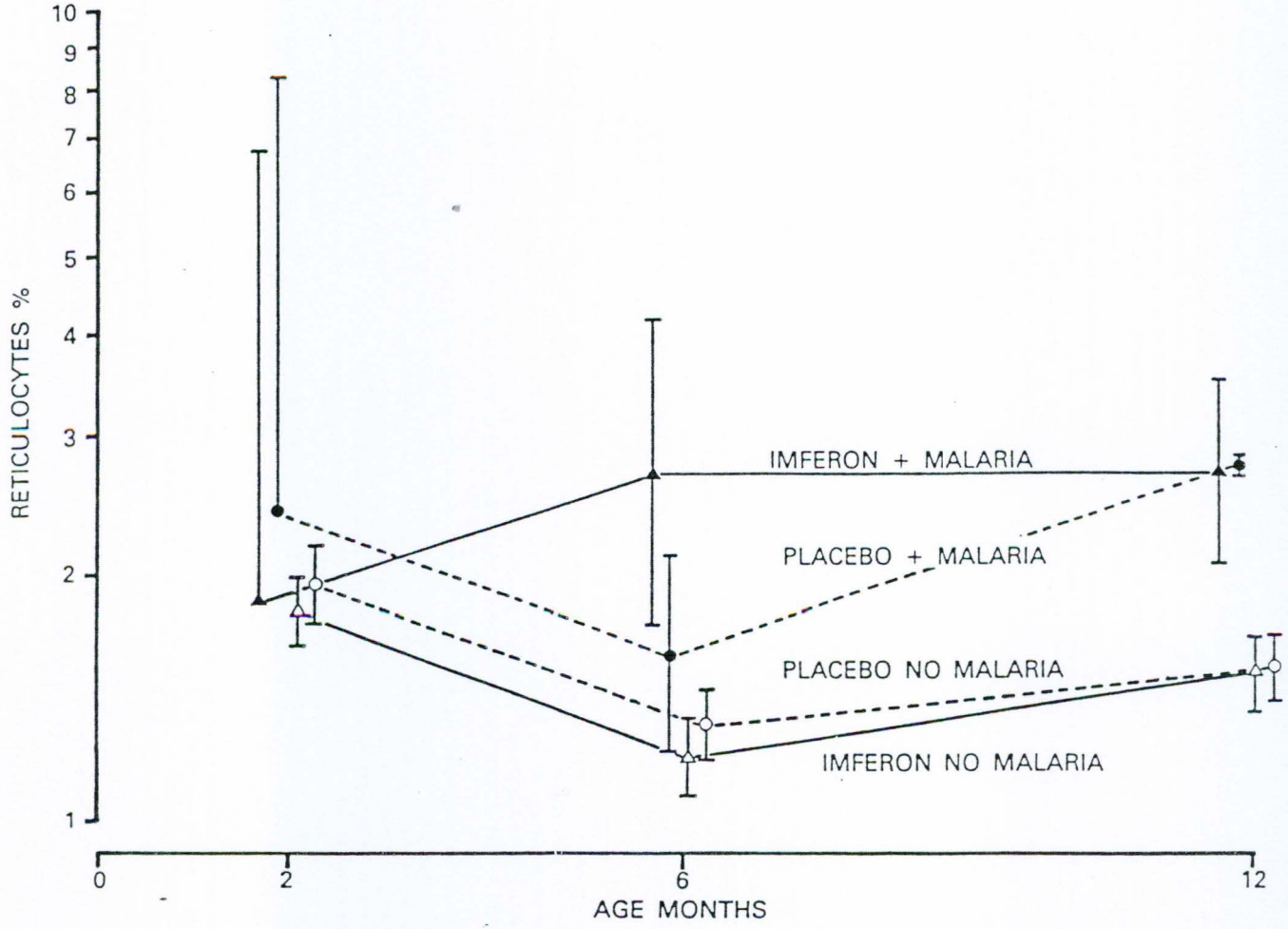

Figure 7 Reticulocyte counts geometric mean (95% confidence interval) at 2, 6, and 12 months broken down by treatment group and malaria slide result at each visit.

TABLE 8

Summary results of analysis of variance of the effects of treatment and malaria slide result (factors) with haemoglobin and spleen size (covariates) on reticulocyte counts $\log_{10}$ (dependent) at 6 and 12 month visits (see Fig. 7)

	6 months		12 months	
Source of variation				
Main effects	F	p	F	p
Haemoglobin (covariate)	33.75	<0.001	21.75	<0.001
Iron dextran (+/-)	2.23	0.137	1.14	0.287
Malaria (+/-)	6.39	0.012	18.42	<0.001
2-way interactions				
Malaria vs injection	1.27	0.261	0.141	0.707
	d.f. = 1;224		d.f. = 1;243	
Total variation explained	21.2%		21.5%	

Analysis of effect of iron and malaria on serum transferrin and transferrin saturation

Serum iron and ferritin levels were found to be influenced by malaria making it necessary to try and separate the iron and malaria effects in analysis (see Methods, Chapter 6.3).

A breakdown of geometric mean serum ferritin by treatment group and malaria slide positivity at birth, 2, 6 and 12 months is shown in Figure 8. As expected the iron dextran group had higher ferritins than the placebo group at 6 and 12 months. A second observation was that within the respective treatment groups malaria slide positivity was associated with higher ferritins at both visits. There also appeared to be an interaction, with the malaria effect on serum ferritin being greater in the placebo than in the iron group. This diagram also shows that in the absence of malaria slide positivity the placebo group had very low mean serum ferritin values by one year (<20 ng/ml). 2-way analysis of variance was performed for the 6 and 12 month visits to assess the significance of these effects. The iron treatment and malaria effects on ferritin levels were independantly significant at both visits while a small interactive effect was detected at the one year visit (Table 7).

The same analysis was performed for transferrin saturation. Fig. 9 shows mean transferrin saturations at the 6 and 12 month visits again broken down by injection group and slide positivity. Malaria and iron treatment were both associated with slightly higher levels of transferrin saturation at both visits. Placebo infants without malaria show a progressive fall of transferrin saturation from two months onwards. The results of analysis of variance of transferrin saturation by treatment group and by malaria slide result are shown in Table 7, indicating small but significant effects associated with iron dextran and malaria at 6 and 12 months with no interactions. Analysis

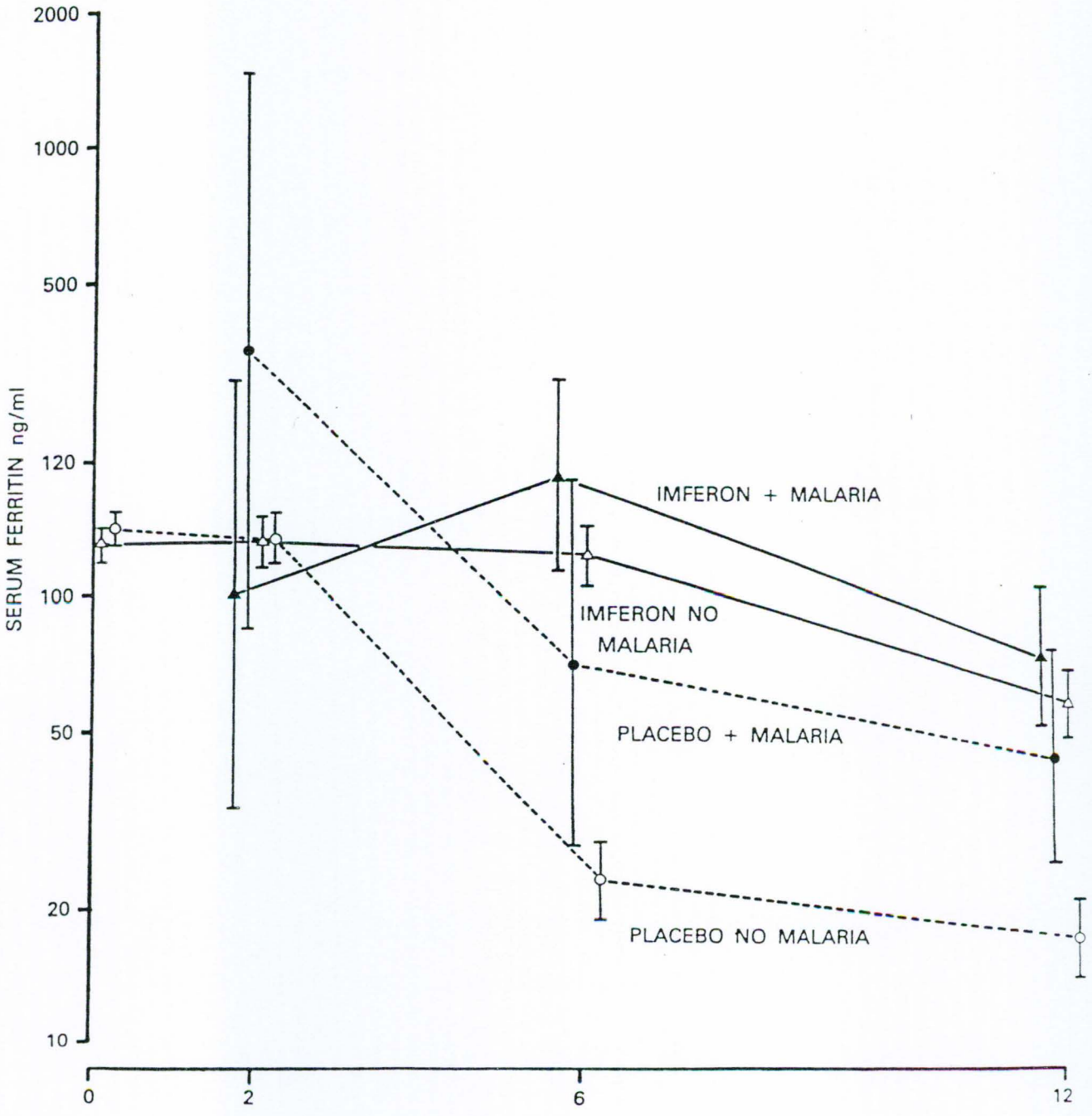

Figure 8 Serum ferritin geometric means (95% confidence interval) at birth, 2, 6 and 12 months broken down by treatment group and malaria slide result at each visit.

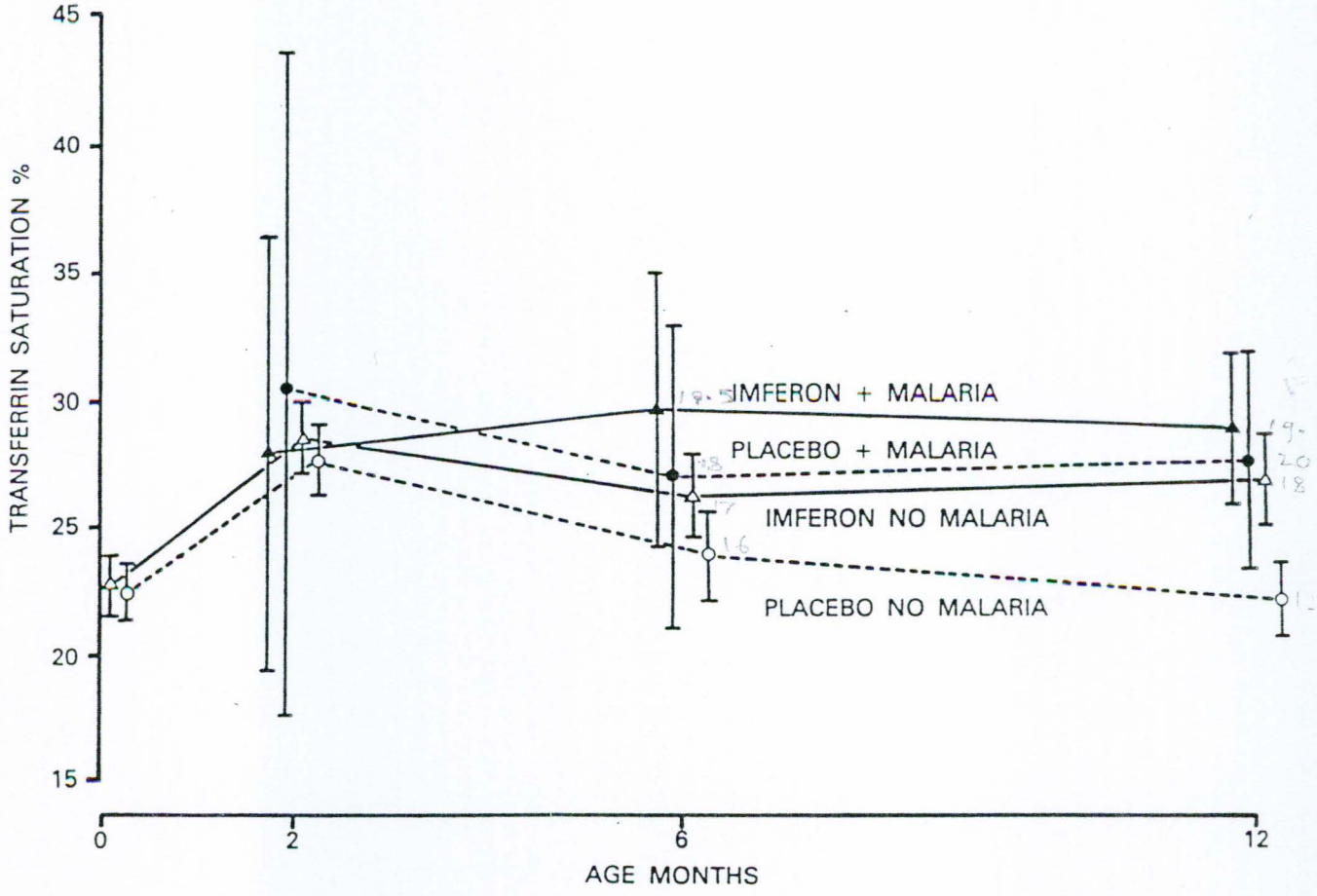

Figure 9 Mean serum transferrin saturation % (95% confidence interval) at birth, 2, 6 and 12 months broken down by treatment group and malaria slide result at each visit.

of serum iron levels gave essentially similar results to transferrin saturation and are not presented here.

Other haematinics, Serum folate and B_{12}, and red cell folate

In a total of 2533 specimens analysed from all three field visits there were only two cases with evidence of folic acid deficiency and none with B_{12} deficiency. These results are discussed in Chapter 13.

Effects of iron and malaria on haematological indices at first admission during trial

Haemoglobin, MCHC, reticulocyte counts, transferrin saturation, serum ferritin (logged), serum and red cell folates (logged), serum B_{12} logged at first hospitalisation were analysed as in the scheduled field visits broken down by injection group and malaria slide positivity (Tables 9-12). Since age was not constant it was controlled for as a covariate in each analysis before factors (malaria slide result and treatment group).

Mean admission haemoglobin was lower in the iron dextran group than at either the 6 or 12 month field visits (Table 9). An analysis of variance of haemoglobin controlling for age and spleen (as covariates) by injection group and malaria slide result showed strong (negative) effects for spleen and malaria slide result but no net effect associated with treatment group.

Mean reticulocyte counts (geometric) were higher in the iron dextran group than at either the 6 or 12 month field visits (Table 10). Analysis of variance of log reticulocyte count controlling for age and haemoglobin (as in the field visits, see Table 8) showed a strong inverse correlation with haemoglobin level, a positive effect associated with injection group and no net effect associated with malaria.

Transferrin saturation was higher in iron dextran patients with malaria than at either field visit; it was also higher in malaria

TABLE 9

Haemoglobin at first admission* broken down by treatment group and malaria slide positivity. Means g/dl (SD) n

	Iron dextran	Placebo
Malaria slide positive	7.18 (2.36) 17	6.89 (2.51) 16
Malaria slide negative	8.99 (1.65) 47	9.56 (1.28) 41

Analysis of variance of haemoglobin controlling for age, spleen size (covariates), treatment and malaria

Source of variation		Sum of squares	d.f.	F	p
Age	covariates	1.73	1	0.616	N.S.
Spleen	covariates	94.5	1	33.7	<0.0001
Treatment	Main effects	0.02	1	0.007	N.S.
Malaria	Main effects	69.4	1	24.74	<0.0001
2-way interactions (Treatment vs malaria)		8.43	1	3.0	N.S.
TOTAL		489.0	116		

Total variation explained = 36%

* After start of trial

TABLE 10

Reticulocyte count (%) at first admission broken down by treatment group and malaria slide result.
Geometric means (coeff var %)* n

	Iron dextran	Placebo
Malaria slide positive	3.5 (97%) 12	2.76 (76%) 9
Malaria slide negative	2.79 (70%) 34	1.35 (251%) 30

* calculated in log transform

Analysis of variance of reticulocyte count % ($\log_{10}$ dependant) controlling for age and haemoglobin (covariates) by injection group by malaria slide result

Source of variation		Sum of squares	d.f.	F	p
Age	covariates	0.154	1	1.21	0.275
Haemoglobin	covariates	0.929	1	7.29	0.008
Treatment	main effects	1.266	1	9.93	0.002
Malaria	main effects	0.110	1	0.87	0.355
TOTAL		12.639	84	-	-

Total variation explained = 19.7%

TABLE 11

Transferrin saturation % at first admission broken down by treatment group and malaria slide result

	Iron dextran	Placebo
Malaria slide positive *	34.2 (14.1) 13	25.0 (14.6) 7
Malaria slide negative	25.5 (8.6) 34	23.9 (8.6) 28

* Overall difference slide +ve vs -ve not significant $t = 1.80$; $p > 0.05$

TABLE 12

Serum ferritin at first admission broken down by treatment group and malaria slide positivity
Geometric mean (coeff var %)* n

	Iron dextran	Placebo
Malaria slide positive	379.5 (16.2%) 13	67.8 (31.6%) 9
Malaria slide negative	179.8 (21.6%) 34	56.0 (33.5%) 28

* calculated in log transform

Analysis of variance of log serum ferritin (dependant) controlling for age and spleen size by injection group by malaria slide result

Source of variation		Sum of squares	d.f.	F	p
Age	covariates	3.81	1	16.6	<0.0001
Spleen	covariates	1.13	1	4.9	0.029
Treatment	Main effects	6.39	1	27.8	<0.0001
Malaria	Main effects	1.07	1	4.6	0.034
2-way interactions (Treatment vs malaria)		0.01	1	0.04	N.S.
TOTAL		29.74	83		

Total variation explained = 40%

positives than negatives although the difference was not significant at the 5% level (Table 11). On analysis of covariance controlling for age and spleen size by injection group and malaria slide result, only spleen size was associated with significant (positive) effects on transferrin saturation ($F = 4.6$; d.f. 1;81, $r^2 = 5.3\%$; $p=0.036$).

Geometric mean serum ferritins were higher in the iron dextran group than at the 6 and 12 month field visits in equivalent cells (Table 12). An analysis of variance controlling for age and spleen size (covariates) showed as expected a strong age effect (inverse), a strong injection effect (positive for iron dextran) but also small positive effects associated with spleen size and malarial slide positivity (Table 12).

Serum and red cell folate results were all in the normal range and are dealt with in detail in Chapter 13.

Serum B_{12} levels were all in the normal range and unaffected by any of the above explanatory variables.

DISCUSSION

The main result observed in this trial was that iron dextran injection at two months of age was followed by an increase in prevalence of malaria at 6 and 12 month follow-up, when compared with the placebo group. This was evidenced both in terms of parasite rates and spleen rates. The risk of parasitaemia was nearly twice in the iron group when compared with the placebo group (Table 2). When infants with evidence of α thalassaemia were removed from analysis this risk increaseed to 14.6. All three species of malaria, P.falciparum, P.vivax and P.malariae were increased. There was also evidence of a trend towards larger spleens in the iron group. Density of parasitaemia in positive cases was unaffected by iron treatment status.

Domicile and season also affected malaria prevalence in the cohort (Figs. 1 and 2); however, the influence of these factors was controlled for in the trial by the method of matching and randomization (pairs of proximate births matched for sex, birth-weight and domicile producing balanced treatment groups (see Chapter 6.1)). Malaria prevalence rates increased with age but this was controlled for by analysing age matched visits separately.

To support a hypothesis that iron deficiency protects against malaria and that the above effects are related to prevention of iron deficiency in the iron treatment group and not due to an unphysiological effect of iron dextran injection, it is necessary to demonstrate firstly that the iron treatment group had adequate iron stores; secondly that the placebo group became iron-deficient; and thirdly that unphysiological effects of iron dextran, such as hyperferraemia, did not persist:

That the iron-treated group had adequate (but not excessive) iron stores is evidenced by serum ferritin levels at the field visits - (Table 6; Fig. 8).

That the placebo group became iron-deficient is evidenced by (a) serum ferritin levels in non-malarious infants at follow-up (Fig. 8), which were low by western standards for those ages (Siimes et al., 1974); (b) haemoglobin levels at follow-up, which were over-all significantly lower than in the iron-treated group at 6 and 12 months (Tables 5 & 7; Fig. 6); (c) mean MCHC levels which were lower than the treatment group at follow-up (Table 5); (d) more hypochromia and microcytosis than in the treatment group at follow-up. (The continuing presence, to some degree, of these features in the iron dextran group may be a reflection of the high prevalence of α thalassaemia in the population (Chapter 11)). An additional observation was that iron-treated infants failed to achieve western iron replete

haemoglobin standards for infants (Burman, 1972). This observation was not apparently due to deficiency of other haematinics (folic acid or B_{12}) but may again have been related to the high prevalence (80%) of alpha thalassaemia in this cohort (see Chapter 7).

Hyperferraemia following intramuscular iron dextran injection may last several weeks but the peak occurs in the first few days following injection (Will, 1968). Significant differences in mean transferrin saturation between treatment groups were observed at follow-up but these were only of the order of only a few per cent (Fig. 9).

Hyperferraemia would be expected at the visit one week after iron injection but there were few malarial episodes observed at this visit, and no significant differences in prevalence between iron treatment and placebo groups noted. In contrast to this, significant differences in malaria prevalence were observed even at the 12 month visit, 10 months after the injections.

Further corroborative evidence of a protective effect of iron lack was seen within the placebo group where birth haemoglobin was positively correlated with malaria parasite rates at follow up (Table 3). Haemoglobin represents the main iron compartment at birth and birth haemoglobin is a major determinant of iron status in the first year of life (Lanzkowsky, 1976).

Given that malaria parasite and spleen rates were higher in iron treated infants one may inquire if the effects of malaria were greater in this group.

There were no cases of cerebral malaria recorded from the cohort in the first year of life. Febrile convulsions occurred with equal frequency in both treatment groups, only one of these being clearly related to malaria (placebo group). Density ranks showed no significant differences between treatment groups in slide positive cases. There were no deaths due to malaria and death rates were

similar in the two treatment groups. These mortality figures may be stated with reasonable confidence since one year survivorship was known for 98% of the trial cohort (Chapter 7), and both of the two district hospitals were covered for admission surveillance.

In the absence of the more severe forms of malaria the one objective approach to assessment of the interaction between iron prophylaxis and severity of malarial morbidity is analysis of the haematological effects at the scheduled field visits (since these are less liable to selective bias than hospital admissions and in effect give an age stratified cross-section of morbidity): in this study, iron prophylaxis was associated with haemoglobin levels one gram higher in the absence of malaria at follow-up. However, the effect of malaria on haemoglobin appeared to be greater in the iron dextran group (reducing the overall improvement in haemoglobin in the treatment group to 0.7 g/dl at 6 months and 0.45 g/dl at 12 months). On analysis of variance the interactive effect was significant at the 6 month visit supporting the concept of greater severity in the iron group. These effects were broadly paralleled inversely in reticulocyte counts at 6 and 12 months.

Two main dynamic effects in malarial anaemia have been suggested: (i) excessive red cell destruction, and (ii) ineffective red cell production (Weatherall & Abdalla, 1982). Anaemia is the main primary stimulus to erythropoiesis. In spite of inherent variability, reticulocyte counts, corrected for haemoglobin, are regarded by some workers as a good guide to effective erythropoiesis (Cline & Berlin, 1963) and in this study, there was a highly significant inverse correlation between reticulocyte counts and haemoglobin levels supporting this view. If malaria adversely affected effective bone marrow response in these infants, one would expect a depressive effect of malaria on reticulocyte counts when controlling for haemoglobin. In fact the reverse was observed, malarial positivity being associated

with elevated reticulocyte counts even after correction for haemoglobin at six months and at 12 months (Table 8). This conclusion differs from another report (Phillips et al., 1986) where reticulocyte response was apparently delayed until parasite clearance in acute cases of malaria. The contribution of the normal delay in reticulocyte response following an acute fall of haematocrit is difficult to assess in this report.

Quantitative assessment and interpretation of haematological indices at admission in relation to the effect of iron treatment is more difficult than that for field visits since admissions are an acute uncontrolled, selected group and in particular more liable to acute confounding effects due to malaria and other infections. Interpretation should therefore be guarded.

Haemoglobin was lower on admission than in the field in the iron dextran group both in malaria positives and negatives. On analysis of covariance explainable variation was entirely related to malaria (spleen size and slide positivity) with no net significant effect associated with injection group (Table 9).

This contrasts with the field haemoglobin results which were associated with a net positive effect of iron dextran and suggests again a differential effect of malaria in the iron group.

Admission reticulocyte counts in contrast did show a significant positive treatment effect when controlling for haemoglobin level and were higher in the iron dextran group than in the field; however, malaria was not associated with any net effect on reticulocyte response.

Admission serum ferritin levels were much higher in the iron dextran group than in the field both in malaria positives and

negatives. How much of this difference was secondary to acute malaria and other infections and how much to an increased risk of high iron status for admission is impossible to say. The effect of malaria on ferritin levels was significant (Table 12), whereas serum ferritins at birth and 2 months were not predictive of admission (see Chapter 10).

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How iron prophylaxis contributes to the higher parasite and spleen rate at 6 and 12 months and increased anaemia of malaria observed in these infants at six months is a matter of conjecture. One hypothesis relates to red cell age. P.vivax can only grow in reticulocytes (Garnham, 1966). Pasvol et al. (1980) showed, using red cells of different ages separated by centrifugation, that P.falciparum preferentially invaded younger cells; the multiplication of parasites and hence the rate of destruction of red cells could be to a certain extent dependent on a continuing supply of younger cells and reticulocytes. The reticulocyte count was greater in iron dextran group infants with malaria at 6 months but this effect disappeared when haemoglobin level was controlled for in the analysis of variance, presumably reflecting the interactive effect of iron and malaria on haemoglobins seen at this visit (Table 7). Given the strong inverse correlation of haemoglobin and reticulocyte counts, these results neither confirm nor refute the above hypothesis. P.chabaudi invades mature red cells so the protection of iron deficiency against this infection noted by Harvey et al. (1985) is less likely to have been related to red cell age effects.

A second hypothesis relates to iron availability. Some observers have shown that increased saturation of transferrin may help bacteria and other micro-organisms to grow (Weinberg, 1974). Most experimental work in this area has used media with transferrin saturation

artificially manipulated by adding inorganic iron. Baltimore et al., (1982) in contrast have shown that good bacteriostatic action of serum is retained by transferrin saturations up to 80% occurring in vivo and that this is only abolished by adding inorganic iron in vitro. Red cells normally lose the capacity to acquire transferrin bound iron at the reticulocyte stage (Harris & Kellermeyer, 1963, Enns et al., 1981). The adult red cell, infected with malaria, would therefore be likely to be dependent on an intracellular iron pool unless specific transferrin receptors were elaborated on the cell surface by the parasite. A recent report suggests that P.falciparum infected cells do indeed have a transferrin receptor (Rodriguez & Jungery, 1986). There is also evidence that desferrioxamine modifies malaria infections (Raventos-Suarez et al., 1982; Fritsch et al., 1984). On the other hand, Peto et al. (1986) suggest that P.faciparum may not take up Fe^{59} labelled transferrin bound iron in vitro if care is taken to ensure specific iron/transferrin binding. These authors further suggest that desferrioxamine may act by another mechanism than binding iron. There were significant differences in transferrin saturation associated with iron dextran treatment and malaria at follow-up in Madang infants but of the order of only a few per cent.

A third hypothesis that microcytosis secondary to iron deficiency affects parasite multiplication (Nurse, 1979) has yet to be studied in depth. Nurse suggested that shortage of intercellular nutrients, e.g. a labile iron pool might result from small poorly haemoglobinised cells. An alternative could be increased susceptibility to oxidant damage in a smaller intracellular environment.

In vitro animal studies need to be extended by in vivo study of the complementary role of the spleen.

The high serum ferritin levels associated with malaria in this study confirm a previous finding which may be partly related to tissue

release (Oppenheimer et al., 1984e, Appendix III.i). It is surprising that this associated effect of malaria is greater in the placebo group. The modest rise in transferrin saturation associated with malaria is in the opposite direction to the effect associated with most pyrexial infections (Beresford, 1971) and could be due to haemolysis.

The results of this clinical trial mitigate against the use of routine iron supplementation in areas with endemic malaria. It is difficult to say from these results whether the same effects would be seen with oral iron; the Murrays' results imply this could be the case (Murray et al., 1978), while Harvey et al. (1987) showed no effect of oral iron in Madang school children. Use of parenteral iron during an acute malarial infection may also be contra-indicated. The routine presumptive use of oral and parenteral iron for treatment of anaemia is a common practice in many malarious areas including Papua New Guinea and may be dangerous if not parallelled by a malaria control programme.

CHAPTER 10.

IRON SUPPLEMENTATION: EFFECTS ON MORBIDITY DUE TO INFECTIOUS DISEASE

SUMMARY

Analysis of field and hospital infectious morbidity in the trial indicated a deleterious effect of iron dextran. Total periods of stay in hospital were increased by 60% in the iron dextran group. Analysis of other factors showed (i) a deleterious effect of low weight for height at the start of the trial; (ii) a significant positive correlation between birth haemoglobin and hospital morbidity rates. A possible association between malarial experience and other infectious morbidity is discussed.

INTRODUCTION

The study area, the cohort profile and the study protocol have been described in detail in Chapters 4, 6 and 7. Malaria rates were increased in the field in the iron dextran treated group (see Chapter 9). Findings related to infectious morbidity from all causes, both in the field and among hospital admissions are reported in this Chapter.

METHODS AND SUBJECTS

Of the 486 infants in the trial cohort six (five iron dextran; one placebo) migrated to another province immediately after the injection, and 2 (placebo) migrated to remote parts of Madang province shortly after injection these 8 were excluded from subsequent analysis. Infants were followed up one week after the injection and full history and examination again performed. This check was carried out on 439 infants (211 in the treatment group). A further 34 infants (17 iron treatment; 17 placebo) were not seen but were reported to be well by the family. 431 infants were seen and examined at 6 months and 392 at 12 months. All follow-up was observer blind (Chapter 7) except in 6 placebo cases and 1 iron dextran case where the code was

broken on request of the government paediatrician to determine appropriate management of anaemia.

Diagnosis and morbidity recording

When sick infants were seen either at field visits or hospital admissions a full history and examination was performed and the results coded on standard proforma which were similar for both hospital and all field visits (Appendix IV). Well infants in the field were all fully examined. In addition to a prose descripton of mother's history, 32 dichotomous (yes/no) direct questions were asked relating to feeding and symptoms of infection. In the examination, 40 physical features were recorded, 33 of which were dichotomous (present/absent) and seven were measurements (nutritional status, pulse, temperature, liver and spleen size). For hospital admissions, blood tests as described above and nose and throat swabs for culture were routine and chest X-ray was performed on 91% of admissions. Other investigations (e.g. lumbar puncture) were performed as indicated. Diagnostic coding for hospital admissions was by the four digit WHO International Classification of Disease (WHO, 1975) while field diagnoses used the three digit WHO tabulation (WHO, 1975).

WHO working committees in the south-western pacific have recommended for epidemiological purposes, a clinical classification of acute respiratory infections which is not dependent on having chest X-ray results (WHO, 1981, 1983). Although chest X-rays were performed on most hospital admissions in this study, and in all cases where pneumonia was suspected, the WHO clinical classification was used to standardize morbidity recording (WHO, 1983) as follows:

Upper Respiratory Infections (URTI)

Ia URTI Mild Nasal discharge, sore throat.

Ib URTI Severe The presence of pus: otitis media, acute sinusitis tonsillitis.

Acute Lower Respiratory Infections (ALRI)

II ALRI Mild "Clinical evidence of ALRI", e.g., history of cough and fever/abnormal sounds on ascultation.

IIIa ALRI Severe As for II but with breathing difficulty: chest indrawing or a rapid respiratory rate.

IIIb ALRI Severe and complicated (Children at special risk): As for IIIa above but in addition: (i) cyanosis, cardiac failure or shock; (ii) severe underlying disease: congenital heart disease, chronic bronchitis etc; (iii) associated specific diseases or symptoms: diarrhoea, measles, malnutrition, pertussis, diphtheria; (iv) very young infants, infants of low birth weight, prematurity, infants who were small for gestational age. (Note local standards have been used for these nutritional parameters).

In the above classification, the main division open to interpretation is presence/absence of "clinical evidence of ALRI". Using logistic discriminant analysis, a discriminant function model was produced based on 12 clinical features (four historical; eight physical) which predicted chest X-ray findings in the cohort infants with 92% sensitivity and 82% specificity (see Appendix I.iv). This function was used to separate groups Ia, Ib on the one hand and II, IIIa, IIIb on the other. Further classification into the WHO subgroups was then made using a simple algorithm based on relevant clinical findings. Since URTI may co-exist with ALRI, the more severe category takes priority thus making the grades mutually exclusive for each episode. In analysis groups IIIa and IIIb are combined with TB to form the category "severe lower respiratory infections" (SLRI).

The reasons for using the above classification rather than depending on chest x-ray results are firstly standardisation within the study, and secondly standardisation with other epidemiological work in the area.

Morbidity surveillance (Episodes and periods)

The scheduled field visits provided the opportunity to study point prevalence of morbidity before and one week after injection and at 6 and 12 months of age. Incidence of infectious disease was estimated from (a) hospital admissions and (b) self-presenting hospital out-patient visits.

The cohort infants living in the catchment area had easy access to Madang base hospital. Cohort infants occasionally presented to Yagaum health centre and were referred to Madang. Usage of health services in Madang area is good. Admissions therefore may be regarded as a good measure of serious morbidity in the cohort. 11 of the 12 deaths in the trial cohort occurred in Madang Hospital. Intercurrent self-presenting out-patient visits on the other hand may have been more influenced by access to hospital.

For the purpose of estimating rates, the period of surveillance for each infant was calculated from the day of injection to the day of the one year visit (or the 365th day of life if no one year visit was made; 86 infants) or death, if before one year (12 infants). Exceptions to this were: infants who migrated to another province later during their first year of life and for whom no information was available for subsequent morbidity (3 iron dextran; 3 placebo); surveillance was calculated to the date of departure for these.

A further 21 infants (12 iron dextran) who migrated from Madang province during their first year were traced after one year and survivorship and length of stay in hospital ascertained. In this group there were five admissions outside Madang Province and clinical

and laboratory details were obtained. This group was assessed on a surveillance period to one year of age. One year survivorship status was ascertained for 470 of the 478 infants with a measurable follow-up period (98%). These 478 infants form the group analysed in this Chapter as being under surveillance.

Mean age (SD) in days at injection was 69.8 (15.1) in the 231 infants in the iron dextran group and 67.9 (11.8) in the 247 infants in the placebo group. Mean post-injection surveillance period (SD) in days was 288.5 (43.9) in the iron dextran group and 288.6 (47.0) in the placebo group.

Total pre-injection stay in hospital in the iron dextran group was 171 days and in the placebo group 170 days. Mean % pre-injection stay in hospital was calculated for each infant as:

$$\frac{\text{Total days in hospital during pre-injection period}}{\text{Age (in days) at injection}} \times 100\%$$

This value was slightly larger in the placebo group (1.03% vs 0.81%), but the difference was not significant.

For the purpose of statistical comparison, percentage post-injection stay in hospital was calculated for each infant as:

$$\frac{\text{Total days in hospital during post-injection surveillance}}{\text{Post-injection surveillance period (days)}} \times 100\%$$

A certain number of babies (5 iron dextran; 9 placebo) received iron dextran and/or blood transfusion subsequent to the two months visit from other health workers. These cases were not removed from the analysis in accordance with MRC recommendations for longitudinal trials (see Chapter 6.1).

Distribution of periods in hospital was not-Normal showing marked skew so a non-parametric rank sum test was used to compare treatment groups - (the Kruskall-Wallis one-way analysis of variance).

Analysis of the risk of admission

Multivariate analysis to determine contributions and interactions of explanatory variables to the risk of admission was performed using logistic analysis on the dichotomous contingency "admitted during surveillance period/not admitted" using Generalised Linear Interactive Modelling (GLIM). Three overlapping categories of admission were analysed separately: 1) all admissions; 2) any admissions with SLRI; 3) any admissions with enlarged spleen and/or positive malaria blood film.

Apart from the main intervention variable (iron dextran vs placebo) a number of other independent pre-trial descriptive explanatory variables were investigated for effects on risk of admission: 1) Birth (24 hr venous) and 2 month haemoglobin g/dl; 2) Birth and 2 month MCHC (g/dl); 3) Birth and 2 month weight (kg); 4) Birth and 2 month serum ferritin ng/ml (log value used); 5) Birth and 2 month transferrin saturation %; 6) Weight-for-length standard deviation score at start of trial (2 months) grouped as: 1-33rd centile (<0.20); 33rd-67th centile (0.20-1.00), 67th-100th centile (>1.00) for the cohort; 7) Domicile at birth: (a) urban residential, (b) urban settlement, (c) rural indigenous, (d) rural settlement, (e) rural institution - these categories are described in detail in Chapter 4; 8) Birth Hb Barts status (see Chapter 11).

All comparisons of incidence of specific infections used 2 x 2 Chi-squared tests, thus avoiding analysis of repeated episodes in individuals (Table 3).

Hosking and Roberton (1981) have proposed a weighted infection episode scoring system to assess immunodeficiency in childhood. This "infection score" has been used as a general guide in Table II to indicate overall prevalence of infectious disease at scheduled visits and in Table III to indicate overall incidence at intercurrent visits

and hospital admissions. Statistical comparisons are not made since the weightings have not been validated.

Computer analysis

All analyses were performed on the Liverpool University IBM 3083 BX computer. Statistical programmes used included Statistical Package or Social Sciences (SPSS) and GLIM (see Chapter 6.3).

RESULTS

1. Mortality

12 infants from the trial cohort of 478 died at some point after the injection and before one year (five iron dextran; seven placebo). These numbers are insufficient for statistical comparison. Among the initial total birth cohort of 565 (531 of whom were traced at one year) there were six deaths before two months of age and 15 deaths from 2 to 12 months, giving an over-all mortality of 21/531, equivalent to a rough infant mortality rate of 40/1000. This is less than the census estimated infant mortality in Madang area of 62/1000 (see Chapter 4) in 1981, however, the figures are not strictly comparable. The 2 to 12 month known mortality in the 'drop-outs' was higher (3/48 = 62.5/1000) than in the trial group (12/471 = 25.5/1000) ($p>0.05$).

Table 1 shows diagnoses and causes of death for the 12 trial deaths, all but one of which occurred in hospital. The history of the home death was highly suggestive of pneumonia following measles. The main cause of death in all cases was pneumonia, usually with intractable cor pulmonale. In two thirds of cases this was secondary to either measles or whooping cough. Exposure to malaria was evidenced by splenomegaly and/or a positive blood slide in six of these deaths. However, in no case was malaria clearly a direct cause of death.

2. Prevalence of infectious diagnoses at scheduled field visits

At the field check one week after injection there were no significant differences in frequencies of any individual infectious diagnosis between the two groups (Table 2).

At the six months visit there were more SLRI's in the iron dextran than in the placebo group (10:2; $X^2 = 4.58$; 1df; $p<0.05$) (Table 2). There were no significant differences in other individual diagnoses between the groups.

TABLE 1

Summary of causes of death in trial cohort

Main causes of death	Secondary to	Associated conditions	Evidence of malaria
IRON DEXTRAN GROUP			
1. Pneumonia + Cor pulmonale	Measles	-	P. falciparum
2. Pneumonia + Cor pulmonale	-	Anaemia	Spleen enlarged
3. Pneumonia + Cor pulmonale	Measles	-	P. falciparum/ Spleen enlarged
4. Pneumonia	-	Thrush, Dehydration	-
5. * Pneumonia	Measles	-	-
PLACEBO GROUP			
1. Pneumonia + Cor pulmonale	Pertussis	-	Spleen enlarged
2. Pneumonia/Lung abscess + Cor pulmonale	Measles	-	-
3. Pneumonia + Cor pulmonale	Measles	T.B.	Spleen enlarged
4. Pneumonia + Cor pulmonale	Pertussis	-	-
5. Pneumonia	-	Gastroenteritis	-
6. Pneumonia	-	-	Spleen enlarged
7. Pneumonia	Pertussis Measles	Gastroenteritis Thrush	-

* Post-mortem history taken at home two weeks after death.

TABLE 2

Infectious diagnoses at scheduled post-injection visits

	Field Check One Week		6 months visit		1 year visit	
	Iron Dextran (n=211)	Placebo (n=228)	Iron Dextran (n=210)	Placebo (n=221)	Iron Dextran (n=191)	Placebo (n=201)
WHO Ia: URTI mild	46	35	48	55	35	29
WHO Ib: URTI severe (otitis media)	12	7	37	37	61	69
WHO II: ALRTI mild	3	4	14	13	10	12
WHO IIIa: ALRTI severe	0	2	5) p<0.05	(2	5	5
WHO IIIb: ALRTI severe + complicated	0	0	5)	(0	2	2
Pertussis	3	2	3	6	0	3
Measles	0	0	4	1	3	5
Chickenpox	0	0	1	0	0	1
Gastroenteritis	3	0	6	9	13	13
Oral candidiasis	8	4	4	2	3	4
Conjunctivitis	3	3	7	12	4	7
Tinea	12	11	14	10	11	19
Infected scabies	45	36	54	50	33	41
Superficial skin infections (impetigo, ulcers)	5	5	11	4	6	11
Deep soft tissue infections (boils, suppurative, lymphadenitis)	0	2	3	3	9	9
Malaria (symptomatic)	6	5	19	10	20	20
P.U.O.	11	13	2	0	3	2
Mean infection score*/visit	1.32	1.09	2.57	2.06	2.94	3.13

n = numbers attending each visit. Respiratory diagnoses Ia, II, IIIa and IIIb are mutually exclusive; other diagnoses may be multiple. *After Hosking and Roberton (1981) (see text).

At the 12 months visit there were no significant differences in any individual category of diagnosis (Table 2).

Commonest diagnoses at field visits in descending order of frequency were skin infections followed by URTIs, and otitis media.

As can be seen from Table 2, numbers of individual diagnoses at scheduled visits are too few to make meaningful comparisons. "Infection scores" using weighted aggregation of all infectious episodes (see Methods) indicate 21% and 25% higher scores in the iron dextran group at the field check and at six months while the score is 6% lower at one year. However, statistical comparison of the difference would be suspect due to the arbitrary (unvalidated) nature of the weighting.

3. Incidence of infectious disease at intercurrent visits

There were 118 iron dextran and 113 placebo self-presenting outpatient visits for intercurrent illness in the surveillance period. Acute otitis media was significiantly more common in the iron dextran group (Table 3). Although most other categories of infectious disease were commoner in the iron dextran group, none of the individual differences were significant. The mean infection score (for the whole cohort) was 26% higher in the iron dextran group).

4. Hospital admissions

68 (29%) of the iron dextran group and 58 (23%) of the placebo group were admitted to hospital at least once during the post-injection surveillance period. There was a total of 100 admissions (average 0.43 per child) in the iron dextran group and 83 admissions (average 0.34 per child) in the placebo group. Numbers of admissions for severe lower respiratory infections (SLRI) were as follows: (a) iron dextran group: 48 infants; 64 admissions; (average 0.28 per infant) and (b) placebo group: 39 infants; 52 admissions (average 0.21 per infant).

60% more days were spent in hospital post-injection by infants from the iron dextran group than by infants from the placebo group (997:623 days). Corresponding figures for the subset of admissions for SLRI were 629:402 days. The frequency distribution of periods of admission is shown in Figure 1. For statistical comparison of the differences observed, "% post-injection hospitalisation" per infant was compared between the two groups (see Methods, this Chapter) and showed significant differences for "all admissions" ($p = 0.04$) (Figure 1) while giving a X^2 value with one degree of freedom of 3.75 ($p=0.053$) for the subset admissions for SLRI.

The average number of admissions per infant requiring admission was similar in the iron dextran and placebo groups (1.47:1.43), but the mean total hospital stay (all admissions) per infant admitted was four days longer in the iron dextran group than in the placebo group (14.7:10.7 days; $X^2 = 5.25$; 1 d.f.; $p=0.022$ using Kruskall-Wallis one-way ANOVA to compare distributions).

Multivariate analysis of factors affecting risk of admission

Risk of admission to hospital (dichotomous dependant) was analysed using logistic regression of all the explanatory variables mentioned in methods, including the following variables: 1) injection group; 2) domicile; 3) weight-for-length "Z" score at the two-month visit; 4) birth haemoglobin (see also Figures 2-4). Of all variables analysed, only weight-for-length "Z" score at 2 months and birth haemoglobin clearly affected subsequent risk of admission:

1) Injection Group. Overall admission risk was significantly greater in the iron dextran group when compared to the placebo group only for the subset "admissions with evidence of malaria" (odds ratio: 1.59) (95% limits 1.44-1.76; $X^2 = 4.2$; $p<0.05$).

2) Domicile. Risk of admission (any category) was not significantly affected by domicile. Figure 2 shows probability of any admission by

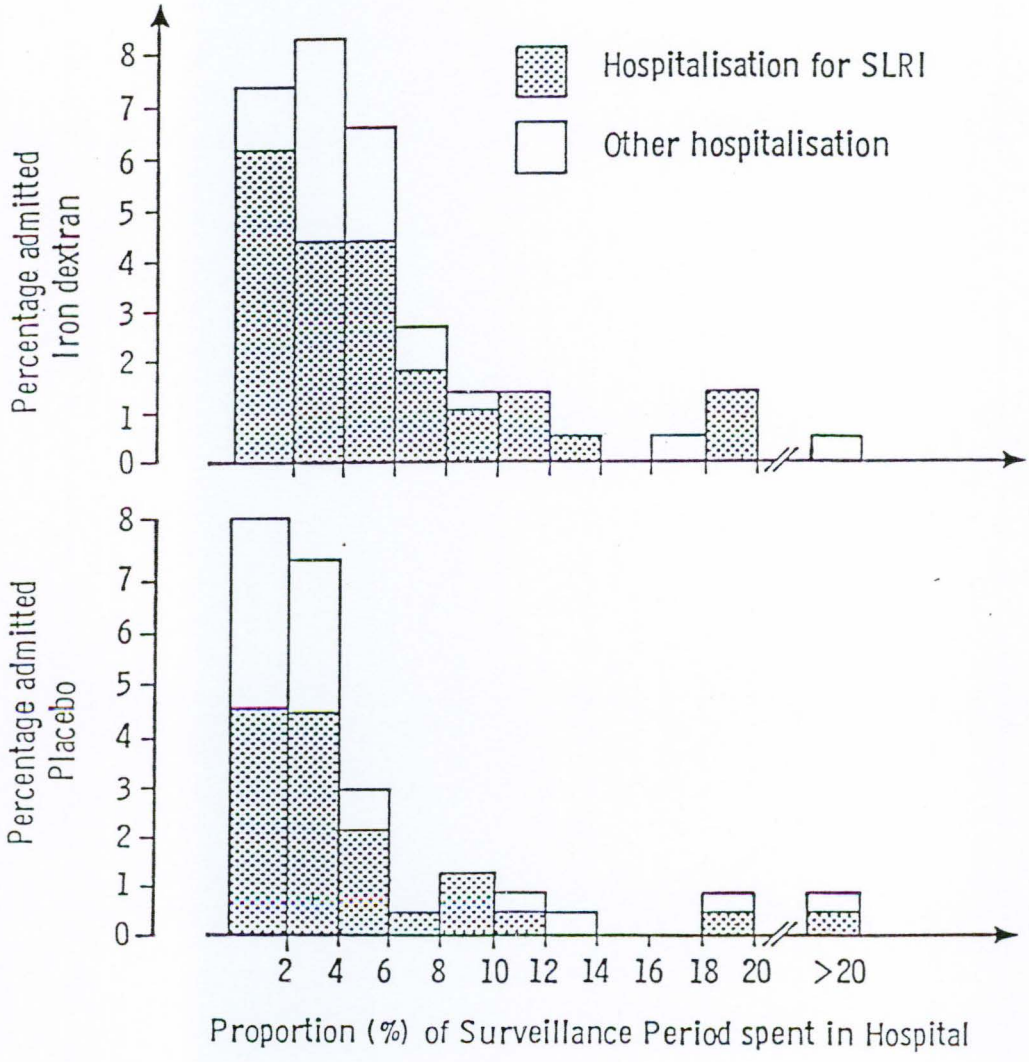


Figure 1 Frequency histogram of total periods as hospital in-patient by treatment group expressed as a percentage of post-injection surveillance period.

Footnotes to figure:

Kruskall-Wallis one-way analysis of variance for comparison of distributions (total periods):

X^2 = 4.20; 1 d.f.; p=0.040

SLRI = Severe Lower Respiratory Infection - WHO

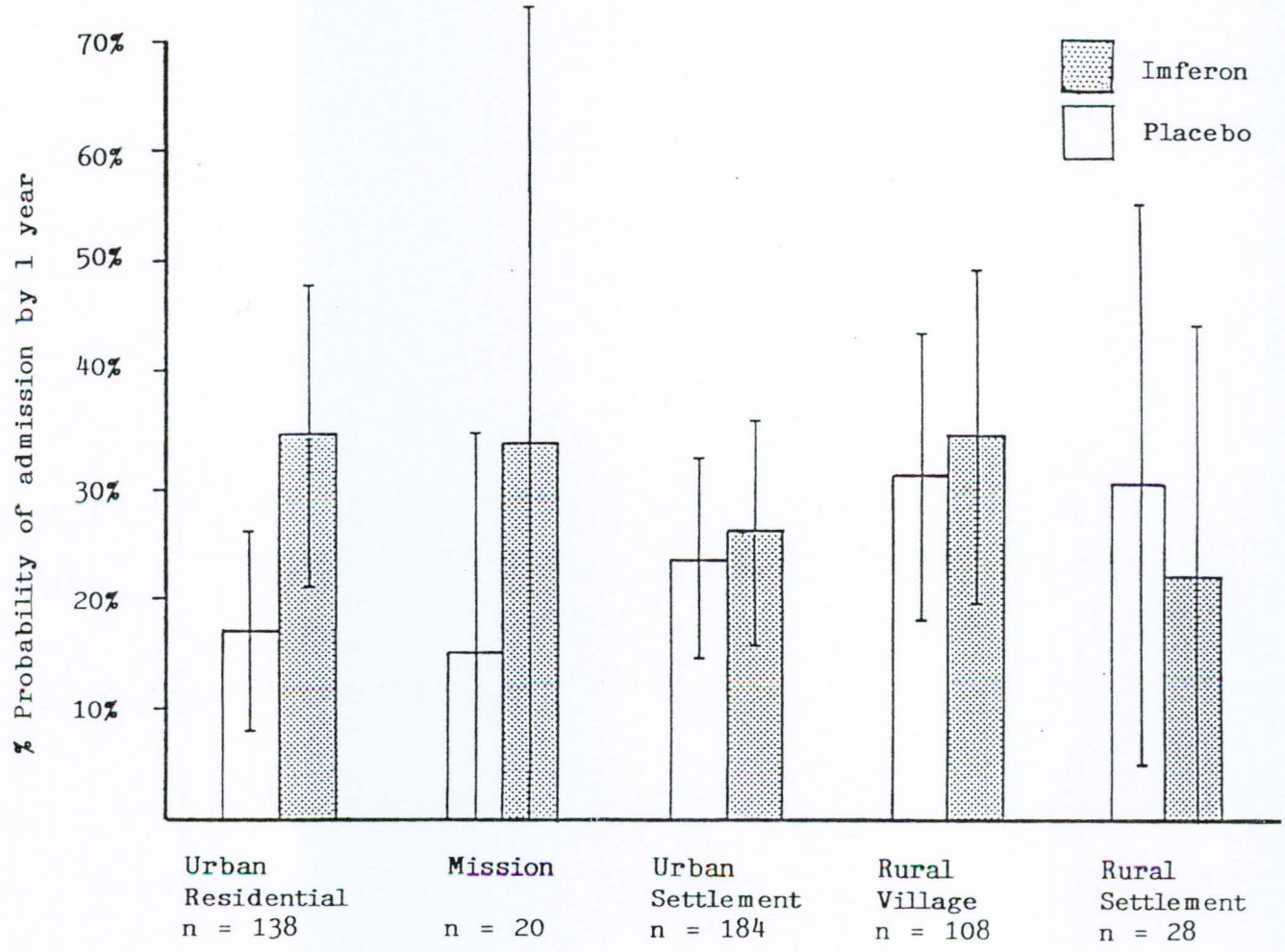


Figure 2 Probability of admission by one year broken down by domicile and by treatment group (± 2SE)

domicile by injection group. There were no significant interactions between injection and domicile on risk of admission.

3) 2 month weight-for-length 'Z' score. Effects of weight-for-length on risk of admission were non-linear with infants with a low score having a higher risk of subsequent admission but no graded effect when the cohort was divided into 3 equal centile groups by the 33rd and 67th centile points (0.20 and 1.00 respectively for this cohort) (Figure 3). Relative risk (odds ratio) for admission was therefore calculated for below versus above the 33rd centile point and was significantly greater than unity for 1) all admissions (odds ratio: 1.73 (95% limits 1.56-1.91); $p<0.025$); 2) admissions with SLRI (Odds ratio 1.87 (95% limits 1.64-2.13); $p<0.01$) (Figure 3). The odds ratio was not significantly greater than unity for the contingency "malaria associated admissions". Figure 3 shows risks of admission with SLRI by 2 month weight-for-length "Z" score by injection group. It may be seen that the risk of admission associated with iron injection is greater in the 2 groups of infants with lower "Z" score. This interaction was tested using GLIM after controlling for the main effects of injection and weight-for-length. The interaction term was significant for all 3 categories of admission: 1) all admissions ($F = 3.26$; d.f. 2, 470; $p<0.05$); 2) SLRI ($F = 3.27$; d.f. 2, 470; $p<0.05$); 3) Malaria associated admissions ($F = 8.75$; d.f. 2, 470; $p<0.001$).

4) Birth venous Haemoglobin. Risk of admissions increased linearly with higher birth haemoglobins. The effect was significant for all admissions ($F = 9.71$; d.f. 1, 474; $p<0.01$), for the subset of those admitted for SLRI ($F = 11.0$; d.f. 1, 1,474; $p<0.001$) and for the subset with evidence of malaria (enlarged spleen and/or positive malaria slide) ($F = 7.7$; d.f. 1, 474; $p<0.01$) (see Fig. 4). The deleterious effect of iron dextran injection was more marked in the high birth haemoglobin group (Fig. 4). This interaction was

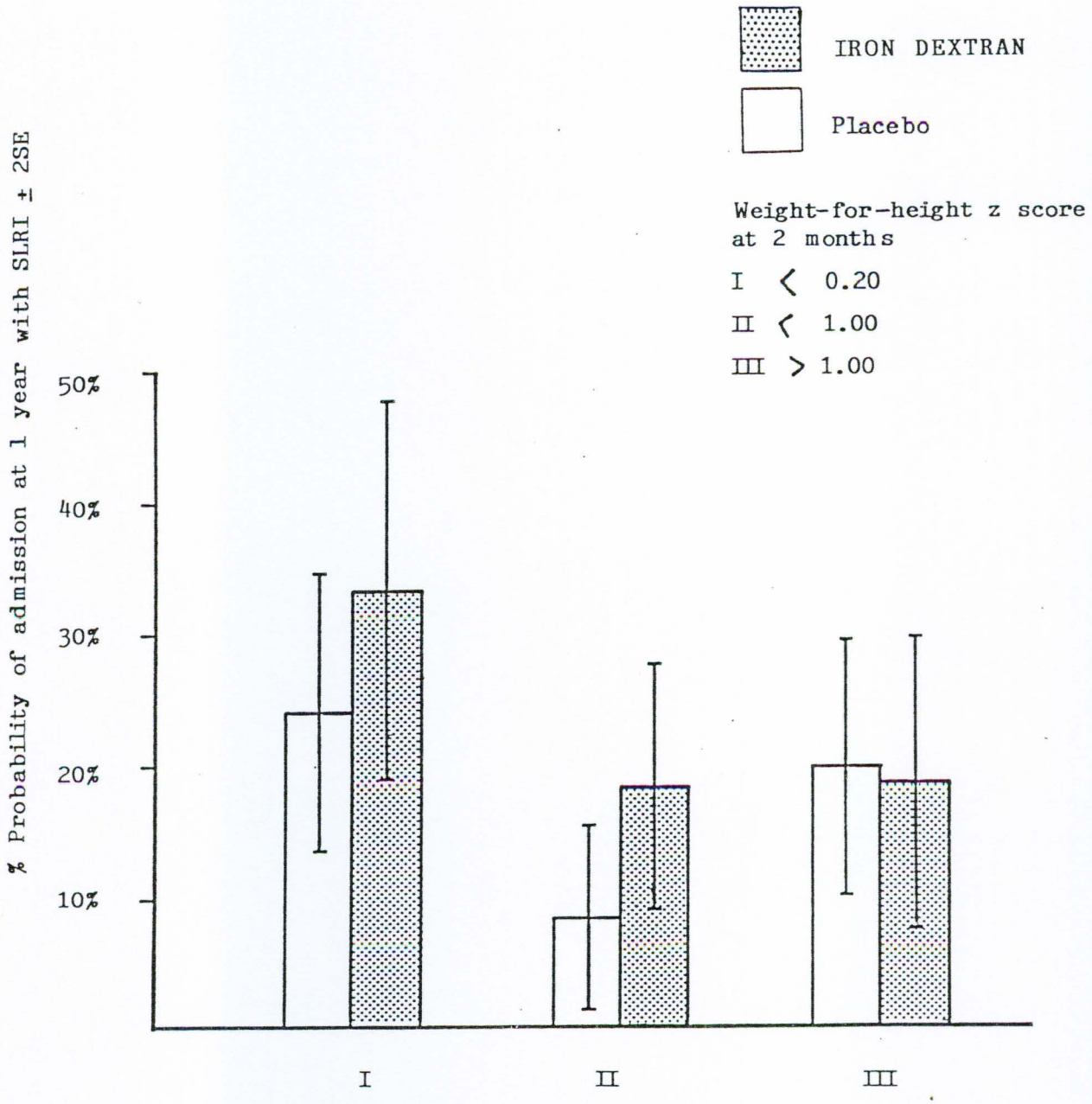


Figure 3 Probability of admission by one year broken down by weight-for-height z score at 2 months and by treatment group.

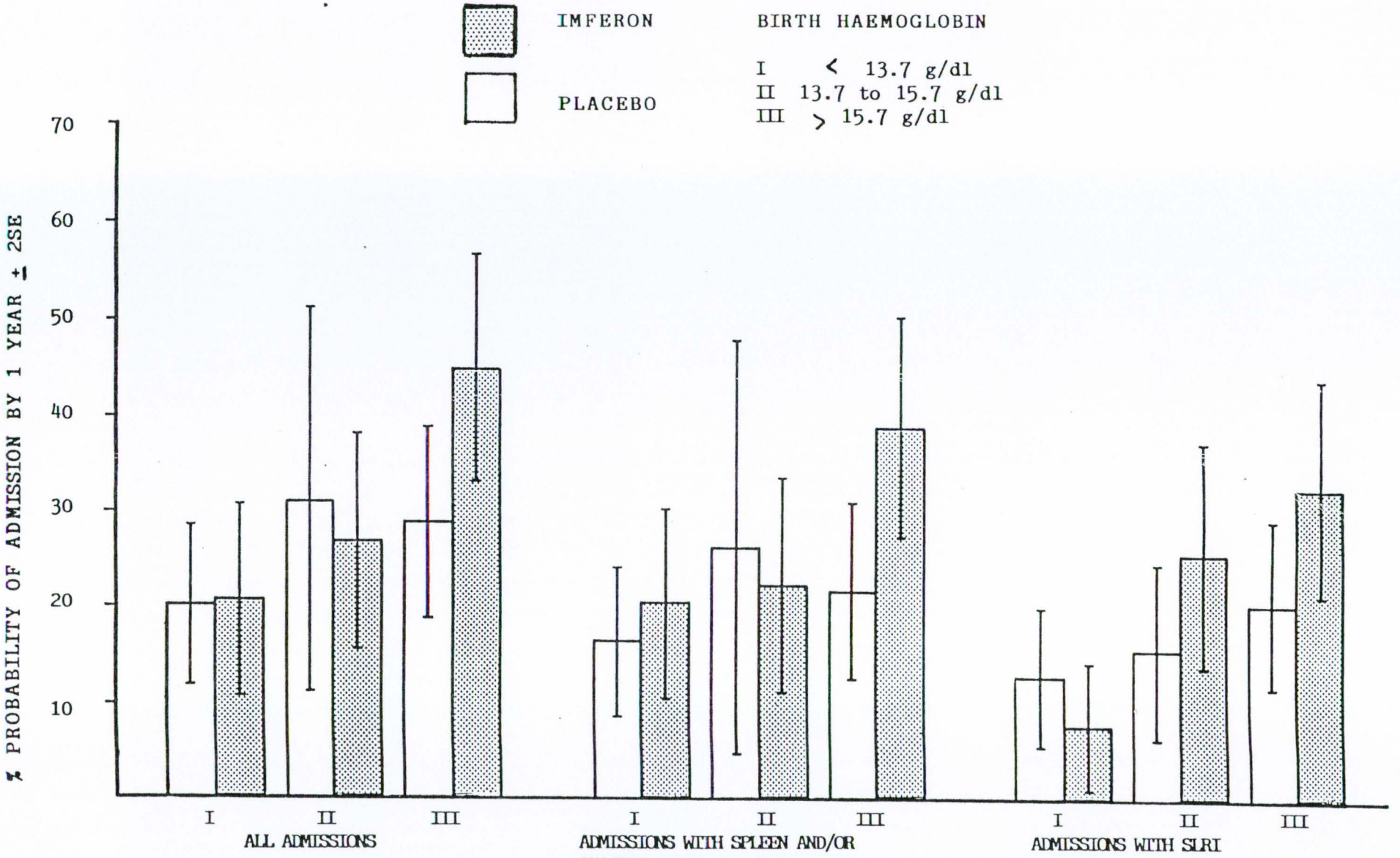


Figure 4 Probability of admission by one year of age (±2SE) broken down by treatment group and by birth haemoglobin (a) for all admissions, (b) admissions with positive malaria blood slide and/or enlarged spleen, (c) admissions for severe lower respiratory infections (SLRI).

significant at just above the 5% level for "all admissions" and both subsets.

Diagnostic categories

A breakdown of diagnoses recorded for admissions is shown in Table 3. Diagnoses were often multiple but lower respiratory infections were the primary reason for admission in over 60% of admissions. There were more admissions associated with otitis media, lower respiratory infections, measles, malaria, malarial anaemia and febrile convulsions from the iron dextran than the placebo group; however numbers were too small for individual statistical comparison. The mean infection score for the whole cohort was 31% higher in the iron dextran group.

Clinical malaria was diagnosed in 42 admissions (25 iron dextran) (Table 3) and was confirmed to be the main reason for admission in only 17 iron dextran and 13 placebo cases. There were no cases of cerebral malaria, 8 cases of severe malarial anaemia (Hb<5.0 g/dl) (Table 4) and five infants (three iron dextran) had febrile convulsions, thought clinically to be secondary to malaria. In five other cases malaria was associated with diarrhoea. No deaths could be directly ascribed to malaria.

Malaria and repiratory infections at first post injection admission

Table 4 shows spleen and malaria parasite rates for all first admissions and separately for all those with severe lower respiratory infection. Malaria parasite rates among admissions for lower respiratory tract infections were similar to those recorded at scheduled field visits and also reflected the trend to higher rates in the iron dextran group seen in the field (Chapter 9). Admission malaria parasite rates were higher than in the field for non-SLRI admissions since some of these had clinical malaria. In contrast to parasite rates, spleen rates were very high in both treatment groups at first

TABLE 3

Infectious diagnosis of hospital admissions and self-presenting intercurrent out-patient visits

	Hospital admissions		Intercurrent visits	
	Iron Dextran (n = 100)	Placebo (n = 83)	Iron Dextran (n = 118)	Placebo (n = 113)
WHO Ia: URTI mild	8	7	35	40
WHO Ib: URTI severe-otitis media	34	19	41 p = 0.05*	21
WHO II: ALRTI mild	8	7	13	8
WHO IIIa: ALRTI severe	31	26	6	17
WHO IIIb: ALRTI severe+ complicated	32	25	2	1
T.B.	4	1	0	0
Lung abscess	0	2	0	0
Pertussis	12	16	5	8
Measles	25 p = 0.05*	13	10	5
Chickenpox	0	0	1	0
Gastroenteritis	12	11	10	4
Oral candidiasis	13	11	2	8
Tinea	3	6	7	4
Skin and soft tissue infections (pyogenic)	9	10	50	31
Malaria (symptomatic)	25	17	12	14
Severe anaemia	3	3	2	0
Severe malarial anaemia	5	3	0	0
Febrile convulsions	7	3	0	0
Meningitis	0	4	0	0
Mean infection score+/ cohort infants	4.12	3.15	3.00	2.38

Note: n = Total number of episodes (e.g., admission) in each group. Respiratory diagnoses in Ia, II, IIIa, and IIIb are mutually exclusive: otherwise diagnoses may be multiple.

* 2 x 2 Chi-squared based on infants with one or more episodes vs none

\+ After Hosking and Roberton (1981) (see text).

TABLE 4

Malariometry at first hospital admission by injection group by SLRI/Non-SLRI

	SLRI		Other		Total	
	Iron Dextran	Placebo	Iron Dextran	Placebo	Iron Dextran	Placebo
Spleen enlarged (%)	37 (82.2)	26 (72.2)	21 (91.3)*	14 (63.6)	58 (85.3)*	40 (69.0)
Malaria slide positive (%)	10 (22.2)	6 (16.7)	8 (34.8)	11 (50.0)	18 (26.5)	17 (29.3)
Neither (%)	7 (15.6)	10 (27.8)	2 (8.7)	4 (18.2)	9 (13.2)	14 (24.1)
Total	45	36	23	22	68	58

* Difference between the iron dextran and placebo groups significant at 5% level

admission (85% and 69% for iron dextran and placebo respectively), (Table 4) and significantly higher than at any of the scheduled field visits (23% at six months visit and 42% at one year (Chapter 9). Spleen rates were also very high for the subset of first admissions with severe lower respiratory infections (Table 4). Over-all spleen rates were significantly higher in the iron dextran group than the placebo group (Table 4).

DISCUSSION

A balanced design is necessary to interpret differences in an intervention trial.

Analysis of social, anthropological and physical descriptive variables showed no significant differences between the two treatment groups and were discussed in Chapter 7. The age of injection, pre-injection morbidity and post-injection surveillance periods analysed here were also closely similar between groups (Table 1).

The results indicate that the iron dextran group had more infectious disease and required more admissions for longer periods than the placebo group. The difference between treatment groups in periods as hospital in-patients following injection was significant. Iron dextran infants who were admitted one or more times required a longer over-all stay in hospital than placebo infants with the same mean number of admissions suggesting an increase in <u>severity</u> of disease in those infants. Similar but smaller effects were present in the large subset of admissions for severe lower respiratory infection (65% of total). The 60% increase in hospitalisation in the iron dextran group may be regarded along with the increase in malaria rates (Chapter 9) as one of the two main outcomes of the study.

Death rates were similar between the two treatment groups, the primary cause of death being identified as lower respiratory infection in every case, and precipitated by measles and/or pertussis in 8 of 12 cases. Numbers were too small to make a valid comparison of

mortality. A surprising fact was that malaria was not identified as a direct cause of infant death in a cohort of this size in an area of high malarial transmission. The possibility of undetected deaths due to malaria is unlikely in view of 98% one year survivorship details in the cohort of 478. All but one of the deaths occurred in hospital. Evidence of malaria (enlarged spleen or positive blood slide) was present in 6 of 12 deaths, but was not apparently a direct cause of death in any case. The possibility that malaria may affect morbidity and mortality indirectly is discussed below.

"Infection scores" at field visits and admissions tended to be increased in the iron dextran group (Tables 2 and 3). Analysis of numbers of episodes for individual disease entities, however, only showed significant increases in the iron dextran group for (a) measles-associated admissions, (b) otitis media in self-presenting out-patient visits, (c) severe lower respiratory infections at the six-month field visit.

Clinical (symptomatic) malaria was not significantly more prevalent at field visits (Table 2) nor had a significantly higher incidence in intercurrent visits or hospital admissions (Table 3) in the iron dextran group when compared with the placebo group, although such a difference would be expected from the parasite and spleen rates noted in the last Chapter. Numbers of clinical episodes were however too small to make valid statistical comparisons of the differences observed.

Malaria associated hospital admissions (slide and/or spleen positive) were, however, significantly more likely in the iron dextran group. Analysis of malariometry at first admission moreover showed overall spleen rates of 78%, which were significantly higher than those recorded in the same cohort at the 6 and 12 month field visits (23% and 42%) (Chapter 9), and significantly higher in the iron dextran than placebo group.

Although malaria was not identified as a major direct cause of morbidity it could be postulated that the effects of iron dextran administration and birth haemoglobin (see below) on admissions were mediated through their observed adverse influence on malarial experience in this cohort. In vitro immune suppressive effects of malaria are well documented (McGregor & Barry, 1962; Greenwood et al., 1972; hence in this context it is worth noting that admissions for severe lower respiratory infections also had very high spleen rates.

Analysis of other factors which could affect admission rates, showed among others no significant effects associated with domicile, birth (or 2 month) serum ferritin or birth-weight. However, infants with a low weight-for-length at the start of the trial had a significantly higher admission rate. This risk was significant for all admissions and for SLRI admissions but not for the subset 'malaria associated admissions'. The risk was also increased interactively by iron dextran injection which might be expected to have greater effects in smaller babies.

Birth haemoglobin was also significantly correlated with admission risk, infants with a low birth haemoglobin being less likely to be admitted. In addition, there was a suggestion of an interactive effect with iron dextran, the risk of admission with iron dextran being greater in infants with a high birth haeomoglobin. The interaction was not significant at the 5% level. These effects were seen for admissions for all infections and for the subsets "severe lower respiratory infections" (65% of admissions) and "admissions with spleen and/or malaria slide positive" (83% of admissions).

The variable thus with most explanatory effect on admission rates for infectious disease was birth haemoglobin level. In Chapter 9 it was shown in the placebo group that this was correlated with chances of a positive malarial blood slide at field visits. Birth haemoglobin is a major determinant of iron status in the first year of life

(Lanzkowsky, 1976), and in this study one of the few independant measures unlikely to be confounded by malaria. The interaction of the risk of admission associated with birth haemoglobin and iron dextran injection suggests that the effect may be iron related. The alternative possibility that birth haemoglobin reflects αthalassaemia phenotype which may affect morbidity is less convincing since there was no correlation between birth Hb Barts level and birth haemoglobin (Chapter 7).

CONCLUSION

Iron dextran prophylaxis in infancy was associated with increased hospitalisation due to infectious disease. There appeared to be an association between malaria and other infectious disease. Caution should be exercised in extrapolating the results of this study from a malarious area to non-malarious areas.

CHAPTER 11

IRON SUPPLEMENTATION: THE INTERACTION OF ALPHA THALASSAEMIA WITH IRON AND MALARIA

SUMMARY

Infants in the trial had a high rate (80%) of α^+ thalassaemia. The neighbouring highland area has a low rate of both malaria and α^+ thalassaemia. The results of clinical and haematological examination of these infants at 6 and 12 months were analysed to determine the relationship between α thalassaemia and susceptibility to malaria. Infants were divided according to haemoglobin Bart's levels found at birth into 3 groups corresponding to probable genotypes. Homozygotes had higher malarial slide positivity and spleen rates at 6 and 12 months than the normal or heterozygote groups. The risk of malaria slide positivity associated with iron treatment was greater in the normals than in the infants with Hb Barts at birth. Analysis of variance of haemoglobin levels showed that the anaemia associated with malaria was greatest in the normals and least in the homozygotes at 6 months. A possible protective mechanism of α thalassaemia is discussed.

INTRODUCTION

In Chapters 9 and 10 the association of parenteral iron supplementation (3 ml iron dextran) to infants at 2 months of age with increased infectious morbidity and increased malaria rates is described. The increase in malaria was associated with more severe malarial anaemia at six months. There was also evidence of a very high rate (80%) of α thalassaemia in the study cohort (Chapter 7). This is an α^+ thalassaemia, mainly of the 4.2 kilobase single α gene deletion type (freq. 0.6) with a much lower frequency of the 3.7 kilobase deletion type (freq. 0.08) (Chapter 7.2 and Flint et al.,

1986 - see Appendix V). α thalassaemia is almost absent from the neighbouring highland populations where malaria is hypoendemic (Chapter 7.2 and Flint et al., 1986 - see Appendix V). This suggested that αthalassaemia has conferred a selective advantage against malaria in the coastal lowland, where malaria transmission is high (Cattani et al., 1983). However, the mechanism of this proposed effect is unknown. The results of an analysis of the interactions of αthalassaemia with malaria and iron supplementation are presented here, which provide preliminary data which it is hoped will contribute to the elucidation of the mechanism of protection in this population. It is also important in relation to the iron study to exclude the possibility that the main effect associated with iron therapy (higher parasite rates) is a result of confounding due to the high prevalence of thalassaemia.

MATERIALS AND METHODS

The study area, disease patterns, the study protocol and the study cohort are described in detail in Chapters 3, 4 and 7.

As detailed in Chapter 7, 486 infants entered the trial cohort at the age of 2 months following explanation and parental consent.

Subjects for αthalassaemia study

A high frequency of α^{+} thalassaemia of the 4.2 (- $\alpha^{4.2}$) and 3.7 (- $\alpha^{3.7}$) kilobase deletion types has been identified in north coastal New Guinea.. No α° thalassaemia was found (Oppenheimer et al., 1984b; Flint et al., 1986 - see Appendix V), which was consistent with the absence of reports of the Hb Bart's hydrops foetalis sydrome from this area (Oppenheimer et al., 1984b).

No DNA analysis was performed on infants in the birth cohort; instead identification and quantification of haemoglobin Bart's in cord blood was performed on 200 infants (see Chapter 7). These are a subset of 217 cord Hb Bart's estimations reported previously

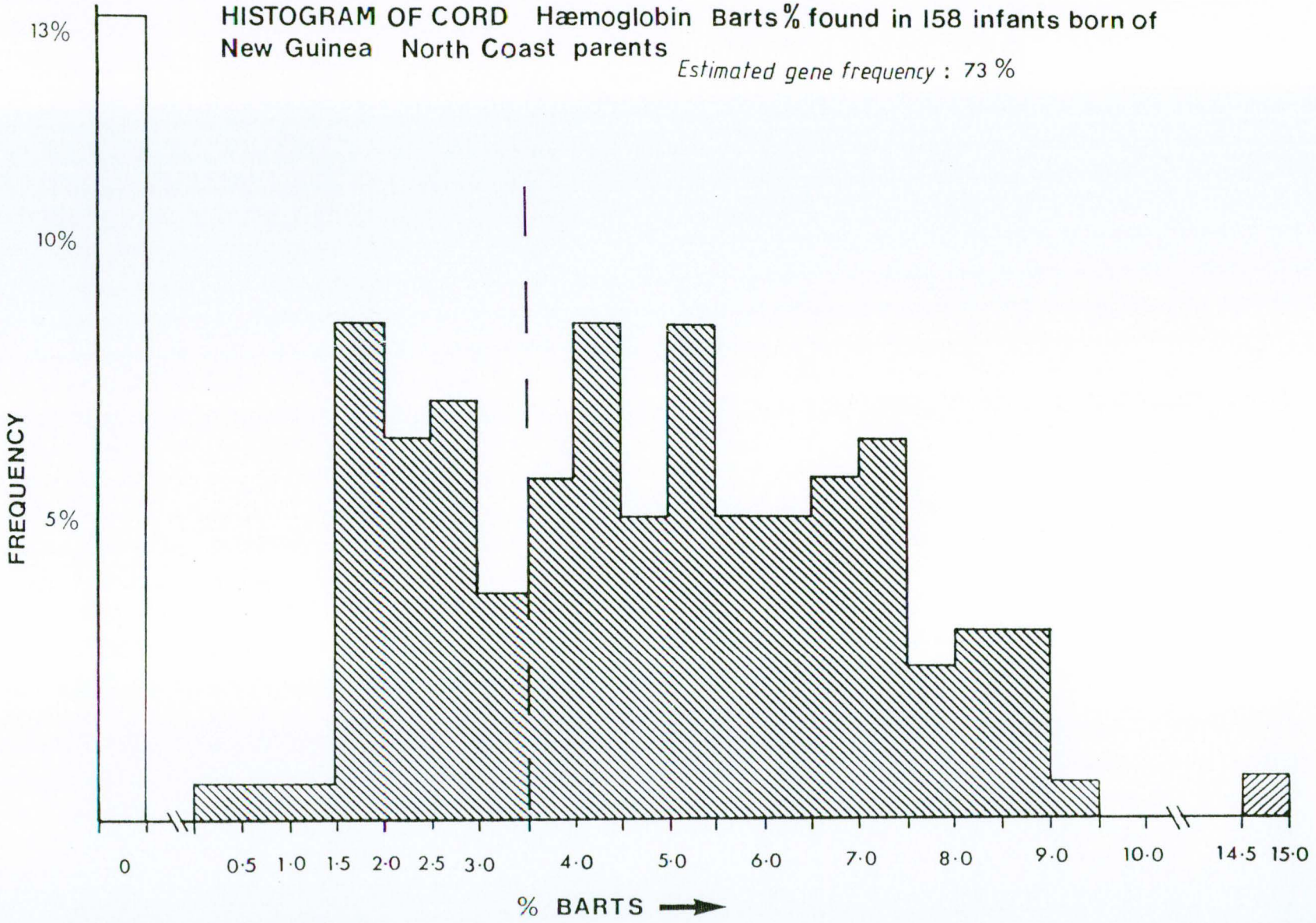
(Oppenheimer et al., 1984b) (see Chapter 7). 174 of these infants subsequently entered the iron trial at 2 months of age. In this paper, all analyses are carried out using this set of 174 infants, 88 of whom were in the iron treatment group and 86 in the placebo group.

Assignment of genotypes based on Hb Bart's levels

Infants are classified into putative genotypes (normals, heterozygotes for α^+ thalassaemia and homozygotes for α^+ thalassaemia) on the basis of the percentage Hb Bart's found in cord blood (Fig. 1). Normals are infants with no Hb Bart's on haemoglobin electrophoresis; "low Bart's" or heterozygotes are those with Hb Bart's <3.5%; "high Bart's" or homozygotes are those with Hb Bart's >3.5%.

This classification is based on the results of previous studies in North American Blacks, Southeast Asians (Higgs and Weatherall, 1983; Lie-Onjo et al., 1982) and, particularly, Melanesians. The most comparable of these is the study of Melanesians, mainly from Vanuatu but also including some samples from Madang, Papua New Guinea (Bowden et al., 1987 - see Appendix V). Hb Bart's levels in both that study and the present one were quantified in the same laboratory. All the $-\alpha^{4.2}$ deletion heterozygotes from Vanuatu had detectable Hb Bart's, so all infants in the present cohort without Hb Bart's have been designated as "normals". Heterozygotes for the 3.7 kb deletion may not show any Hb Bart's but they are relatively uncommon in Madang (8%) (Flint et al., 1986 - see Appendix V; Hill et al., 1985 - see Appendix V; also see Chapter 7). The cut-off level of Hb Bart's which distinguishes α^+ thalassaemia heterozygotes from homozygotes is not well defined. The level of 3.5% Hb Bart's is used here, a relatively high value based on the predominance of $-\alpha^{4.2}$ deletions in north coastal Papua New Guinea (60% gene frequency), because this deletion produces more Hb Bart's at birth than the $-\alpha^{3.7}$ deletion (Bowden et

Figure


HISTOGRAM OF CORD Hæmoglobin Barts % found in 158 infants born of New Guinea North Coast parents
Estimated gene frequency : 73 %
13%
10%
5%
FREQUENCY
0
0·5
1·0
1·5
2·0
2·5
3·0
4·0
5·0
6·0
7·0
8·0
9·0
10·0
14·5
15·0
% BARTS

al., 1987; see Appendix V). The gene frequency of - ∝ north coastal indigenes calculated using this value is 0.73 (Fig. 1), close to the 0.68 found in this population using DNA genotyping (Flint et al., 1986 - see Appendix V). Use of lower and higher cut-offs (3% or 4%) made little difference to the results reported here.

Hb Bart's levels alone provided a measure of the severity of the globin chain deficit at birth. Therefore, the classification into high, low and absent Hb Bart's groups is sufficient to allow assessment of the effect of ∝ thalassaemia on other variables.

Statistical methods

The data were processed using the Liverpool University computer. Results were analysed using Student's 't' tests, Fisher's exact test and comparison of relative risk, where applicable. Analysis of variance was performed using SPSS ANOVA programs designed for use with unequal cell sizes produced by experimental attributes (such as malaria) (see Chapter 6.3). Each analysis includes only infants for whom all variables were present for that analysis. Sample sizes are indicated.

RESULTS

Malaria and birth Bart's status

The results of malariometry (malaria slide positivity and spleen rates) at 6 and 12 months, broken down by Bart's Group and iron dextran vs. placebo, are shown in Table 1. In the placebo group, which is representative of the natural situation, slide positivity is higher in the "high Bart's" group than in either the "low Bart's" or "normal" groups both at 6 and 12 months. Table 2 shows the results of 6 and 12 months visits combined, with infants categorized as at least one positive slide or no positives. Fisher's exact test for the contingency "High Bart's" vs the rest is significant (p=0.006). A

TABLE 1

Malaria slide positivity and spleen rate at 6 and 12 months

MALARIA		6 months malaria slide Number positive	Number examined	Parasite rate	12 months malaria slide Number positive	Number examined	Parasite rate
Iron dextran	"Normals"	5	16	31%	6	13	46%
	"Low Bart's"	1	18	6%	2	14	14%
	"High Bart's"	11	36	31%	11	31	35%
Placebo	"Normals"	0	12	0%	1	12	8%
	"Low Bart's"	1	15	7%	0	11	0%
	"High Bart's"	7	42	17%	10	35	29%
SPLEENS		6 months spleen Number palpable*	Number examined	Spleen rate	12 months spleen Number palpable*	Number examined	Spleen rate
Iron dextran	"Normals"	5	17	30%	9	12	75%
	"Low Bart's"	1	17	6%	6	12	50%
	"High Bart's"	13	36	36%	17	32	53%
Placebo	"Normals"	1	12	8%	3	11	27%
	"Low Bart's"	1	15	7%	6	11	55%
	"High Bart's"	10	44	23%	15	31	48%

* Hackett's grade 1 or greater

TABLE 2

Malaria slide positivity rate (6 and 12 month visits combined)

	"Normals" (Negative Bart's)	"Low Bart's" (Bart's <3.5%)	"High Bart's" (Bart's >3.5%)
Iron dextran			
At least one positive slide	9	3	16
Total infants	16	18	36
Placebo			
At least one positive slide	1	1	16
Total infants	14	15	43

Statistical analysis:

(a) "High Bart's" vs "Normals" + "Low Bart's" (placebo group only) : Fisher's exact test p = 0.006 (2 tailed)

(b) Iron dextran vs placebo ("Normals"): risk (odds ratio) (95% confidence limits) = 14.63 (1.55 - 138.2); Fishers exact test p = 0.011 (2 tailed)

(c) Iron dextran vs placebo ("Low Bart's" plus "High Bart's"): risk (odds ratio) (95% confidence limits) = 1.31 (0.59 - 2.90)

(d) All "Normals" vs "All Barts": risk (odds ratio) (95% limits) = 1.06 (0.73 - 1.53)

Comparison of risks (b) vs (c) significance: t = 1.99; df:112; p<0.05

similar trend is seen with spleen rates in the placebo group at 6 months (Table 1).

An increase in malarial positivity and spleen rates in infants who received iron dextran at 2 months has been described in Chapter 9 (Oppenheimer et al., 1986). The same effect was present in this subset of the larger trial. When categorized by Hb Bart's status at birth, there was a difference in the effect of iron dextran between the normal and thalassaemic groups: the relative risk (iron dextran vs. placebo) of having malaria at either 6 months or 12 months, or both, was greater in the normals (14.63) than in the thalassaemics (1.31) (Table 2). Thus the normals were more disadvantaged by iron treatment with respect to slide positivity than the thalassaemics. The difference in relative risk was significant ($p<0.05$) (see Discussion). No meaningful separate estimate could be made of the relative risk of iron dextran in the low "low Bart's" group because of small numbers of positives. A similar trend was seen in spleen rates at the 2 visits but was not significant.

Parasite species and density

Plasmodium falciparum and P.vivax were found in relative proportions of 77% and 23% respectively. There was no evidence of significant difference between these proportions in infants of the three Bart's categories, nor between parasite densities on positive slides in the three Bart's categories.

Effect of Hb Bart's %, malaria and iron on haemoglobin at 6 and 12 months.

In the analysis of the whole cohort a negative effect of malaria and a positive effect of iron prophylaxis on haemoglobins at the 6 and 12 months visits was seen (Chapter 9). At 16 months there was also an interaction between iron treatment and malaria, with the negative effect of malaria being greatest in the iron treatment group (Chapter

TABLE 3

Breakdown of means and 3-way analysis of variance of the effect of both Hb Bart's status and malaria on haemoglobins at 6 months

Haemoglobin g/dl at 6 months	Mean (SD)		
Malaria slide	"normals"	"low Bart's"	"high Bart's"
Positive	7.2(1.29) n = 5	8.8(0.0) n = 2	8.9(1.58) n = 18
Negative	9.8(1.45) n = 23	9.7(0.95) n = 31	9.44(1.02) n = 60

3 way analysis of variance of haemoglobin at 6 months by malaria, treatment and Bart's status

Source of variation	Mean square	df	F	Significance
1. Malaria	26.26	1	19.79	p< 0.0005
2. Injection group	19.27	1	14.53	p< 0.0005
3. Bart's group	0.20	2	0.15	p = 0.860
2-way interactions				
(1) vs (2)	0.32	1	0.25	p = 0.622
(1) vs (3)	6.98	2	5.26	p = 0.006
(2) vs (3)	0.35	2	0.26	p = 0.770
Residual	1.33	129		

Total variation explained: 26%

9). When the analysis was repeated for this subset in which the three categories of birth Hb Bart's were known, as a 3-way ANOVA, the malaria/iron interaction was replaced by a significant malaria/ thalassaemia interaction (Table 3). Malaria had a graded effect depending on Bart's category: the greatest difference in haemoglobin between malaria positive and negative cases was seen in the normals; lesser effects were seen in the "low Bart's", and even smaller effects in the "high Bart's" group (Table 3). No such interactive effect on haemoglobin was seen at the 12 months visit.

DISCUSSION

The geographical distribution of the thalassaemias has for a long time prompted speculation that they provide some selective advantage against malaria. Some evidence in support of this hypothesis has been advanced for the beta thalassaemia trait (Willcox et al., 1983a; 1983b; Siniscalco et al., 1966). Because of the relative difficulty in screening for the α^+ thalassaemias, it has only recently been recognised that in some populations they are very common. For example, in the South Pacific islands of Vanuatu α^+ thalassaemia is found at a gene frequency of 25% (Bowden et al., 1982 - Appendix V). In north coastal New Guinea, where malaria is meso-holoendemic (Cattani et al., 1983) a 68% gene frequency of a single α globin gene deletions, mainly of the 4.2 kb type is found. This abnormality appears to be absent from the neighbouring eastern highlands province (Chapter 7 and Flint et al., 1986 - Appendix V) where there is no regular malaria transmission (Peters et al., 1958). This large gene frequency difference provides circumstantial evidence for gene selection rather than genetic drift.

In this study malariometry and haematology were performed on infants at 6 and 12 months; 174 of these infants had a quantitative

assessment of haemoglobin Bart's made at birth, which is the best phenotypic method of screening for αthalassaemia.

The main finding was that the "high Bart's" group (homozygotes) had much higher malaria parasite rates than the "normals" and "low Bart's" (heterozygotes) groups both at 6 and 12 months. Spleen rates showed a similar trend at 6 months. Hence, α^+ thalassaemia homozygotes who, on the basis of the geographical distribution data, should be protected against malaria are more likely than the normals to show parasites on peripheral blood smears during the first 12 months of life. This finding might suggest a homozygote disadvantage, which would, however, be incompatible with the observation that, in this population with stable high malaria transmission, homozygotes apparently outnumber both heterozygotes and normal individuals (Chapter 7 and Flint et al., 1986 - Appendix V). One speculative explanation for this apparent paradox may be as follows. If one assumes equal rates of infection in each group, infected red cells in homozygous α^+ thalassaemics may sequester from the peripheral blood more slowly than in normals. This could be due to a slower maturation rate of parasites in red cells homozygous for α^+ thalassaemia, so that infected thalassaemic red cells spend longer in the peripheral circulation than non-thalassaemic infected cells. Hence, a homozygous α^+ thalassaemic might be more likely to show parasitaemia at any time than a normal, but should have less severe infections. This growth impairment model should be testable in vitro. There is some recent evidence for growth impairment in HbH disease which has a more severe globin chain defect than homozygous α^+ thalassaemia (Ifediba et al., 1985; Brockleman et al., 1986).

The observations on the effect of malaria on haemoglobin levels suggest that malarial infections are indeed least severe in the high Bart's group. The decrease in haemoglobin associated with malaria in

this group at 6 months was less than in the other 2 groups, the greatest effects being seen in the normals (Table 3). This suggests that, in terms of the morbid effects of malaria, the homozygotes may be relatively protected. The lethal effects of malaria in non-immunes are the end result of the uncontrolled geometric multiplication of parasites: detection of parasites in the peripheral blood is not in itself evidence of rapid multiplication, since immune populations may show high parasite rates. Hence, these observations, although on relatively small numbers, suggest that the protective effect of thalassaemia may act by altering the severity rather than the patency of infections by malaria. HbF levels at one year were lower in cases with Hb Barts at birth (see Chapter 7) so any potential protective advantage of this feature which may operate in β thalassaemia could not operate here.

Another finding of malariometry was that the association of iron prophylaxis with higher parasite rates (Oppenheimer et al., 1986; and Chapter 9) was more marked in the normals than the thalassaemics and the odds ratio was significantly greater than unity for the normal group alone (Tables 1 and 2). This finding validates the conclusion on the overall effect of iron therapy on malaria slide positivity rates (Chapter 9) but implies that the effect would be greater in a population with less thalassaemia. This interaction also provides circumstantial evidence supporting a hypothesis put forward in 1979 by Nurse (1979). He suggested that a common potential selective advantage of iron deficiency and the thalassaemias against malaria was the small poorly haemoglobinized red cells which occur in these conditions. A prediction of this hypothesis is that, in a population with mixed iron deficiency and thalassaemia, iron therapy which only reverses microcytosis due to iron deficiency would affect malaria rates more in the normals than in the thalassaemics.

In conclusion, the influence of α thalassaemia on parasite and spleen rates in infants living in a region of holoendemic malaria has been analysed. Remarkably, α^+ thalassaemia homozygotes had higher malarial parasite rates than the non-thalassaemics. However, the latter group appears to have malaria associated anaemia of greater severity, suggesting that α^+ thalassaemia may protect by reducing the severity rather than the patency of malarial infection.

This preliminary report is based on relatively small numbers and putative genotypes. A larger longitudinal study using genotypes based on DNA analysis of cord blood would clearly be needed to investigate clinical protection further.

CHAPTER 12.

G6PD AND PYRUVATE KINASE DEFICIENCY, OVALOCYTOSIS AND IRON AND MALARIA

INTRODUCTION

Genetic abnormalities found in the birth cohort were described in Chapter 7. The commonest abnormality was the presence of Hb Barts in cord blood (80%). Effects associated with haematology, iron and malariometry for this abnormality are discussed in Chapters 7 and 11. This chapter summarises findings associated with pyruvate kinase deficiency, G6PD deficiency and ovalocytosis. Also mentioned in this section are effects associated with HbF and HbA_2 levels at one year.

Cases and methods

Methods used for identification of these traits are described in Chapter 6. Criteria for inclusion in analysis are described below:

1) Pyruvate Kinase deficiency

Using a SIGMA Kit No. 205 a qualitative separation into "full" deficiency and "partial deficiency" is obtained. There was no sex association of the defect. 10/491 (2%) showed full deficiency and 20 (4%) showed partial deficiency. None of 58 babies of highland mothers tested, (including those from the Finisterre range), showed deficiency (Fishers exact for babies of highland mothers vs coastal: $p = 0.0396$; 2-tailed). 24 of those identified with some degree of deficiency subsequently entered the trial and were seen at least once during their first year.

2) Glucose-6-Phosphate dehydrogenase deficiency

Using SIGMA Kit. No. 202 a qualitative screen for full deficiency (17/431; 4%) was obtained (see Chapter 6). It is a much less reliable screen for partial (heterozygote) deficiency. None of 50 babies of highland mothers tested (including those from the Finisterre range) showed any deficiency. 13 males and 1

female with full deficiency were seen at one or more follow-up visits after the commencement of the trial. Deficiency noted at birth was always confirmed at follow-up in these cases.

Frequency of partial deficiency is a clear underestimate of the heterozygote trait (Chapter 7) and these cases are not analysed here.

3) Ovalocytosis

Ovalocytosis in Melanesia may constitute more than one polymorphism (Castelino et al., 1981) but is characterised by a benign high frequency (5-20%) abnormality probably inherited as an autosomal recessive (Amato & Booth, 1977). Characteristic morphological features apart from ovalocytes include stomatocytes, knizocytes, bacillary shapes and lack of rouleaux formation. Percentage of oval cells range from 20-90%. Amato and Booth suggest that although low percentages may represent variable penetrance, 50% should be the cut-off point for identification of the condition. Oval features may not be present at birth.

47/557 (8.4%) babies in the trial cohort showed some degree of ovalocytosis. Overall % ovals in each individual was slightly less at the 2 month visit than at 6 and 12 months visits, but results for the latter 2 visits were largely consistent for each individual. Distribution of mean % ovals (average of 2nd and 3rd visit) in the 47 cases was bimodal 22 being less than 50% (mode 20-30%) and 25 being over 50%. Those with over 50% ovals also consistently showed at least one of the features: absent rouleaux, knizocytes pencil cells and stomatocytes. In contrast those with % ovals less than 50% rarely showed any of these features. None of 64 babies of highland mothers tested

(including those from the Finisterre range) showed ovalocytosis. The highest rate detected was in Austronesians (7%).

Analysis of ovalocytes here includes only those 24 individuals with mean % ovals greater than 50%.

4) HbF levels at one year

Pasvol et al. (1977) have shown that raised HbF levels retard parasite growth in red cells. 241 estimations of HbF were performed at one year (see Chapter 7) at the same time as malariometry was performed. 5% of estimations showed HbF greater than 5%.

5) HbA_2 levels at one year

189 estimations of HbA_2% were performed at one year (see Chapter 7). 2 values were over 3.8%.

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As described in Chapter 6, full haematology and malariometry were performed at 2, 6 and 12 month visits for infants attending these visits.

RESULTS

Haematology

To determine any potential confounding effects (for the trial) on haematological variables associated with genetic abnormalities, 3-way analysis of variance was performed separately for all haematological variables (dependant) by injection group, by malaria slide, by presence or absence of the genetic trait (PK deficiency; G6PD deficiency; ovalocytosis). The only genetically associated haematological effects detected in this analysis were 1) at the 2 and 6 months visits ovalocytics had significantly lower haemoglobins: these differences after adjusting for the other explanatory factors were respectively: 1.05 g/dl ($F = 21.2$; d.f. 1; 416; $p<0.001$) at 2 months, and 0.74 g/dl ($F = 10.8$; d.f. 1; 409; $p=0.001$) at 6 months;

and 2). At 2 months, pyruvate kinase deficients had raised reticulocyte counts ($F = 10.4$; d.f. 1; 263; $p=0.001$). Haemoglobin levels in pyruvate kinase deficiency cases were marginally higher than non-deficients at 6 and 12 months but the differences were not significant.

Malariometry

Spleen: no significant effects on spleen size were associated with any of the genetic markers discussed in this section.

Malaria slide positivity

Pyruvate Kinase deficiency (PK)

There were no positive blood slides in 22 PK deficient cases examined at 2 months. The rate for known non-deficients was 13/371 (3.5%).

Slide positivity rates were lower for both full and partial PK deficiency cases both at 6 and at 12 month visits when compared with known non-deficient cases (Table 1). Differences were not significant either individually or when full and partial deficiency were combined or when 6 and 12 month visits were combined in the same infants to give the contingency: "Both visits malaria negative/at least one visit malaria positive". The group "iron dextran with any PK deficiency" had no malaria slides positive at any visit. This result was significantly less than expected given the rate of slide positivity in iron dextran non-deficients (Fishers exact test for 6 and 12 month infants visits combined: $p=0.046$; two-tailed) (Table 2).

G6PD Deficiency

Malaria slide positivity rates were similar at 2 and 6 months in fully G6PD deficient individuals when compared with non-deficient individuals and slightly higher at 12 months. The difference was not significant (Table 3). As with PK deficiency the parasite rates associated with G6PD deficiency in the iron dextran group were lower

TABLE 1

Malaria slide positivity rate by visit by injection group by pyruvate kinase status

Non-deficient

6/12

malaria slide	I	P	
+ve	30	23	53
-ve	140	159	299
	170	182	352
	18%	13%	15%

12/12

malaria slide	I	P	
+ve	52	33	85
-ve	100	133	233
	152	166	318
	34%	20%	27%

PK partially deficient

malaria slide	I	P	Total
+ve	0	1	1
-ve	7	7	14
Total	7	8	15
	0%	13%	7%

malaria slide	I	P	
+ve	0	1	1
-ve	6	4	10
	6	5	11
	0%	20%	9%

PK fully deficient

malaria slide	I	P	
+ve	0	1	1
-ve	4	3	7
	4	4	8
	0%	25%	13%

malaria slide	I	P	
+ve	0	1	1
-ve	3	2	5
	3	3	6
	0%	33%	17%

Part deficient and deficient combined

malaria slide	I	P	
+ve	0	2	2
-ve	11	10	21
	11	12	23
	0%	17%	9%

malaria slide	I	P	
+ve	0	2	2
-ve	9	6	15
	9	8	17
	0%	25%	12%

TABLE 2

Malaria slide positivity at 6 and 12 month visits combined (infants present at both visits: any positive vs both visits negative) in iron dextran group only, by pyruvate kinase status (any deficiency vs no deficiency).

		PK deficient	Non deficient	
malaria slide	+	0	102	102
	-	9	191	200
		9	293	302
	Rate	0%	35%	34%

Fishers exact test; P = 0.046; two-tailed

TABLE 3

Malaria slide positivity rate by visit by injection group by G6PD status

Normals

6/12

malaria slide	I	P	
+ve	27	23	50
-ve	126	156	282
	153	179	332
	18%	13%	15%

Odds ratio 1.45
(95% limit 1.0-1.75)

12/12

malaria slide	I	P	
+ve	40	32	72
-ve	99	132	231
	139	164	303
	29%	20%	24%

Odds ratio 1.667
(95% limit 1.44-1.93)

G6PD deficients

6/12

malaria slide	I	P	
+ve	1	1	2
-ve	8	4	12
	9	5	14

Odds ratio: 0.5

12/12

malaria slide	I	P	
+ve	2	2	4
-ve	4	2	6
	6	4	10

Odds ratio: 0.5

TABLE 4

Malaria slide positivity rates at 6 and 12 months by presence of ovalocytosis

6 months

		Ovalo cytic	Non ovalo cytic	
malaria slide	+ve	3	57	60
	-ve	21	323	344
	Total	24	380	404
	Rate	13%	15%	15%

Fishers exact: Not significant

12 months

		Ovalo cytic	Non ovalo cytic	
malaria slide	+ve	1	89	90
	-ve	23	236	259
	Total	24	325	349
	Rate	4%	27%	26%

Fishers exact test: P = 0.012; 2 tailed

than in iron dextran non-deficients at 6 and 12 months. This is reflected in the odds ratios (Table 3). Differences, however, were not significant at either 6 or 12 months or in 6 and 12 month visits combined.

Ovalocytosis

At 2 months there were no positive slides in 22 ovalocytic cases compared with 14/385(4%) of non ovalocytics.

At 6 months slide rates were similar in ovalocytics and non ovalocytics.

At 12 months slide rates were lower in ovalocytics than non ovalocytics. The difference was significant (Table 4). No interaction was seen with the risk of iron dextran administration.

HbF% - at one year.

Malaria slide positivity was not affected by HbF% at one year. There was no effect of HbF% at one year on ring density of malaria infection.

HbA_2%

In two cases where one year HbA_2% was greater than 3.8% blood slides were taken at 2, 6 and 12 months. One slide was positive at 12 months (light infection).

DISCUSSION

The reason in this study for screening for genetic anomalies was to detect and if necessary control for confounding effects associated with haematological and other variables. This meant that tests used (e.g. Sigma Kits) were for qualitative screening rather than accurate classification of genotypes.

All genetic anomalies detected were equally distributed between iron dextran and placebo groups (see Chapter 7); so in measuring the main effects associated with iron treatment, any independant confounding genetic effects should tend to cancel out. It is

therefore only in relation to inter-active effects that controlling for genetic anomalies is potentially important, and in practice this only becomes relevant statistically if the anomaly was very common as with α thalassaemia (see Chapter 11).

Of the traits discussed in this section, none had a frequency greater than 4% and only ovalocytosis was associated with any degree of anaemia (Hb 1.05 and 0.74 g/dl lower at the 2 and 6 month visits). The potential confounding effects on the main study are therefore likely to be small.

It is worthwhile, however, to speculate on the possible protective effects of these traits which may explain why they are present in frequencies not generally found in non-malarious areas. Interpretation of results should be guarded though, given the small numbers involved.

Pyruvate kinase deficiency has not been previously reported in Melanesians. In this study it was not found in babies of mothers from highlands regions where malaria is hypoendemic, suggesting malaria as a possible selective factor as in the case of α thalassaemia. Malaria slide positivity rates were in fact less in the PK deficient cases at field visits, however the difference was only significant if one looked at the iron dextran group separately.

No significant effects on slide positivity were associated with full G6PD deficient cases although again the risk associated with iron dextran was reduced (not significant). As in a previous study (Gorman & Kidson, 1962), G6PD deficiency was confined to the coastal population.

In vitro studies have demonstrated a resistance of melanesian type ovalocytes to merozoite invasion (Kidson et al., 1981) and this has been related to the selective suppression of blood group antigens (I^T) found in this condition (Booth et al., 1977).

Clinical studies have had more difficulty in demonstrating protection. Ovalocytics certainly contract malaria. Lower rates and densities of P.vivax and P.malariae were noted by Sergeantson et al. (1977) associated with ovalocytosis in the Madang area. However, the authors suggested that larger sample sizes would be necessary for more conclusive results.

Slide positivity rate in the present study was significantly lower in ovalocytics at the 12 month visit. However, again the low sample size indicates caution in interpretation.

Ovalocytosis rates observed by Sergeantson et al. (1977) in Austronesian populations were higher (12%) than in the present study (4%) which included only 27% Austronesians (Chapter 7) who had a rate of 7%. Again, babies of highlanders showed no ovalocytosis echoing previous studies.

Amato and Booth (1977) have stated that ovalocytosis found in Melanesia is not associated with significant anaemia. In the present study, younger infants had haemoglobins ¾ - 1 gm lower than normals although the effect disappeared at one year.

Little can be said in relation to HbF and HbA_2 levels at one year because of very small numbers; however, the rate of raised HbA_2 (1%) is rather lower than previously reported on the north coast of New Guinea (Curtain et al., 1965), however, numbers are too small to draw conclusions.

In conclusion, 3 inherited disorders of red cell metabolism (PK deficiency; G6PD deficiency; and ovalocytosis) have been found at rates of around 4% in the study population. These traits are absent from babies of highland extraction and may confer protection against malaria although no conclusive evidence is presented here.

Effects associated with these traits are unlikely to materially affect the conclusions of the main study.

CHAPTER 13.

SERUM AND RED CELL FOLATE LEVELS ASSOCIATED WITH MALARIAL PARASITAEMIA

INTRODUCTION

There is some doubt if human plasmodia can utilize exogenous folic acid (Ferone, 1977; Hurly, 1959). Findings are reported here suggesting an association between high red cell folates and malaria parasitaemia in man.

In the longitudinal study of morbidity in infants in north coastal New Guinea thick and thin blood films were examined for presence, density and species of malaria at scheduled visits at two, six and 12 months of age. Details of study design and numbers are described in Chapters 4 and 7. Serum and red cell folates were estimated on venous blood using an immuno-radiometric assay (Becton Dickinson SIMULTRAC). This test measures total immuno-reactive folic acid after adding ascorbic acid. Results are tabulated by visit and by blood slide (positive or negative) for malaria. Since both variables showed marked skew, geometric means (coefficient of variation) are shown. 't' tests were performed in the log transform.

RESULTS

The results (Table 1) indicate that total red cell folate (RBCF) was higher in infants with malaria-positive blood slides at all three visits. The effect was significant at less than the 0.1% level at one year and at the 7% and 9% levels at two and six months respectively (Table 1). Significant effects of the same magnitude were associated both with Plasmodium vivax and P.falciparum infections at one year. At six months P.falciparum alone was associated with a significant effect ($t=2.14$; $p=0.033$; d.f.:197) (Table 2). To test if the malaria/folate association was temporary or long lasting, red cell folate levels were compared at one year in infants with (a) a positive

TABLE I

Serum and red cell folate levels at field visits at 2, 6, and 12 months of age by malarial slide positivity

Age	Red Cell Folate* µg/l of packed cells				Serum Folate* µg/l			
	Malaria Blood Slide				Malaria Blood Slide			
	Positive	Negative	't'	p	Positive	Negative	't'	P
2 months	675.3 (9.3%) n = 10	468.5 (10.2%) n = 288	1.82	0.07	7.05 (13.6%) n = 10	7.93 (17.8%) n = 232	1.0	0.32
6 months	646.1 (9.5%) n = 31	546.4 (7.8%) n = 177	1.68	0.09	8.40 (22.1%) n = 27	8.50 (19.65) n = 163	0.13	0.89
12 months	728.3 (8.0%) n = 56	489.1 (8.3%) n = 187	5.05	<0.001	7.54 (10.6%) n = 55	7.52 (18.7%) n = 173	0.05	0.96
First Admission to Hospital	792.7 (9.9%) n = 20	579.7 (9.2%) n = 57	1.96	0.05	9.00 (22.3%) n = 19	9.00 (18.1%) n = 52	0.00	1.00

* All results are geometric mean (coefficient of variation % in log transform).

TABLE 2

Red cell folate levels at field visits at 6 and 12 months by malaria species (P.falciparum and P.vivax only)

				vs negatives	
Age	Slide result	Red cell* folate	n	t	p
6 months	Negative	546.4 (7.8%)	177	-	-
	P.falciparum	704.2 (10.2%)	21	2.14	0.033
	P.vivax	665.4 (5.0%)	7	1.55	0.165
	Mixed P.f P.v	329.8 (5.4%)	3	-1.77	0.079
12 months	Negative	489.1 (8.7%)	187	-	-
	P.falciparum	700.5 (8.2%)	40	3.98	<0.001
	P.vivax	730.5 (5.8%)	9	2.31	0.022
	Mixed P.f P.v	1026.1 (10.1%)	5	3.15	0.002

* All results are geometric mean (coeff. var. % in log transform).

slide at six months; (b) a negative slide at six months. Group (a) had a geometric mean RBCF of 655.4 µg/l and group (b) 498.1 µg/l at one year ($t=2.49$; $p<0.02$; d.f.:118). This effect remained even after excluding infants positive for malaria at one year from analysis ($t=2.1$; $p<0.05$; d.f.:90). Red cell folate levels did not correlate with treatment group after controlling for reticulocyte count.

Younger red cells in particular reticulocytes contain higher levels of folic acid (Hoffbrandt et al., 1966). Malaria may as a result of haemolysis reduce the mean age of the red cell population and increase reticulocyte percentages. To control for this, analysis of covariance was performed controlling for reticulocyte counts (as covariate). The results are shown in Table 3. Malaria slide positivity was still a significant term after controlling for log reticulocyte count at 12 months, although contribution to total variation by malaria was decreased from 10.6% to 5.3%. At the 6 month visit, log red cell folate correlated with log reticulocyte count ($r^2 = 4\%$; $p=0.004$) and there was no significant malaria effect.

It should be noted that using ranges established in the clinical laboratory where the tests were carried out (combined Dept. of Haematology, Royal Liverpool Hospital) only two of the 749 RBCF tests performed showed evidence of deficiency, mean values for all groups being high.

No effects on serum folate were associated with malaria positivity at field visits (Table 1). No serum folate measurements were below the normal range (<2 µg/l).

Similar results to the field visits were seen for a subset admitted during the study who also had folate assays. Red cell folate at first admission was higher in those with positive blood slides ($p=0.05$) and serum folate was unaffected by malaria. Both of these measures tended to be higher among admissions than in the field. When

TABLE 3

Analysis of variance of $\log_{10}$ red cell folate (dependant) controlling for reticulocyte count (logged as covariate) by malaria at 12 month visit

Source of variation	Sum of squares	d.f.	F	p
log Reticulocyte count (covariate)	0.875	1	17.67	<0.005
Malaria Slide positivity (main effect)	0.717	1	14.48	<0.005
Total	13.33	239		

Total variation explained = 12%

log reticulocyte count was controlled for, the association of red cell folate with malaria was no longer significant.

DISCUSSION

These results indicate an association between malaria slide positivity and high total red cell folates.

Most of the folate in red cells is in the form of N^5 methyltetrahydrofolate polyglutamates and it seems to be agreed that at least in this form it cannot be used by plasmodia (Ferone, 1977). Several studies have shown an apparent increase in total blood folate in malaria in animal cells (Reid & Friedkin, 1973); Trager, 1959). One of these (in vivo) showed an increase mainly in the proportion of oligoglutamated folic acid (Reid & Friedkin, 1973) and it was suggested that rather than an actual increase in total folate (implying parasite de novo synthesis) there was simply a shift to folate oligoglutamates or other breakdown products which may possiby be used by the parasite (Ferone, 1977; Reid & Friedkin, 1973).

It is difficult to see how in vivo results could be explained by de novo parasite folate synthesis since the proportion of cells actually infected in man is much lower than in culture situations. To show the relatively large differences seen in total RBC folate, uninfected red cells would need to acquire parasite folate during erythropoiesis (Izak et al, 1968) - a slow process. Also there is evidence of the effect being maintained over six months.

Another hypothesis could be that pre-existing high red cell folate favours parasite growth. However, this would suppose that only a small proportion of the excess of folate already present in uninfected cells is available for parasite metabolism. This is in fact supported by a recent report by Watkins et al (1985) which shows strong antagonism of sulphadoxine antimalarial activity by exogenous

folic acid and also suggests that parasites may be able to use only a small pool of intracellular folic acid oligoglutamate.

A third hypothesis is that the reticulocytosis resulting from malarial infection gave a young cell population with higher mean cell folates (Hoffbrand et al, 1966). Some evidence for this hypothesis is seen in the results of the analysis of variance at the 12 month visits (Table 3), where controlling for reticulocyte counts reduces (but does not abolish) the malaria effect. However, a red cell age effect would not adequately account for the apparent duration of the malaria effect (six months).

CHAPTER 14

DISCUSSION

Aims:

The main aims of the study (Chapter 3) were to investigate under controlled conditions the relationship of iron deficiency and iron supplementation to incidence, prevalence and severity of infection.

Introduction:

Detailed discussion of individual results appears at the end of each results section and in appendices. An overview of results in the context of the main study will be discussed in this section.

Design:

Much of the criticism levelled at previous clinical studies of iron deficiency, iron supplementation and infection was related to design, control and methods of recording. Extra emphasis has therefore been made on protocol (Chapter 6), choice and description of study area (Chapters 4 and 5), development and evaluation of laboratory methods (Appendix I) and comparative description of the cohort (Chapter 7).

Protocol:

The study was designed as a prospective randomised, double-blind placebo controlled trial of intramuscular iron administration to 2 month old infants. Mothers' knowledge of treatment type may be regarded as a breach of the double-blind, thus for the first 109 infants (22%) entering the trial before coded supplies became available, follow-up was single (observer) blind. Analysis of withdrawals showed no evidence of bias between treatment groups and single and double-blind periods were combined for analysis. Apart from this unforeseen problem the protocol was followed closely. Excluding the eight immediate post-injection migrations from the Madang area, 470 (98%) of the resulting trial cohort of 478 were

traced at one year; 439 (92%) were seen at the field check; 431 (90%) were seen at 6 months (412 with blood slide results); 392 (82%) were seen at the one year visit (367 with blood slide results). The proportion Iron dextran:Placebo seen and/or bled remained constant at each visit and the same as in the initial trial cohort (0.94-0.95) indicating no differential response.

Evidence for iron deficiency:

The other aspect of the study which could not be predicted but was implicit in the aims, was that the placebo group would become relatively and differentially iron deficient. Pilot studies in Madang (Chapter 5) indicated evidence of iron deficiency over 2 months of age, and a more detailed analysis of haemodynamic changes in the first four months of life showed a rapid fall of haemoglobin to subnormal levels by six weeks which could be related to low mean birthweight and rapid post-natal weight gain (Appendix III.i). A likely compounding contribution to low birth body iron in this population was low birth haemoglobin (Chapter 7). Transferrin saturation fell progressively to subnormal levels by three months in Madang infants (Appendix III.i).

Evaluation of iron status in the trial at the follow-up was to some extent confounded by the effect of malaria on haemoglobin, serum ferritin and transferrin saturation.

In the placebo group with negative blood slides, ferritin levels were low at follow-up. Mean MCHC was also significantly lower and there was significantly more hypoochromia and microcytosis noted in the placebo group. The best evidence however for iron deficiency in any population is the haemoglobin response to iron (Garby, 1970) and in this population at follow-up visits, the malaria slide negative infants of the placebo group had mean haemoglobins around a gram lower than the slide negative infants in the iron dextran group (Chapter 9).

The results of analysis of variance confirmed the significance of the effects of iron dextran on measures of iron status.

Maternal influence on birth iron status:

Chapter 8 makes an opportunistic analysis of maternal determinants of birth weight and birth haematological status in this cohort. Gestation, maternal weight, parity and maternal haemoglobin, all affected birth weight (in that order). Birth haematological variables appeared to be largely independant of maternal variables and the low birth haemoglobin levels found could not be related to any variable tested (including the Hb Barts level at birth - see Chapter 7).

Total dose iron infusion (TDI) to the mother was associated with higher neonatal ferritins but the small difference (5% of the log mean) was offset by lower haemoglobins. Thus in this cohort the main maternal influence on birth total body iron would appear to have been manifested through birth weight which is very low in this population (Chapter 8).

Iron in pregnancy: The effects noted in the mothers in relation to TDI were complex and must be interpreted with caution because of incomplete maternal information and lack of control inherent in an observational study. Risk (odds ratio) of slide positive post-natal malaria associated with TDI was 5.5 in primipara but close to unity (1.1) for multipara implying an increased susceptibility in primipara receiving TDI. This is a new finding. A possible confounder to this was that mothers contracting gestational malaria might have been more anaemic and therefore have received more TDI, but this risk should also have applied to multipara. Primipara who as adults should have solid immunity in a stable endemic malarious area appear to suffer a lapse of defence which is not seen in later pregnancies (Brabin, 1983; Macgregor et al., 1983). One intepretation of the findings in this study is that any malaria enhancing effect of iron treatment is more

likely to be manifest in those with lowered defences. In this context there is an interesting analogy with Harvey's study (1987) which found no effect of oral iron on malaria rates in Madang schoolchildren (who are likely by virtue of age to have better specific immunity to malaria than the infants followed in this study).

When booking haemoglobin was controlled for in an analysis of covariance of post-natal haemoglobin, TDI did not appear to be associated with any net haematological improvement for the mothers. The high rate of use of TDI in pregnancy noted in this study is mirrored in many other tropical countries and should be reviewed.

Discussion of effects of TDI on pregnant mothers leads on to an evaluation of the main results of the study.

Iron and Infection

Morbid effects associated with measures of iron status, iron treatment and infectious disease may be divided firstly into those directly attributable to malaria and secondly those due to other infectious aetiologies.

Malaria prevalence and incidence: Iron dextran injection was associated with 1) higher prevalence rates of malaria slide positivity and spleen enlargement at scheduled follow-up field visits four and ten months later (Chapter 9); and 2) higher incidence/risk of any admission with either malaria slide positive and/or spleen enlarged (Chapter 10). These were the most unequivocal results of the trial, and represent new findings since in previous observations increases in malaria rates were closely related in time to treatment (Murray et al., 1978; Masawe, 1974; Byles & D'Sa, 1970).

Birth haemoglobin was correlated with 1) slide positivity rate at field follow-up in the placebo group; and 2) risk of any admission with slide positive malaria and/or spleen enlarged. (Although the

latter effect was also seen for admissions as a whole). This again suggests a correlation between iron status and morbidity and is a new finding.

High serum ferritin and transferrin saturation noted in association with malaria slide positivity both at field visits and on admission to hospital (Chapter 9) cannot be taken as evidence that these levels predispose to malaria since they may have been secondary to malaria.

Malaria severity: The effect of iron dextran treatment on severity of malaria was difficult to assess.

Firstly apart from severe malarial anaemia (8 cases), severe malaria as defined on strict criteria (WHO, 1986), was not a feature of admissions from this cohort of infants, although a high proportion (of admissions) had some evidence of malarial infection. Of the 12 deaths (5 iron dextran), none could be directly attributed to malaria. No effect of iron dextran was detected on ring density of positive malarial infections either in hospital, or at the field visits. No cases of cerebral malaria were noted; five infants (3 iron dextran) had febrile convulsions thought to be secondary to malaria. 5 of the 8 cases of severe anaemia (Hb<5 g/dl) associated with malaria were from the iron dextran group (not significant). At the six month visit there was, however, a significiant interaction between the effect of malaria and iron dextran on haemoglobin levels on analysis of variance with the difference in haemoglobin between positives and negatives being greater in the iron group (indicating greater severity). This effect was inversely reflected in reticulocyte counts at this visit. No such effect was, however, seen at one year. Admissions showed no net beneficial effect of iron dextran treatment on haemoglobin levels, in contrast to the field results, possibly reflecting a more severe malarial effect in the treatment group (Chapter 9).

There was a significant trend to larger spleens at field follow-up in the iron dextran group which may also reflect severity.

* * * * * * * * *

The effect of iron dextran was thus more clearly related to prevalence or patency of malarial infections than to severity although this difference may only reflect methods of measurement.

The longevity of the iron dextran effect (up to 10 months), the differential effects noted in the placebo group, and the lack of hyperferraemia and iron overload at follow-up suggest that one is looking at effects related to variation in equilibrium iron status rather than iron treatment artefacts, although there still is the possibility of long term reticuloendothelial blockade by the dextran complex. It should be noted that no significant effects were detected at the one week field check where acute treatment effects were most likely.

Possible cellular mechanisms for the effects have been discussed in Chapter 9. Disorders related to merozooite invasion, schizont maturation, and red cell survival could all equally have accounted for the results observed, since all of these are likely to affect asexual multiplication and hence both patency and severity of infection.

Other Infections

Prevalence: Although the "infection score" tended to be higher in the iron dextran group at scheduled field visits the only significant difference noted in prevalence of individual disease categories was for severe lower respiratory infections (SLRI) at the 6 month visit (Chapter 10).

Incidence: Otitis media had a significantly higher incidence in the iron dextran group at self presenting outpatient visits while measles associated admissions were also significantly commoner with iron

dextran. Otherwise there were no significant differences in incidence of individual disease categories.

63% of all admissions had severe lower repiratory tract infections (SLRI). 69% of all infants admitted had one or more episodes of SLRI while 18% of the whole cohort had one or more admissions with SLRI making this the most important cause of severe infectious morbidity.

More infants were admitted for "any diagnosis" and "with SLRI" from the iron dextran group than from the placebo group. The respective risks associated with iron treatment were not significant, however they were in the opposite direction to the four previous longitudinal studies on iron supplementation and infectious morbidity in infancy in non-malarious areas (Mackay, 1928; Adnelman & Sered, 1966; Cantwell, 1972; Salmi et al., 1963).

Analysis of factors affecting risk of admission in the post-injection period showed a significant positive correlation with birth haemoglobin for "any admissions", "SLRI associated admissions" and "malaria associated admissions". Apparent positive interactive effects of birth haemoglobin associated with iron dextran injection, however, failed to reach 5% probability for all 3 subsets. Low weight-for-height 'Z' score at the start of the trial significantly increased risk for "any admission" and "SLRI associated admissions" (but not "malaria associated admissions"). Low weight-for-height infants receiving iron dextran had a further increased risk of admission. This interaction was significant for all 3 subsets. This may relate to a greater effect of the fixed dose of iron in thinner babies.

Mortality: There was no significant difference in mortality between the treatment groups: however numbers were small. The dominance of SLRI as the main cause of infant death with the lack of "severe

malaria" deaths is noteworthy in an area of high malaria transmission. Evidence of malaria in half of the deaths means that a contributing role cannot be ruled out.

Severity: Severity of infectious disease, particularly respiratory disease, is difficult to separate from incidence and prevalence of the more severe episodes. For instance, at the 6 month field visit, iron and placebo groups had nearly equal overall prevalence of respiratory tract infections (Table 2, Chapter 10), but in the iron dextran group more were severe. Admission to hospital is also an indication of severity; this risk has already been discussed under the heading of incidence.

Another approach to assessment of severity is to examine the period of hospitalisation. Iron dextran infants spent 60% more of the surveillance period in hospital with infectious disease than the placebo group (Chapter 10). Looked at in a slightly different way, taking only infants who were admitted one or more times, mean numbers of admissions were the same between treatment groups but average total stay in hospital was four days longer in the iron group ($p = 0.022$) indicating greater severity.

In terms of measurable cost to the child these effects of iron dextran are probably more important than malaria slide positivity rates and have not been noted before.

Interaction with malaria: While the finding of a very high rate of splenic enlargement in admissions including those with SLRI (a rate which was higher in the iron dextran group) (Table 4, Chapter 10) is not in itself direct evidence of a role of malaria in decreasing susceptibility to other infections, it is sufficient to raise that possibility and the further suggestion that the effects of iron dextran associated with non-malarial morbidity could have been mediated through a primary malaria effect. One should therefore

express caution before generalising the results of this study to non-malarious areas.

Confounding variables

Genetic abnormalities: Screening for genetically determined blood disorders was performed in the first place to detect and if necessary control for (in analysis) other causes of anaemia and potential confounders to the main study.

Apart from effects on haemoglobin levels, other potential confounding effects of genetic anomalies were: 1) iron overload; 2) increased morbidity; 3) decreased morbidity; and 4) interactive effects which could be synergistic or inhibitory.

Apart from a lower mean haemoglobin at 2 and 6 months in ovalocytics, no other main effects were detected on any haematological variables at follow-up in association with any of the five genetic anomalies found.

It should be noted that given the anomalies detected were equally randomised between treatment groups (Chapters 11 and 12), non-interactive effects on morbidity should have less relevance to interpretation of the results of the main study, than interactive effects which would become important if the anomaly was common.

Alpha thalassaemia detected as Hb Barts in cord blood was very common in the cohort (80% of those tested) which was a new finding. The rate in Papuan north coastal infants was 95%, which is the highest yet found in the world. If one compares overall slide positivity at field follow-up between those with and those without Hb Barts at birth there is no significant difference (Table 2, Chapter 11). Hb Barts status at birth was not associated with detectable significiant effects on risk of admission to hospital (Chapter 10). Haematological measures used in this study at follow-up also showed no detectable direct correlation with evidence of α^+ thalassaemia at birth.

On the other hand some evidence for interactive effects was found: 1) the depressive effect of malaria slide positivity on haemoglobin level was less at 6 months in those with Hb Barts at birth than in "Normals"; and 2) the risk (odds ratio) of malaria positivity at field visits associated with iron dextran was significantly less in those infants with Hb Barts detected at birth (1.31) than in "Normals" (14.63). The risk for "Normals" alone was thus not only significantly greater than unity but higher than that for the whole cohort. Given these results one would expect if anything to find a more pronounced iron treatment effect in a population with less α thalassaemia.

Examination of the other genetic anomalies, G6PD deficiency, pyruvate kinase deficiency, ovalocytosis and β thalassaemia trait (raised HbA_2 at one year) showed firstly relatively low rates of detection for any anomaly (<4%), secondly some evidence of lower parasite rates at follow-up in PK deficiency and ovalocytosis, and thirdly a possible negative interaction between PK deficiency and iron dextran treatment. Given 1) balanced randomisation; 2) the low rates for these anomalies; and 3) that main and interactive effects detected were negative, these anomalies are unlikely to seriously affect the overall interpretation of the results of the iron study. The higher than normal rate of pyruvate kinase deficiency observed in this population is a new finding and suggests that it may be worth investigating in relation to distribution of malaria.

Folate: Effects on red cell folate levels associated with malaria were potentially confounding, however no effect on red cell folate was detected in association with iron treatment.

Growth

No overall effect of iron dextran was detected in relation to growth in the first year of life, although malaria slide positivity was associated with significant negative effects on anthropometric

measurements (Appendix II). The lack of effect of iron on growth contrasts with findings by Judisch et al., (1966) and Tonkin et al., (1979).

CONCLUSIONS

Iron supplementation in the form of a single iron dextran intramuscular injection to 2 month old infants in a malarious coastal area of Papua New Guinea was associated with:

1) Higher mean haemoglobin levels at 6 and 12 month follow-up.

2) A higher prevalence of malaria in the first year of life as measured by spleen rates and blood slide parasite positivity.

3) A higher incidence of malaria associated admissions during the first year of life.

4) An interaction with the effect of malaria on haemoglobin levels implying increased severity in the iron group at six months.

5) Longer periods of hospitalisation with infectious disease during the first year of life.

Birth haemoglobin levels correlated with risk of admission with infectious disease for the whole cohort and with prevalence of malaria at follow-up in the placebo group.

Low weight-for-height at the start of the trial was associated with increased risk of admission with infectious disease.

RECOMMENDATIONS

I. Public Health*

a) Taking firstly the context of the study design, area and age group, iron dextran prophylaxis to infants in a malarious area should be contraindicated.

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* Full results of the study have been communicated to the Department of Health in Papua New Guinea.

Oral iron supplementation in the same age group in a malarious area may also be contraindicated. Preliminary results from Harvey et al., (1987) however, suggest that oral iron supplementation to older children in the Madang area may not be associated with adverse effects.

b) The treatment of anaemia at any age in malarious areas with total dose parenteral iron replacement (TDI) should be reviewed urgently. The practice should be contraindicated in infancy. The practice of TDI for anaemia in pregnancy was very common in Madang and may be contraindicated as it was not clearly associated with any benefit to the mother while some evidence of risk of malaria was seen in primipara. Modification of practice would need to take account of local factors and should preferably be based on controlled clinical studies.

c) Iron treatment for anaemia in a malarious area should be covered (preferably preceded) by effective antimalarial therapy.

d) Policies of standard treatment of anaemia at the primary health care level in malarious areas need to be reviewed with particular reference to age group and identifiable causes.

e) The results of the study cannot be generalised to older children and cannot be generalised to non-malarious areas where further study is needed.

II. Research

Clinical: The need for further controlled prospective clinical studies of iron supplementation in other age groups and in non-malarious areas has been mentioned.

Epidemiological and some clinical evidence was obtained that thalassaemia may protect against malaria in Melanesia. Further prospective study in this area would need accurately known genotypes and larger numbers.

Laboratory: In spite of *in vivo* animal evidence that iron deficiency protects against malaria, *in vitro* work has failed to provide a convincing mechanism. Further work is needed to confirm the presence or absence of functional transferrin receptors on parasitised red cells and to confirm the presence or absence of an utilisable iron source within the red cell; the importance of marrow reticulocyte response to the multiplication of different species of *plasmodium* should be testable *in vivo*; the relevance of red cell size and/or globin content to parasite invasion or growth should also lend itself with suitable controls to *in vitro* study.

APPENDIX I.

DEVELOPMENT AND EVALUATION OF CLINICAL AND LABORATORY METHODS FOR THE STUDY

Appendix I.i. LABORATORY EVALUATION OF MINIATURE PORTABLE APPARATUS FOR ESTIMATION OF RED CELL INDICES FOR USE IN THE FIELD

Two miniature pieces of equipment for the measurement of red cell indices have recently been marketed by Miles Laboratories Ltd. (Ames Division), Slough. They were primarily intended for use in operating theatres and ward side rooms. It was decided to examine their potential for field work.

The COMPUR M1000 miniphotometer (weight 340 g) measures haemoglobin by a modified cyanmethaemoglobin method (Drabkin and Austin, 1932), using a 5 µl capillary sample. Red cell counts are determined on the same instrument by nephalometry of a red cell suspension in Gower's solution. The COMPUR M1100 minicentrifuge (weight 845 g) is essentially a battery-operated microcentrifuge which uses 9 µl capillaries with a direct PCV read out (see Fig. 6).

This study compares the results obtained by using these two instruments on a range of normal and abnormal bloods (n = 34), with results obtained with the same specimens using a Coulter 'S' electronic counter.

The correlations and corresponding regression equations obtained are indicated in Figures 1-5. Figure 1 shows a high degree of correlation of Hb estimation over a wide range of values (r = 0.997). Figure 2 shows a high degree of correlation between physical (COMPUR M1100) and electronic (Coulter 'S') methods of estimation of PCV (r = 0.974). PCV tended to be slightly overestimated using COMPUR M1100 in the microcytic bloods. Figure 3 shows results obtained for RBC by the

two methods. In normocytic cases there was good correlation (r = 0.970). In microcytic cases COMPUR M1000 underestimated RBC and vice versa in macrocytic cases. Figure 4 indicates the correlation of the derived values of MCHC obtained by both methods (r = 0.910). The slope and intercept indicate that the physical method tends to exaggerate the degree of hypochromia when present (see England et al., 1972). Using the accepted lower limit of 30g/dl (Dacie and Lewis, 1975) for MCHC by physical methods, the eight points below this level on Figure 4 include seven out of eight cases with an MCV less than 76 fl and one normocytic case. Figure 5 shows the derived MCV by both methods. Overall, there was no significant correlation, the underestimation of the RBC in microcytosis and overestimation in macrocytosis tending to cause inaccuracy of MCV by the COMPUR method.

On replicate testing (n = 11) of the COMPUR system the following reproducibility was found: Hb, mean 15.5 g/dl, SD = $\pm$ 0.20; RBC, mean 4.96 x $10^{12/l}$, SD = $\pm$ 0.15; PCV, mean 45.9%, SD = $\pm$ 0.20. These values are well within the limits of acceptable precision (Dacie and Lewis, 1975).

The results obtained for Hb and PCV were well within acceptable limits of accuracy of conventional bench methods (Dacie and Lewis, 1975). The RBC count was accurate by COMPUR 1000 only when using normocytic blood (as stated by suppliers). The MCV showed no significant correlation with the Coulter 'S' values. The MCHC by the "COMPUR" method correlated well with the Coulter 'S', and also demonstrated the effect, previously reported by England et al., (1972), that increased plasma trapping in microcytic blood exaggerates the fall in MCHC in microcytic anaemias when measured by physical methods. Using the accepted lower limits of 30 g/dl for MCHC by physical methods (Dacie and Lewis, 1975), the COMPUR method showed a

high specificity (96.3%) in predicting severe microcytosis (MCV < 76 fl by Coulter 'S').

In conclusion, the COMPUR M1000 and COMPUR M1100 provide an accurate portable haematological survey kit suitable for the estimation of Hb, PCV and MCHC but not for RBC or MCV, as inaccuracies caused by abnormal red cell size cannot be predicted in the field.

ACKNOWLEDGEMENTS

The author wishes to thank Professor A.J. Bellingham, Mr. C. Strange and the combined Department of Haematology, Royal Liverpool Hospital, who made the Coulter 'S' and routine blood specimens available.

Figures 1-5. Correlation of red cell indices as estimated by COMPUR M1000 and M1100 equipment against Coulter 'S' electronic counter on 34 samples of blood [(●) 100 fl > MCV > 80 fl; (▼) MCV < 80 fl; (■) MCV > 100 fl].

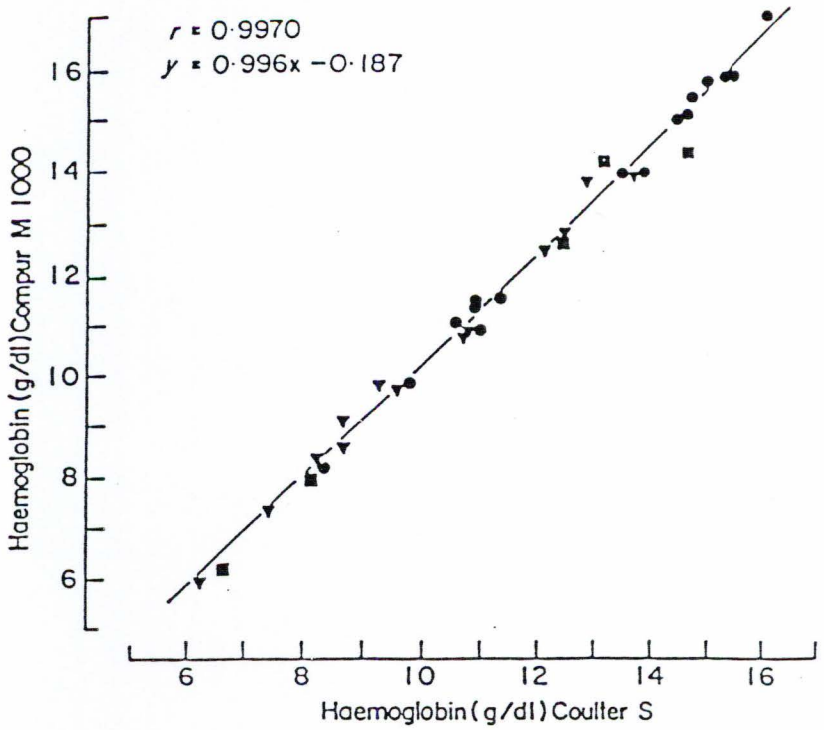


Figure 1 Correlation of haemoglobin estimated using COMPUR M1000 and Coulter 'S'.

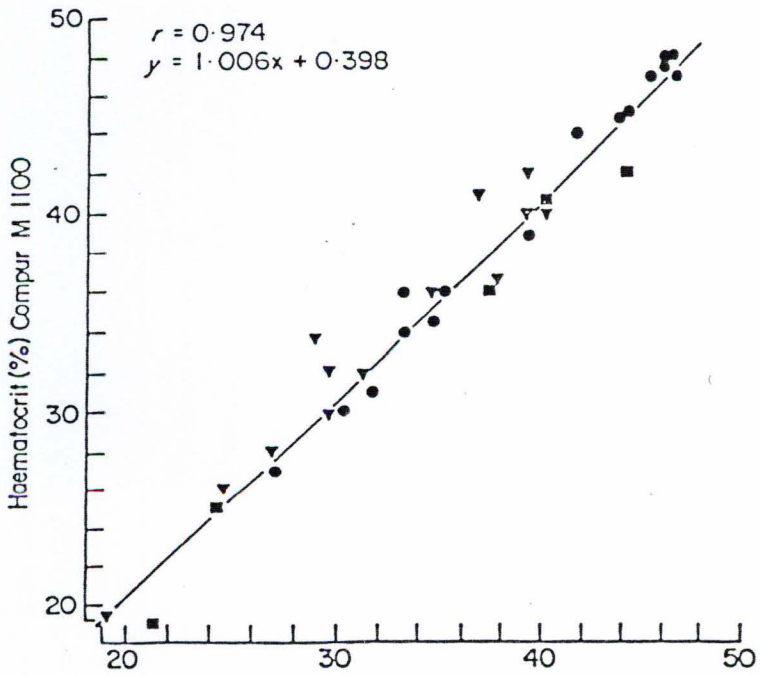


Figure 2 Correlation of haematocrit (PCV%) using COMPUR M1100 and Coulter 'S'.

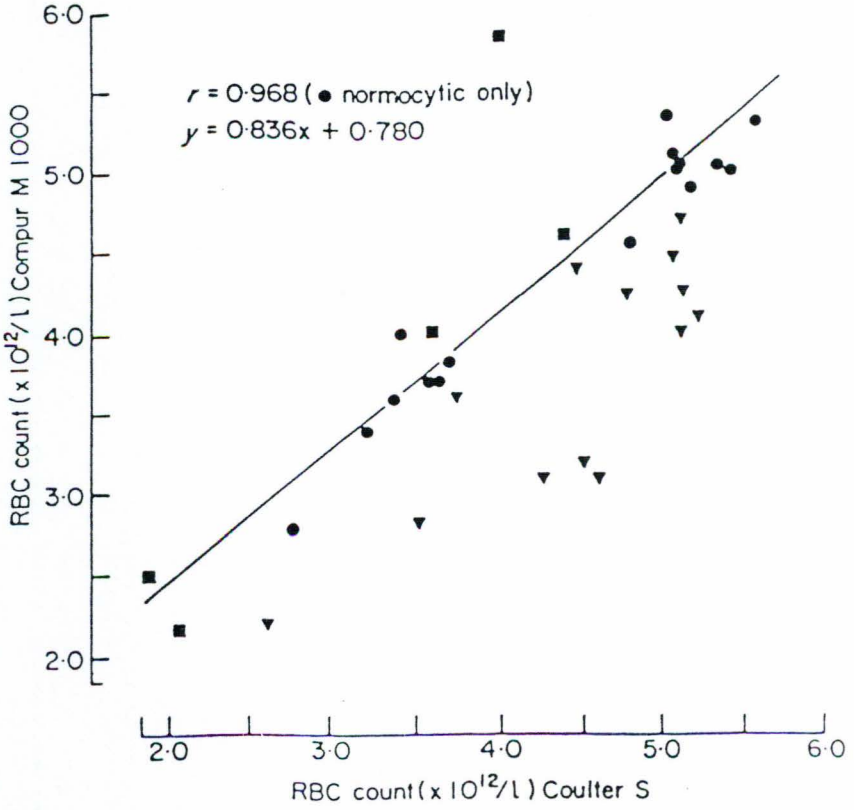


Figure 3 Correlation of RBC count using a COMPUR M1000 and Coulter 'S' (Regression for normocytic cases only - see Fig. 5).

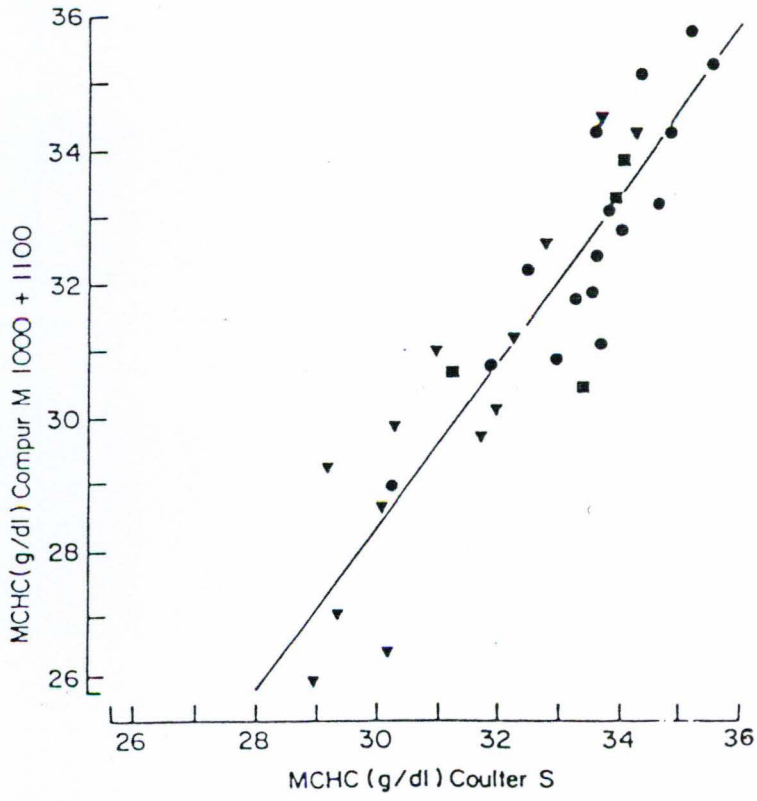


Figure 4 Scattergram of MCHC derived from COMPUR M1000 and M1100 and Coulter 'S'.

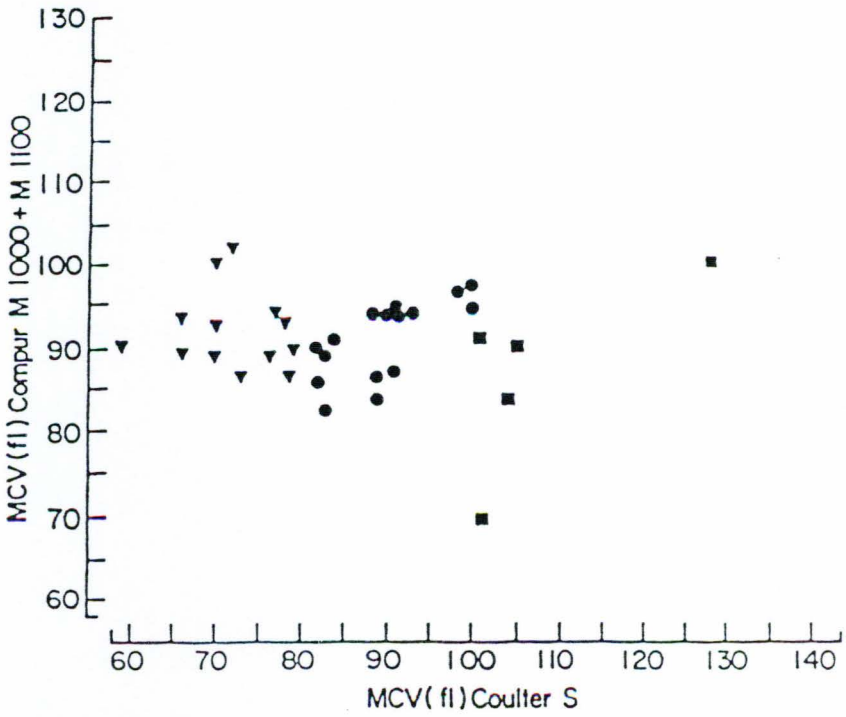


Figure 5 Scattergram of MCV derived from COMPUR M1000 and M1100 and Coulter 'S'

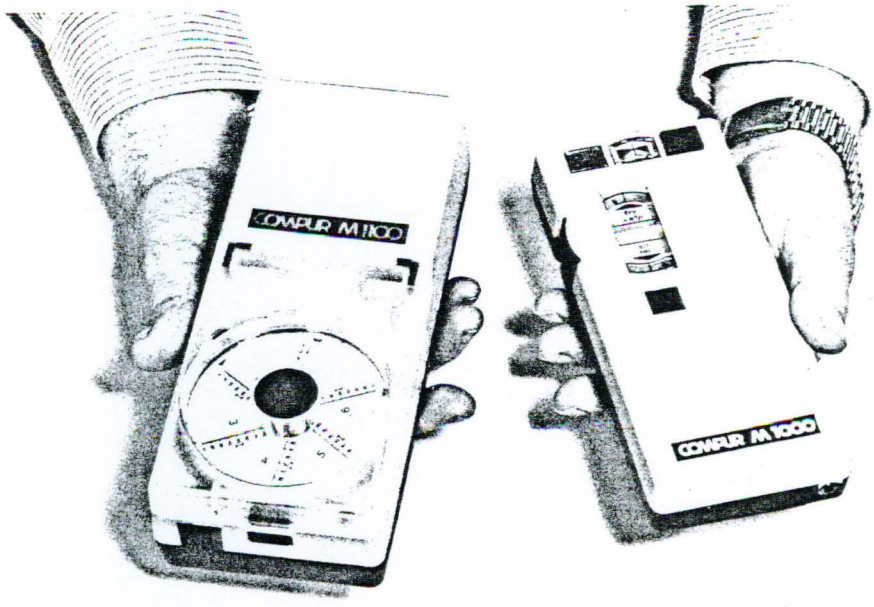


Figure 6 The minicentrifuge and miniphotometer

Appendix I.ii. DEVELOPMENT OF A MICRO METHOD FOR THE DETERMINATION OF SERUM IRON AND IRON-BINDING CAPACITY (UTILISING FERROZINE)

SUMMARY

A spectrophotometric method for the determination of serum iron and TIBC is described which requires only 75 µl of serum. The assay is comparable with conventional methods as regards sensitivity, reproducibility and accuracy.

INTRODUCTION

Conventional methods of serum iron and total iron binding capacity measurement require relatively large sample volumes. When venepuncture is not possible or desirable, for example in screening procedures, in paediatric practice, or as field studies in Madang, an assay on the much smaller sample for instance obtained by skin stick is needed. A method for determining serum iron and TIBC requiring only 75 µl of serum is described. This has been developed from the procedures which utilize the iron complexing properties of ferrozine, a sulphonated derivative of phenyltriazine (Carter, 1971; Persijn et al, 1971). Control sera of known iron and TIBC levels from two different sources were used to validate the accuracy and reliability of this adaptation.

METHODS

Assay Principle (Persijn et al., 1971)

1. Total serum iron determination

Transferrin bound serum iron dissociates to form ferrous ions at acid pH and in the presence of a suitable reducing agent. When reacted with ferrozine, the ferrous ions form a magenta coloured complex with an absorption maximum near 560 nm. The difference in colour intensity at 560 nm before and after addition of ferrozine is proportional to the serum iron concentration.

2. Serum unsaturated iron-binding capacity determination (UIBC)

At a slightly alkaline pH, ferrous ions when added to serum bind specifically with transferrin at the unsaturated iron binding sites. The remaining unbound ferrous ions will react with ferrozine to form a magenta coloured complex absorbing maximally at 560 nm. The difference between the residual unbound iron and the total amount added is equivalent to the quantity bound by transferrin and represents the UIBC.

3. Serum total iron-binding capacity (TIBC) calculation

The TIBC is the sum of measured total iron plus the UIBC.

Reagents and equipment

Iron and Total Iron Binding Capacity kit, number 565 (Sigma Chemical Company Ltd, Poole, UK).

Spectro Plus Spectrophotometer (MSE Scientific Instruments Ltd., Crawley, UK).

Water Bath at 37°C.

Micro-cuvettes, 0.9 ml total volume, 10 mm light path, disposable (W. Sarstedt Ltd., Leicester, UK).

"Ferrifree" Iron free detergent (J.T. Baker, Mast Laboratories, Liverpool, UK). (Used to wash all glassware and reusable cuvettes).

Control Sera (RIA Products Inc., Washington, Tyne & Wear, UK and Wellcome Reagents Ltd., Dartford, UK).

Method for Micro-Assay

1. Total serum iron

A 50 µl sample of serum or iron standard solution (5 µg/ml) was added to 250 µl Iron Buffer Reagent in a micro-cuvette and mixed thoroughly. The optical density, (OD_1) of the solution read against a water blank at 560 nm was noted. 10 µl of the Color Reagent was added and after mixing, the sample and standard

cuvettes were incubated at 37°C for 10 mins. The optical density was again noted, (OD_2). The change in optical density, OD, was calculated. The concentration of iron present in the sample was calculated from the formula:

$$\left(\frac{\text{OD sample}}{\text{OD standard}}\right) \times 500 = \text{iron concentration ug/dl}$$

2. The unsaturated iron-binding capacity

The Unsaturated Iron-Binding Capacity (UIBC) of the unknown sample was estimated by adding 25 µl sample, 50 µl Iron Standard and 25 ul water to 200 µl UIBC Buffer Reagent in a micro-cuvette. The reference cuvette contained 75 µl Iron Standard, 25 µl water and 200 µl UIBC Buffer Reagent. The contents of the cuvettes were mixed thoroughly and the optical density at 560 nm noted, (OD_1). 10 µl of Color Reagent was added to each cuvette then incubated as before and the OD_2 noted. The OD was calculated. The UIBC was calculated as below:

$$500 - \left(\frac{\text{OD sample}}{\text{OD reference}}\right) \times 500 = \text{UIBC µg/dl}$$

3. Total iron binding capacity (TIBC)

TIBC µg/dl = Total Iron µg/dl + UIBC µg/dl

Method for macro assay

This was carried out essentially as for the micro-assay except that the volumes of reagents and samples added to the cuvette were as follows:

1. Total iron assay: 0.5 ml sample or iron standard solution was added to 2.5 mls Iron Buffer Reagent. 50 µl of the Iron Color Reagent was added to each cuvette. Total volume = 3.05 mls.

2. UIBC: 0.5 ml sample or water was added to 0.5 ml iron standard solution and 2.0 mls UIBC buffer, followed by 50 µl Iron Color Reagent. Total volume = 3.05 mls.

RESULTS

The following validity studies were carried out in order to test this adaptation of the method.

1. Comparison with the full scale assay was performed using sera of previously unknown iron and UIBC concentrations. Sera from different subjects were assayed in duplicate by each method on three separate days. The relative precision of the two methods was compared by considering the ratio (between the methods) of the variance between duplicate estimates for each method. This ratio was close to unity for both

   Iron $\frac{\text{var micro}}{\text{var macro}} = \frac{96.0}{82.3} = 1.17$ (df = 20,20; $p>0.1$)

   and UIBC $\frac{\text{var macro}}{\text{var micro}} = \frac{73.7}{69.3} = 1.06$ (df = 16,20; $p>0.1$)

   Estimates of UIBC differed slightly between the methods with the micro method yielding higher values at the lower end of the range and lower values at the upper end. Further analysis of these results suggested that this was likely to be due to an increase in variation of the pH values in the micro test (see Section 2) rather than to inaccuracies due to scaling down of the assay. No such effect was noted in the comparisons of the micro- and macro-iron assay.

2. Because of the known sensititivy of both the serum iron and the UIBC determination to pH, the pH in the cuvette during both the micro and macro assays was measured using à micro-electrode. 17 paired pH measurements were made for the iron assay and 16 for UIBC. The micro method for UIBC involved the addition of a disproportionately large volume of acidic iron standard solution compared with the macro-assay and although the resulting mean pH differences were not significant, the variance in the case of the micro-assay was greater (Table).

Table

Comparison between the pH of Serum Iron and UIBC in the micro- and macro-assay

	i) Micro-assay pH value			ii) Macro-assay pH value		
	Mean	SD	n	Mean	SD	n
Serum Iron	4.482	0.035	17	4.522	0.033	17
UIBC	6.938	0.291	16	7.085	0.015	16

3. The accuracy and reproducibility of the micro method was examined by performing multiple determinations of the iron concentration and UIBC (and calculation of the TIBC), on 14 different quality control sera of given mean iron and TIBC levels. A total of 270 estimations of iron concentration and 138 estimations of TIBC were made and a regression curve of the mean values obtained for each control serum + 2SE against the given mean was plotted for the serum iron and TIBC results. The correlation coefficient, r for serum iron was 0.9994 and for TIBC 0.9947 (Figs. 1 and 2).

4. Within and between assay variation was examined using quality control sera. When n = 12 for serum iron, the mean, (SD; coef. var.) values obtained were 29.71 µmol/l (1.19; 4%) and for TIBC, mean 63.12 µmol/l (4.59; 7%).

5. The linearity and sensitivity of the micro-assay for serum iron was tested using a serially diluted standard iron solution. Results were plotted as the measured iron concentration against the expected value. The lowest concentration of iron which could be accurately measured was found to be equivalent to 2.27 µmol/l (Fig. 3).

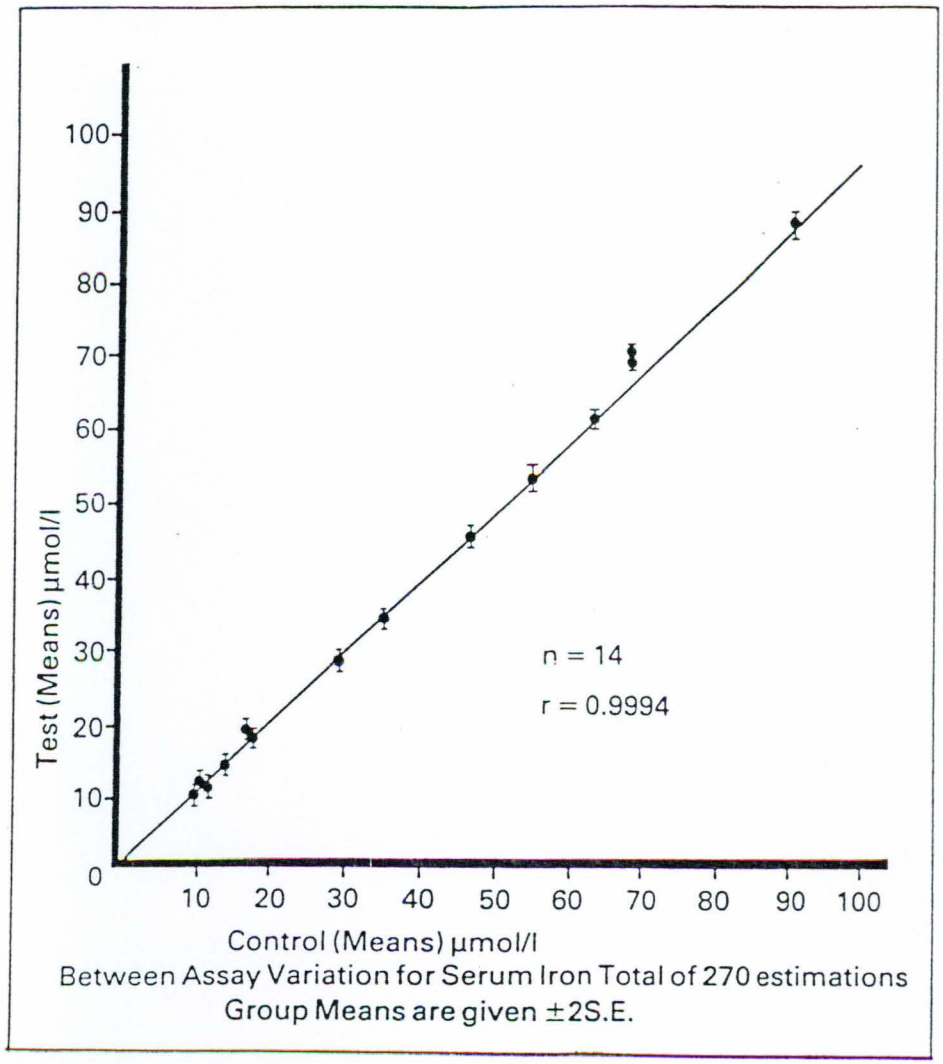


Figure 1 The accuracy and reproducibility of the microassay for serum iron were determined by multiple analyses of 14 different quality control sera of given means for serum iron (totalling 270 estimations).

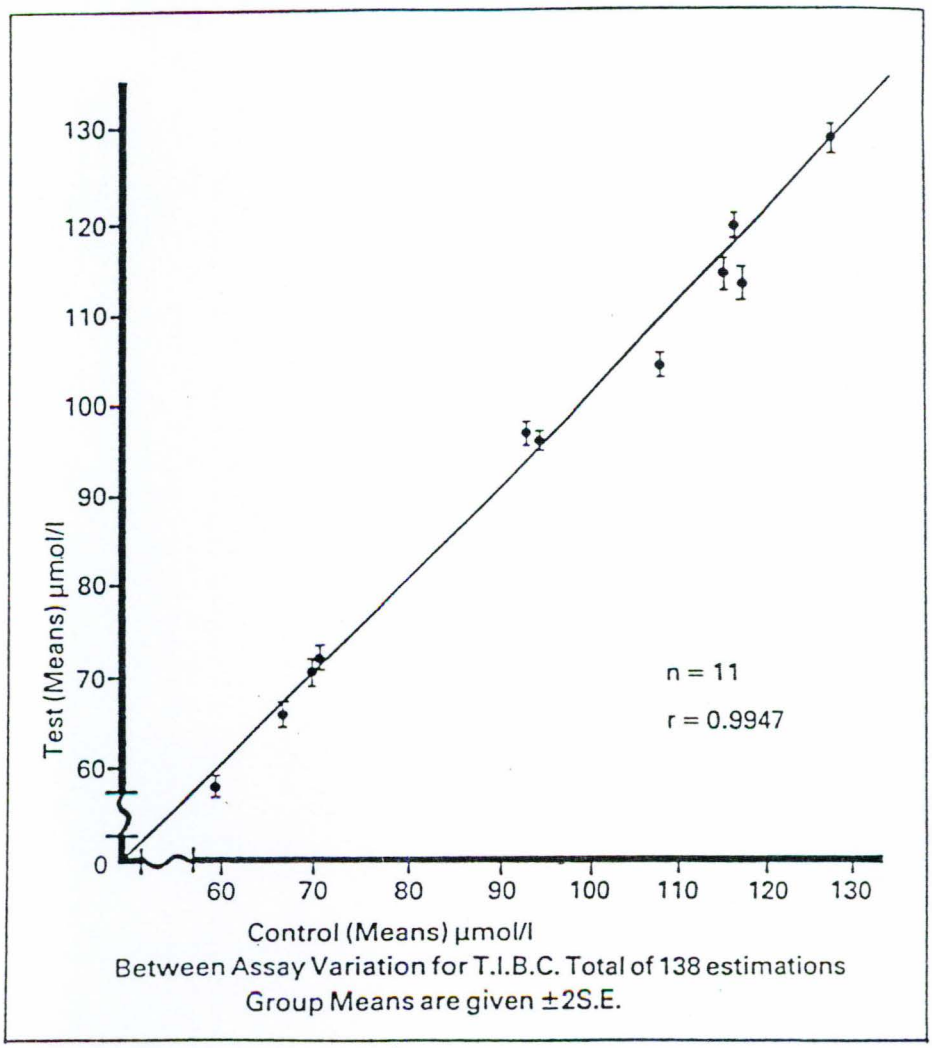


Figure 2 The accuracy and reproducibility of the microassay for TIBC were determined by multiple analyses of 11 different quality control sera of given means for TIBC (totalling 138 estimations).

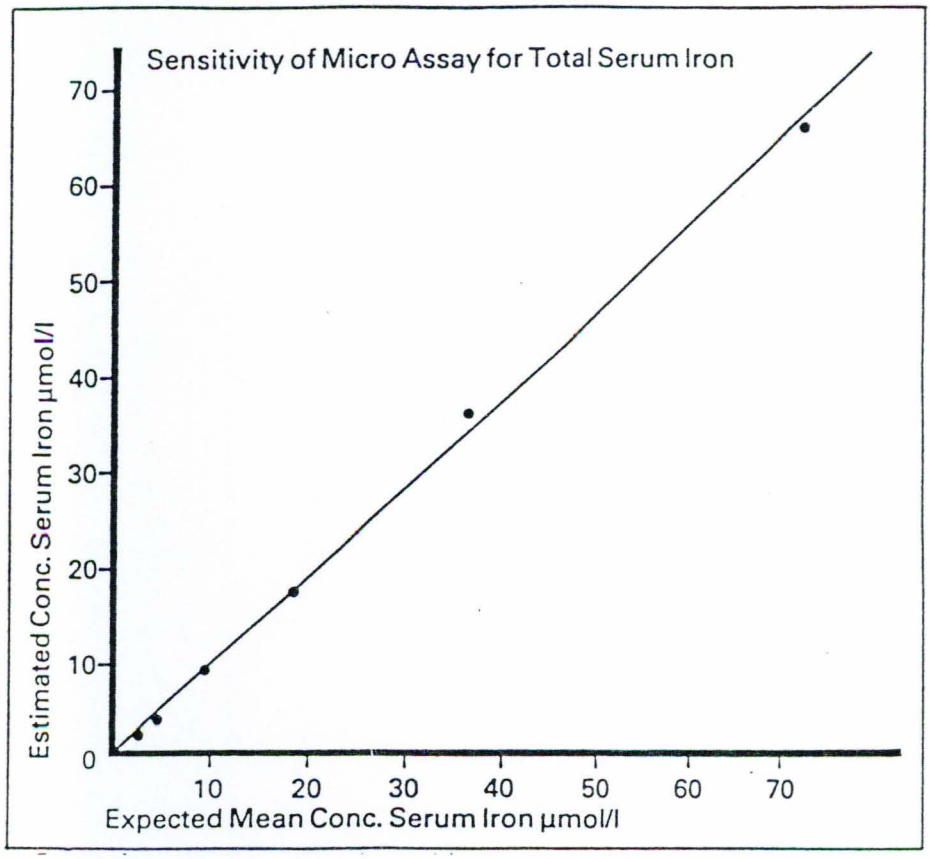


Figure 3 The linearity and sensitivity of the microassay for serum iron were tested by serial dilution of a standard iron solution.

DISCUSSION

This validation study of the micro adaptation has shown that the proposed alterations to the established method have not led to any loss in sensitivity or reproducibility of the assay and that good correlation is obtained between the micro and full scale assay methods. An increase in variance of TIBC estimation by the micro-assay appeared to be related to the pH differences resulting from the addition of the disproportionately high volume of iron standard. Precision of the iron assay was not affected by scaling. The micro-method was found appropriate for use in the Madang field study but is equally applicable to paediatric practice where small sample volumes are preferable or small numbers of tests preclude the use of auto-analysers. The saving in reagent cost is appreciable.

ACKNOWLEDGEMENTS

The author would like to thank Mrs. Thelma Williams (Senior Technician, Department of Tropical Paediatrics, Liverpool School of Tropical Medicine) who had the idea for the miniaturisation of the Sigma Test for use in the Madang field study and designed the test. The author carried out the bench work for sections 1 and 2 of results while in Liverpool. Ms. Cathy Harrison (Senior Technician, Department of Tropical Paediatrics, Liverpool School of Tropical Medicine) carried out all the assays on the field samples and quality control sera (sections 3-5). Tony Fulford and Barry Moody (Department of Tropical Paediatrics, Liverpool School of Tropical Medicine) advised on the statistics.

Appendix I.iii. DEVELOPMENT OF A SOLID-PHASE ENZYME-LABELLED IMMUNOSORBENT ASSAY FOR SERUM FERRITIN: EVALUATION OF METHOD AND COMPARISON WITH AN IMMUNORADIOMETRIC ASSAY

SUMMARY

An enzyme-linked immunosorbent assay is described for the measurement of serum ferritin. The assay utilizes small volumes (10-20 µl) suitable for capillary samples and is comparable with the conventional immunoradiometric assay with respect to sensitivity and reproducibility.

INTRODUCTION

Ferritin is the iron-storage protein of the body and is present in all tissues. It has been established that the measurement of serum ferritin provides an important contribution to the estimation of the body's iron stores (Munro & Linder, 1978; Boemisch, 1976).

Radioimmunoassay (RIA) and immunoradiometric assay (IRMA) techniques are now available for detecting serum ferritin, and provide sensitive methods for analysis of this compound. However, compounds labelled with gamma-emitting isotopes have a relatively short shelf-life, are expensive, present a health hazard and are not always suitable for the routine clinical laboratory. Enzyme conjugates provide an attractive alternative to isotopic labelling and the introduction of the enzyme-linked immunosorbent assay (ELISA) has brought many analyses within reach of any laboratory that can make spectrophotometric measurements.

An ELISA is described for serum ferritin, using commercially available reagents, which is not only sensitive and reliable, but requires a very small sample size.

Apparatus and Materials

Rabbit anti-human ferritin (IgG fraction) and peroxidase conjugated to human ferritin were obtained from DAKO Diagnostics Ltd.,

Bucks. 2,2' Azino-di-[3 ethylbenzthiazoline sulfonate (6)] (ABTS) and bovine serum albumin Fraction V were obtained from Boehringer Corp (London) Ltd., Sussex. All other chemicals (Analar grade) were obtained from BDH Chemicals Ltd., Poole. The following working solutions were prepared: phosphate buffered saline (PBS), pH 7.4, 40mM, 0.5M sodium chloride bovine serum albumin (BSA), 20g/L in PBS, working substrate, containing 9.5ml citrate buffer pH 4.0, 100mM, 0.04ml hydrogen peroxide, 30% w/v, and 0.5ml aqueous ABTS solution, 20g/L freshly prepared.

Consecutive routine clinical serum specimens were analysed by an IRMA assay (Becton Dickenson) by the Combined Haematology Department, the Royal Liverpool Hospital, Liverpool, then repeated by this ELISA technique for comparison.

All ferritin control sera (RIA(UK) Ltd., Washington, Tyne and Wear) and serum specimens were diluted with heat inactivated normal rabbit serum (Flow Laboratories Ltd., Ayreshire) 10% w/v in PBS buffer.

The reaction was carried out using Immulon M129A flat-bottomed Microelisa plates and the resultant absorption measured using a Microelisa Auto Reader MR580, which were obtained from Dynatech Laboratories Limited, Sussex.

Method

1. Preparation of antibody-coated microelisa plates

0.2 mls of coating antiserum (diluted 1:1968 in PBS buffer) were added to each well and incubated at room temperature for 1½ hrs. The plates were aspirated using a water vacuum pump and washed 7 times with PBS using a wash-bottle to fill each well. Excess moisture was removed by blotting the plates, which were then allowed to dry in air. Plates were stored at 4°C for up to one month.

2. Assay procedure

Plates were pre-coated with 0.2ml BSA. buffer (extreme outside wells were not used due to occasional distortion of wells during manufacture) and incubated at room temperature for 30 minutes. They were emptied by inversion (no need to aspirate) and blotted dry.

0.1 ml sample, standard or control, was added to each well at timed intervals and the plates were incubated at 37°C for 2 hours. Each well was aspirated after exactly 2 hours, then washed 6 times with BSA buffer followed by a final wash with PBS buffer to avoid bubbles forming before blotting dry.

0.1 ml peroxidase-conjugate (diluted 1:1000 in BSA buffer) was added to each well at timed intervals and incubated at room temperature for 2 hours. The wells were aspirated after exactly 2 hours, washed 7 times with PBS buffer and blotted dry.

0.1 ml of working substrate was added (as quickly as possible) to each well. Then the plate was incubated at room temperature for 1 hour, following which it was gently agitated and the absorbance read using the Microelisa Auto Reader. Dual wavelengths of 405 nm and 630 nm were used, to eliminate optical variation not specific to the antigen-antibody reaction. Plates were zeroed on two wells containing only citrate buffer. A set of standards was included on each plate to produce a standard curve, from which the concentrations of ferritin in the sera were calculated. Two control sera were also assayed on each plate.

Analyses Performed to Obtain Optimal Assay Conditions

i) Antibody coating concentration, time and temperature

The concentration of antiserum required to occupy the sites on the plate was determined by obtaining standard curves from data on wells coated with concentrations varying from 1:10 to 1:2000. An

excessive number of available binding sites may result in a loss of effective sites, due to the so-called 'carpet' effect (Worwood, 1980). The optimum concentration was obtained at a dilution of 1:1968.

Several plates were coated for different lengths of time ranging from 30 minutes to 17 hours and at 3 different temperatures: 4°C, room temperature and 37°C. Optimum conditions were obtained at room temperature for 90 minutes. These conditions were adopted for all subsequent assays.

ii) Pre-assay coating conditions

To prevent non-specific binding of antigen to the antibody coated plates, assays were performed with and without pre-assay coating with BSA buffer. Optimum conditions were obtained with the BSA, as without the pre-assay coating an unacceptable level of variation was found.

Several plates were assayed using the pre-assay coating buffer (BSA) at times varying from 15 - 90 minutes and at 4°C, room temperature and 37°C. The best conditions were found to be at room temperature for 30 minutes. These conditions were then adopted for all subsequent assays.

iii) Sample dilution and incubation

Standards were assayed at dilutions ranging from concentrated to 1:200 and in varying proportions of diluent from PBS alone to 20% NRS in PBS and varying incubation conditions of time and temperature. It was found that there appeared to be a 'cushioning' effect with undiluted samples being under-estimated. The best conditions were obtained with 10% NRS in PBS and at a sample dilution of 1:19 incubated for 2 hours at 37°C. Subsequently, these conditions were adopted for all assays.

iv) Washing procedures after sample incubation

The plates were tested with 3 x 5 minute washes and 5 or 7 consecutive washes with BSA buffer. Washing was performed by flooding the wells using a wash-bottle, shaking excess moisture from plate and blotting to dryness. Frequency of washes and the presence of BSA in the washing buffer were found to be critical in reducing background and variation in the assay. 7 washes with BSA were found to be adequate. This method of washing was adopted for all subsequent assays.

v) Conjugate concentration, time and temperature

Varying dilutions of conjugate were added to plates ranging from 1:10 to 1:5000 in BSA buffer. Optimum conditions were reached at a dilution of 1:1000. Using this dilution plates were assayed at incubation times ranging from 15 to 180 minutes and at temperatures of 4°C, room temperature and 37°C. Optimum conditions were attained at room temperature for 2 hours. This method was adopted for all subsequent assays.

vi) Washing procedures after conjugate incubation

Once again the plates were tested with 3 x 5 minute washes and 5 or 7 washes, but this time with PBS buffer. 7 washes again proved to be sufficient to obtain optimum assay conditions. Excess moisture was shaken from plates and they were blotted to dryness. This method of washing was adopted for all subsequent assays.

vii) Substrate concentration, time and temperature

To ensure zero-order kinetics during the peroxidase reaction it is essential to have an excess of the two substrates in the reaction. To establish whether the kinetics for this reaction were zero-order, the highest standard i.e. 25 µg/l was incubated for 1 hour at 37°C on one plate, followed by a 50-fold diluted

conjugate for 1 hour at room temperature on a second plate. After washing both plates with PBS, 0.1 ml working substrate was added to each plate, in which either the concentration of hydrogen peroxide or ABTS was varied while keeping the concentration of the other constant. The absorbance was measured. The amount of colour generated during the enzyme reaction increased to an optimum with an increase in the amount of hydrogen peroxide or ABTS. At concentrations greater than that chosen for the assay final optical density readings were lower.

The incubation times that were tested ranged from 15 to 120 minutes and the temperatures were 4°C, room temperature and 37°C. Optimum assay conditions were achieved at room temperature for 1 hour. This method was adopted for all subsequent assays.

RESULTS

1. Batch to batch variation was examined on six different RIA controls totalling 129 separate estimations. The correlation coefficient was found to be 0.992 (Fig. 1). The standard deviation for the ELISA estimates was always lower than that given for the RIA(UK) controls (Table).

Table

Batch to batch variation for ELISA estimates using six commercial controls RIA(UK)

R.I.A. control No.	R.I.A. Mean Conc. Ferritin (µg/l) (SD) (n not given)	ELISA Mean Conc. Ferritin (µg/l) (SD)
1	10.50 (3.10)	10.20 (1.98) (n = 26)
2	9.15 (2.05)	7.92 (1.38) (n = 31)
3	25.70 (3.70)	24.75 (3.04) (n = 32)
4	36.50 (6.50)	36.67 (5.28) (n = 23)
5	27.25 (6.75)	26.38 (5.24) (n = 12)
6	260.00 (42.00)	215.20 (13.24) (n = 5)

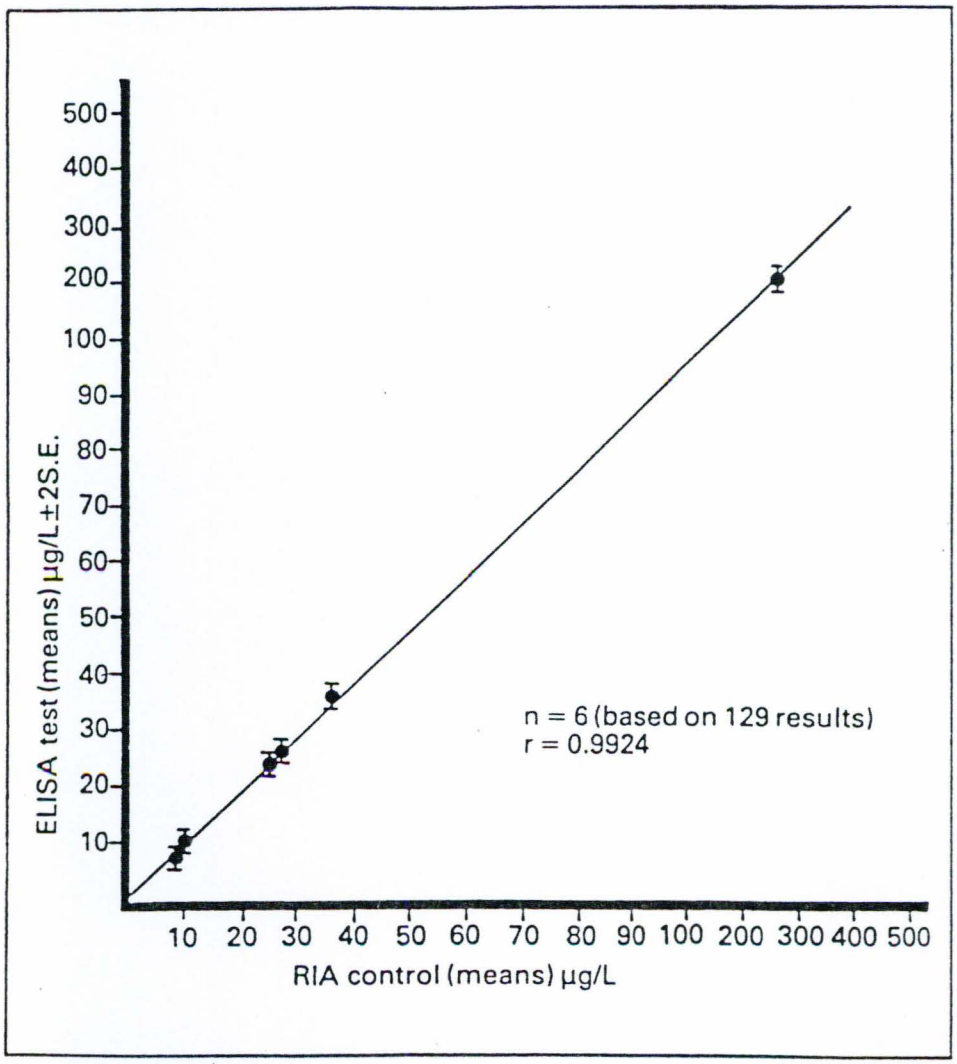


Figure 1 Batch to batch variation of six different RIA controls (129 estimations) measured by ELISA

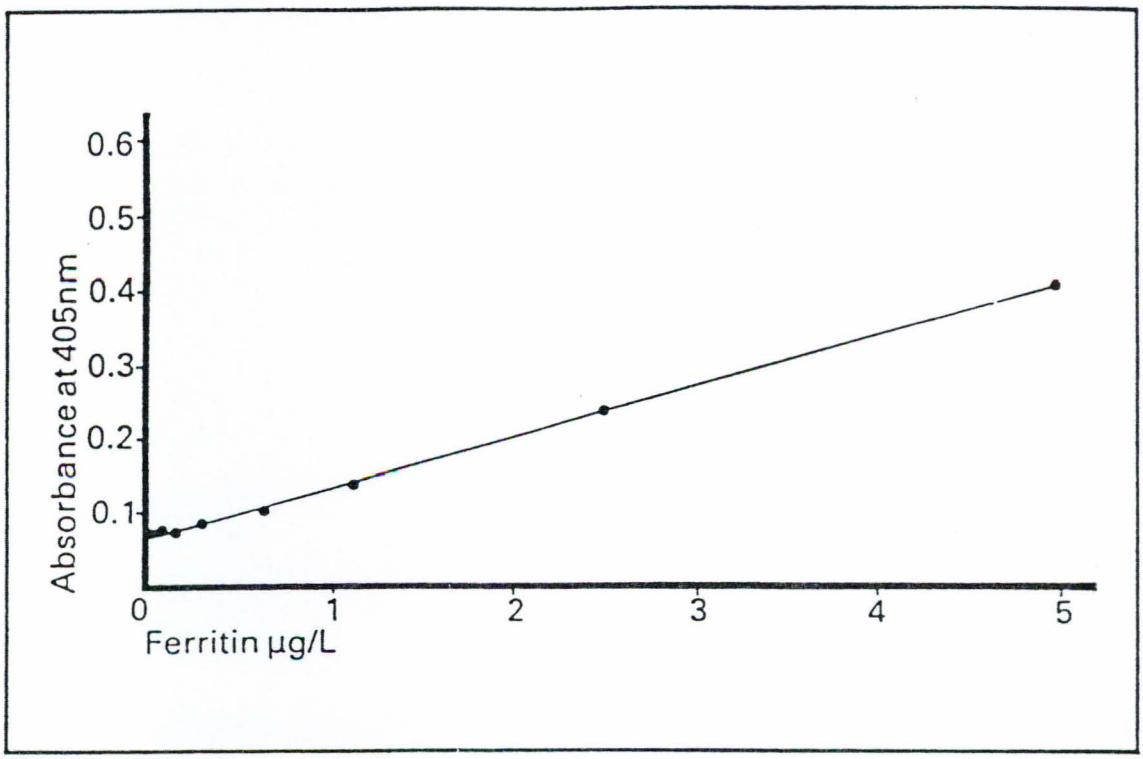


Figure 2 The sensitivity and linearity of the ELISA for determining serum ferritin was measured by serial dilution of a 5 µg/l RIA (UK) control.

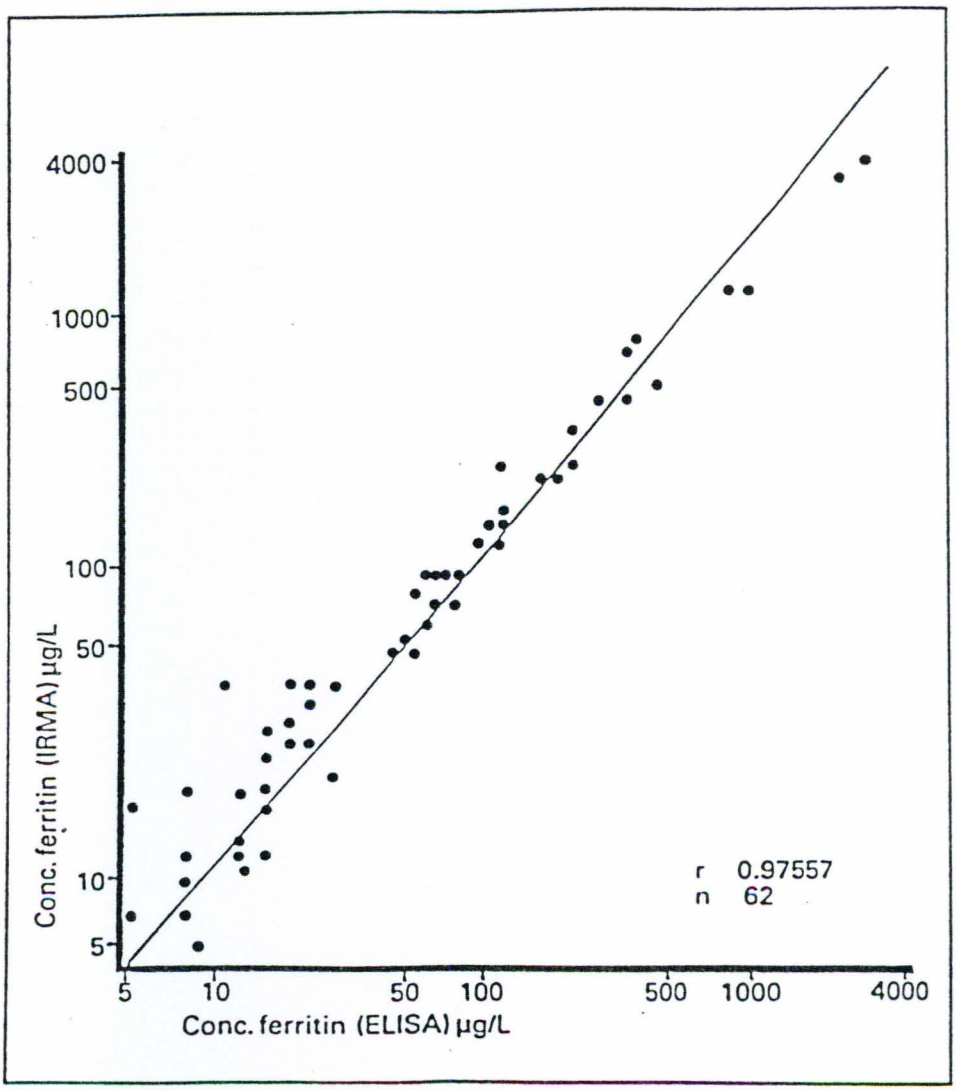


Figure 3 Comparison between the ELISA and IRMA for the determination of serum ferritin.

2. One serum sample was assayed for serum ferritin in twenty-three different positions on one plate (excluding the marginal wells) to examine across the plate variations. The mean serum ferritin for this test was 161.39 µg/l with a coefficient of variation of 2%, implying no positional effect.
3. The sensitivity of the ELISA was measured by a serial dilution of a 5 µg/l RIA(UK) control and was accurate down to levels of 0.3125 µg/l (Fig. 2).
4. When increasing concentrations of an RIA(UK) ferritin standard (range 5-15 µg/l) were added to an RIA(UK) control (in triplicate) the resulting mean percentage recovery was 81% with a coefficient of variation of 2%.
5. The serum ferritins of 62 patients were determined using both ELISA and IRMA methods. Taking the IRMA reading as the independent variable a correlation coefficient of 0.976 was obtained (Fig. 3). A paired 't' test of the results showed no significant differences ($t=0.66$); d.f. 61; $p>0.5$.

DISCUSSION

In spite of the overall high correlation between the IRMA and ELISA techniques over the whole range, there is an apparent lack of correlation between methods at concentrations below 30 µg/l (Fig. 3). It is impossible to say whether the variation was introduced by the IRMA test or the ELISA, although the overall high performance of the ELISA in the low range i.e. accuracy, sensitivity and linearity would suggest the former.

The particular advantages of the ELISA technique for serum ferritin described here are small sample size, low cost, simplicity and adaptability to semi-automation. It thus was well suited for analysis of the numerous small volume samples obtained in the Madang field study.

ACKNOWLEDGEMENTS

The author would like to thank: Mrs. Thelma Williams, (Senior Technician, Department of Tropical Paediatrics, Liverpool School of Tropical Medicine), who, with Ms. Cathy Harrison, designed and developed this ELISA; Ms. Cathy Harrison (Senior Technician, Department of Tropical Paediatrics, Liverpool School of Tropical Medicine), who performed all the analyses on field specimens and the bulk of the quality control work quoted in the results. The author collaborated with Thelma Williams at the bench in development of the assay prior to leaving for Papua New Guinea to carry out field work. Mr. Phil Cashin of the Combined University Department of Haematology, Royal Liverpool Hospital, performed the IRMA tests for comparison with ELISA.

APPENDIX I.iv.

DISCRIMINANT ANALYSIS IN THE CLINICAL DIAGNOSIS OF ACUTE LOWER RESPIRATORY TRACT INFECTION IN INFANTS: DEVELOPMENT OF A STANDARDISED MODEL FOR EPIDEMIOLOGICAL USE

SUMMARY

Discriminant function models are constructed using clinical criteria to predict chest x-ray findings relating to acute lower respiratory infection (ALRI). The subjects consist of 210 infant admissions to hospital from the cohort study, for whom x-rays had been taken and a questionnaire completed recording responses for 12 features indicative of ALRI. 71% of these infants had ALRI as indicated by a chest x-ray. A model is proposed which includes the six features: cough, respiratory difficulty, intercostal recession, bronchial breathing, crepitations and other adventitious chest sounds. Using a relative risk of 1.0 the error, sensitivity and specificity rates are 12%, 93% and 79% respectively. A second model for use by paramedical staff is proposed and includes only four features: respiratory difficulty, intercostal recession, simple cough and audible grunting. A positive response for 2 or more of these resulted in error, sensitivity and specificity rates of 22%, 81% and 71% respectively when the model was tested on an independent group of 23 outpatients. The use of such models for epidemiological and clinical use is discussed.

The 12 variable model is used in Chapter 10.

INTRODUCTION

World Health Organisation working committees on acute respiratory infections have recommended a standardised clinical classification for epidemiological recording and case management policies (WHO, 1981; WHO, 1983). The classification agreed in 1983 (WHO, 1983) is based on

the use of clinical signs and symptoms rather than on results of investigations such as chest x-ray or sputum culture. It is recognised that the balance of criteria may vary in different countries and in different age groups, but it is recommended that the principle of classification should remain the same (WHO, 1983):

I (a) mild upper respiratory tract infection (URTI),

I (b) severe URTI (presence of pus: otitis media, tonsillitis),

II mild acute lower respiratory tract infection (ALRI) (clinical evidence of ALRI but no respiratory difficulty),

III (a) severe ALRI (as for II but with evidence of respiratory difficulty),

III (b) severe and complicated ALRI (as for III (a) but with one or more specified complicating features e.g. cyanosis).

Given this classification the main problem is to decide which combinations of clinical features give adequate evidence of ALRI.

ALRI was studied in a prospective study of morbidity in the first year of life in a birth cohort recruited in Madang, Papua New Guinea (Oppenheimer et al., 1984). Hospital admissions had chest x-rays as a routine, but at the regular field visits chext x-rays were not taken unless the examination took place at the hospital out-patient clinic. In both situations a range of signs and symptoms were recorded either by a doctor or by a state registered nurse using the same format. Consistency was required in the diagnosis of ALRI and for this purpose the hospital data were used to develop a function of the various signs and symptoms which could discriminate between positive and negative x-ray findings indicative of ALRI. The function was then used to define ALRI in conjunction with the WHO clinical classification in both the hospital and in the field situations.

The results of the analysis provide a simple standardised method for diagnosing ALRI in infants suitable for use by paramedical

personnel without access to x-rays which it is hoped will find wide general application.

METHODS

Chest x-rays were performed routinely on 224 out of 246 infants admitted to hospital from the cohort. These 224 were used for establishing the discriminant function. The model was subsequently tested on a group of 23 infants who attended as out-patients and who had x-rays but who were not admitted.

Signs and symptoms

Twelve respiratory related signs and symptoms (or features) were recorded as present or absent for each patient.

The historical features recorded were all in the form of direct questions:

(F1) Recent fever? (colloquial "skin hot")

(F2) Simple cough? (colloquial "cough nothing")

(F3) Paroxysmal cough? - (colloquial "pulling cough"); spasms of continuous coughing with or without whoop;

(F4) Respiratory difficulty or dyspnoea? (colloquial "short wind").

When necessary these symptoms were elaborated by imitation; however in practice the majority of mothers were objective unambiguous historians.

Physical features recorded included:

(F5) Nasal flaring with respiration;

(F6) Nasal discharge;

(F7) Intercostal recession with respiration;

(F8) Audible grunting with respiration;

(F9) Crepitations heard on auscultation;

(F10) Bronchial breathing heard on auscultation;

(F11) Rapid respiration assessed subjectively. There was better consistency between observers when respiration

was assessed subjectively than when the respiratory rate was counted and a cut-off point used.

(F12) Other chest sounds. These included adventitous sounds on auscultation such as coarse rhonchi and expiratory wheeze.

Features F9, F10 and F12 involving auscultation are normally only included in the training of doctors and clinical assistants (syn. Health Extension Officers) but were included in the initial training of the nurses in this study.

Chest x-rays

Chest x-rays were used in the diagnosis of the patients. These were performed within 24 hours of recording signs and symptoms. All x-rays were subsequently reviewed independently (without access to clinical data) by a consultant paediatric radiologist (HC) for the purposes of this work. The following classification was used:

	ALRI Class (CXR)
No evidence of ALRI (n = 62)	
No evidence of lower respiratory infection	0
Evidence of ALRI (n = 151)	
Mild patchy consolidation	1
Moderate patchy consolidation	2
Severe patchy consolidation	3
Nodular consolidation or Bronchopneumonia	4
Lobar pneumonia	5
Other chest x-ray findings (n = 6)	
Cardiac changes but not pneumonia	-
Changes suggestive of tuberculosis	-
Doubtful chest x-ray (n = 5)	
Poor x-ray quality	-
Vague markings which did not permit definitive classification	-

Amongst the 224 hospital admissions there were 62 with no evidence of ALRI, 151 with evidence, 6 with other x-ray findings and 5 with doubtful chest x-ray. After exclusion of the cases with "doubtful" or "other" x-ray classification and three further infants in whom one or more clinical variables were missing, 210 cases were available for analysis, 149 showing evidence of ALRI. Amongst the out-patient group there were 7 with no evidence and 16 with evidence of ALRI.

Statistical methods

All variables (features) (F1) to (F12) were dichotomous and the method of analysis used was logistic regression with a model of the form:

$$\frac{P(TT_1/X)}{1-P(TT_1/X)} = \exp[-(Bo + B'X)], \quad B' = (B_1 B_2 \ldots B_{12})$$

The left hand side of the equation represents the relative risk of being a member of the ALRI population (TT_1) given a set of observations for the features X (having a value of 0 if the feature was absent and a value of 1 if it was present). Bo is a constant and B' is a vector of parameters associated with the various signs and symptoms.

Variables were selected for the model in a stepwise fashion using the maximum likelihood procedure of Generalised Linear Interactive Modelling (GLIM) (Nelder & Wedderburn, 1972). The criterion for entry at each step was to select the variable which most reduced the model deviance. The change in deviance introduced by including that variable was compared with theoretical X^2 values at the 5% level with one degree of freedom. Only variables which significanty reduced the model deviance were included in the selected model. Maximum likelihood parameter estimates were produced for the selected model. An infant was then allocated to the ALRI population if the relative risk of having ALRI was greater than unity given its observations for X.

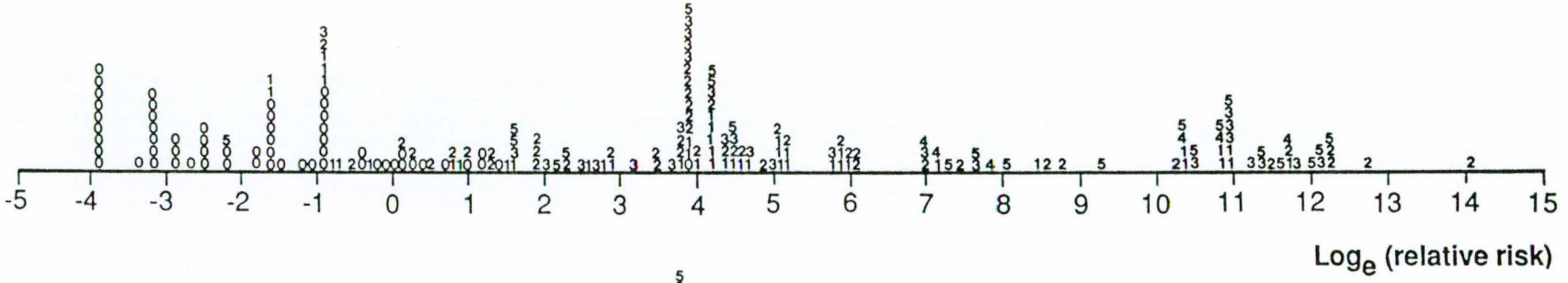
The performance of a model was measured by the error rate (percentage of total infants who were wrongly classified), the sensitivity rate (percentage of those known to have ALRI who were allocated to that group) and the specificity rate (percentage of those known not to have ALRI who were not allocated to the ALRI group).

RESULTS

Table 1(a) shows the reduction in deviance achieved by the introduction of each of the 12 variables in the model. The full 12 variable model was used in subsequent morbidity analysis in the cohort study (see Chapter 10). The error rate, sensitivity and specificity rates for this model were 11%, 92% and 82% respectively. Table 2 shows the actual chest x-ray findings broken down by the discriminant function prediction of ALRI/not ALRI using the 12 variable model. The distribution of the scores is shown in Figure 1(a). There was clear separation of ALRI cases for values of relative risk greater than 4 (with one exception) and non ALRI infants for values of less than 0.1 but between that range there were 56 infants of which 21 were ALRI. The ALRI cases in this group consisted of one with lobar pneumonia, one with severe patchy consolidation and 19 with mild or moderate patchy consolidation.

The selected model included only six features: crepitations, respiratory difficulty, bronchial breathing, other chest sounds, simple cough and intercostal recession. The model parameters are given in Table 1(b). Interaction terms were considered. Only one interaction, when added to the 6 feature model, significantly reduced its deviance: simple cough by rapid respiration. This interaction was not included in the model because the intention was to keep the model as practical as possible. The error sensitivity and specificity rates for this model were 11%, 93% and 79% respectively. Figure 1(b) shows the distribution of the relative risks when the 6 feature model was

Figure 1a Histogram plot of predicted relative risk of ALRI using 12 feature model. Numbers represent actual CXR category allocated by radiologist.



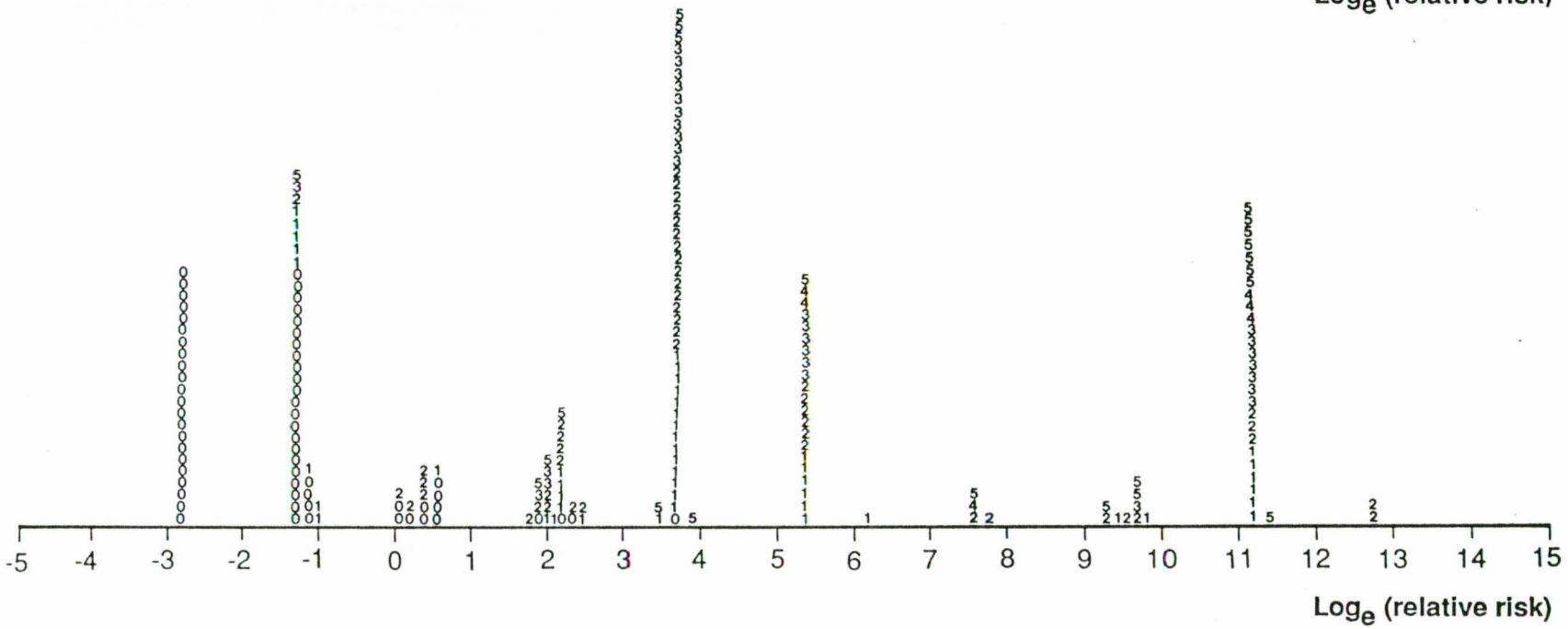


Figure 1b Histogram plot of predicted relative risk of ALRI using 6 feature model. Numbers represent actual CXR category allocated by radiologist.

Table 1(a)

Analysis of deviance for all 12 features

No. of variables included	Model	Deviance	Degrees of Freedom
	Grand mean	253.1	209
1	+ (F9) Crepitations	156.5	208
2	+ (F4) Respiratory difficulties?	131.3	207
3	+ (F10) Bronchial breathing	117.7	206
4	+ (F12) Other chest sounds	111.3	205
5	+ (F2) Simple cough?	105.4	204
6	+ (F7) Intercostal recession	99.2	203
7	+ (F1) Recent fever?	95.7	202
8	+ (F6) Nasal discharge	94.1	201
9	+ (F8) Audible grunting	92.8	200
10	+ (F3) Paroxysmal cough?	92.4	199
11	+ (F11) Rapid respiration	92.2	198
12	+ (F5) Nasal flaring	92.2	197

Table 1(b)

Parameter estimates for the selected 6 features model

	Term	Estimate	Standard Error
	Constant	-2.887	0.619
(F9)	Crepitations	1.423	0.664
(F4)	Respiratory difficulties?	1.828	0.601
(F10)	Bronchial breathing	7.495	8.321
(F12)	Other chest sounds	1.685	0.716
(F2)	Simple cough?	1.510	0.615
(F7)	Intercostal recession	1.709	0.689

For each feature: 0 = absent 1 = present

Table 2

Chest x-ray categorisation by discriminant function prediction using all 12 features and a threshold of prior probability of 0.5

		Discriminant Model Prediction	
Class	Chest x-ray findings	ALRI	Not ALRI
0	No evidence of ALRI	11	50
	Evidence of ALRI		
1	Mild patchy consolidation	38	8
2	Moderate patchy consolidation	45	2
3	Severe patchy consolidation	27	1
4	Nodular consolidation	6	0
5	Lobar pneumonia	21	1

Table 3(a)

Analysis of deviance for 9 features suitable for field work

No. of variables included	Model	Deviance	Degrees of Freedom
	Grand mean	253.1	209
1	+ (F4) Respiratory difficulty?	161.6	208
2	+ (F7) Intercostal recession	139.0	207
3	+ (F2) Simple cough?	126.5	206
4	+ (F8) Audible grunting	122.3	205
5	+ (F11) Rapid respiration	120.3	204
6	+ (F1) Recent fever	117.5	203
7	+ (F6) Nasal discharge	115.7	202
8	+ (F3) Paroxysmal cough?	115.5	201
9	+ (F5) Nasal flaring	115.3	200

Table 3(b)

Parameter estimates for the selected 4 feature model

	Term	Estimate	Standard Error
	Constant	-2.729	0.606
(F4)	Respiratory difficulty?	2.129	0.526
(F7)	Intercostal recession	2.227	0.581
(F2)	Simple cough?	2.080	0.606
(F8)	Audible grunting	1.536	0.792

For each feature: 0 = absent 1 = present

Table 4

Relative risk of having ALRI for different combinations of responses using the 4 feature model

No. of positive responses	Respiratory difficulty?	Intercostal recession	Simple cough?	Audible grunting	Not ALRI	x-ray findings ALRI Class					Relative risk of having ALRI
	(F4)	(F7)	(F2)	(F8)	0	1	2	3	4	5	
0	A	A	A	A	24	1	0	0	0	0	0.06
1	A	A	A	P	1	0	0	0	0	0	0.30
	A	A	P	A	25	6	5	1	0	2	0.52
	P	A	A	A	0	1	0	0	0	0	0.55
	A	P	A	A	1	0	1	0	0	0	0.61
2	A	A	P	P	1	0	1	0	1	0	2.43
	P	A	A	P	0	1	0	0	0	0	2.55
	A	P	A	P	1	0	1	0	0	0	2.81
	P	A	P	A	4	5	3	3	0	3	4.38
	A	P	P	A	1	2	3	0	0	2	4.84
	P	P	A	A	1	1	2	0	0	1	5.08
3	P	A	P	P	0	0	0	0	0	0	20.28
	A	P	P	P	0	0	1	1	0	1	22.49
	P	P	A	P	0	2	3	1	0	1	23.61
	P	P	P	A	2	16	17	16	2	9	40.68
4	P	P	P	P	0	11	10	6	3	4	189.00

A = Absent P = Present

evaluated for the 210 cases which were used in its derivation. There was clear separation of ALRI cases for values of relative risk greater than 1.7 (with 4 exceptions) and non ALRI infants for values of less than 0.25 but within that range were 52 cases of which 17 were ALRI. These consisted of one case with lobar pneumonia, one with severe patchy consolidation and 15 with mild or moderate patchy consolidation.

The analysis was repeated omitting the three variables (F9, F10 and F12) which are arguably best recorded by a doctor. The results are given in Tables 3(a) and 3(b). The selected model included only four variables: respiratory difficulty, intercostal recession, simple cough and audible grunting. The error sensitivity and specificity rates were 12.9%, 88.6% and 83.6% respectively. The equation is evaluated in Table 4 for different combinations of answers to the four variables. Responses of 'yes' to two or more variables results in allocation to the ALRI group for posterior probabilities ranging from 0.38 to 0.70.

The performance of the four feature model for field use was assessed using the group of 23 infants who had x-rays at outpatient visits on clinical suspicion but were not subsequently considered sick enough to be admitted. All 16 cases of ALRI in this group showed mild or moderate patchy consolidation. The error rate sensitivity and specificity were 22%, 81% and 71% respectively, using the criterion of any two positive responses.

DISCUSSION

Few clinicians would make the clinical diagnosis of acute severe lower respiratory infection in a child without also performing an x-ray if the facilities were available. A chest x-ray is used as confirmation of an opinion, resolution of a doubt and sometimes as an indication of aetiology. Although it is possible to miss the

diagnosis in, for example, neonates and lingular inflammation, this investigation is still regarded as the single most discriminating clinical finding. In developing countries there are many situations where the chest x-ray cannot be used. This affects the ability of all cadres of health workers - doctors, clinical assistants, MCH nurses, dispensers, and aid post orderlies - to make a diagnosis and start presumptive treatment. The diagnosis needs to be sensitive to ensure the adequate treatment of ALRI and specific to avoid missing other serious disease. Without standardisation of useful and simple diagnostic criteria it is difficult for the doctor to train another cadre to make the diagnosis of ALRI.

In this section logistic discriminant models have been developed using clinical signs and symptoms to predict ALRI as determined by chest x-ray results. The data relate to a group of 210 infant hospital admissions to Madang hospital, Papua New Guinea, 71% of whom had ALRI. These models all gave reasonably high sensitivity and specificity and low error rates when their performance was judged using this set of data.

The 12 feature model has been used to categorise ALRI episodes in hospital and in the field in our longitudinal study of infectious morbidity in Papua New Guinea. Given that the model predicts with acceptable accuracy whether ALRI is present or not, further categorisation into WHO respiratory infection grades (WHO, 1983) described in the introduction became relatively straightforward. Although the 12 feature model was used in this study the 6 feature model gave an equally powerful prediction and would be adequate and more appropriate for general epidemiological use.

It is arguable for certain cadres of paramedical primary health workers, that it is not possible to rely on results of auscultation. In fact a four feature model excluding auscultatory features gave an

acceptable prediction with error rate, sensitivity and specificity of 13%, 89% and 84% respectively. Positive response for two or more of the four features resulted in allocation to the ALRI population. Of the 57 radiologically serious cases (severe patchy consolidation nodular consolidation or lobar pneumonia) in the sample 95% would have been detected.

The chief objection to generalising the use of these models is that they are based on hospital admissions with a high prior probability of ALRI. For this reason one of the models (4 feature) was tested in a group of 23 outpatients where diagnosis was in doubt clinically and it performed comparably. The other objection to generalisation is that the variables may have different power in different populations. Validation and calibration of the model would be needed in different contexts; however the principles should remain the same.

In conclusion, it is possible that use of discriminant models of this type may obviate the need for chest x-rays in certain situations and help to standardise the diagnosis of ALRI on objective simple clinical criteria.

ACKNOWLEDGEMENTS

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APPENDIX II

EFFECTS OF IRON AND MALARIA ON GROWTH

INTRODUCTION

Judisch et al., (1966) reported improved weight gain in iron deficient infants receiving iron. Tonkin (1970) reported that Maori infants receiving iron dextran at birth had a greater weight gain than controls although no statistical analysis was performed. In contrast, Andelman and Sered (1966) and James and Coombs (1960) found no anthropometric differences between iron supplemented and control infants in prospective studies. This section deals with anthropometric measurements made in the study cohort at the 6 and 12 month scheduled visits. Birth measurements are described in Chapter 7 and showed no significant differences between treatment groups. The same applies to the 2 month visit.

METHODS

Infants when seen at 2, 6 and 12 month visits were weighed naked using Salter hanging scales. Length was measured lying using a standard wooden length frame with vertical head board and a sliding vertical foot rest. Occipito-frontal head circumference was measured using a paper tape measure.

Variables used for analysis included 1) actual anthropometric measurements at the three visits; 2) 'Z' scores which were derived from the 1983 WHO NCHS standards (1983) using the standard computer programme supplied for this purpose. 'Z' scores used were: weight-for-age; height-for-age; and weight-for-height. Actual chronological age was used for the 'Z' scores; 3) velocities for the 3 anthropometric measurements were calculated between the 2 and 6 month and between the 6 and 12 month visits.

Analysis was performed on the Liverpool University computer using the SPSS X statistical package. One way and 2 way analysis of variance (by injection group and malaria slide result) were performed (see Chapter 6). Analyses include only cases for whom all relevant variables were recorded.

RESULTS

At the 6 month visit, weight and weight-for-height were significantly lower in the iron dextran group (Table 1). Two way analysis of variance by treatment group by malaria slide result showed no effect of malaria. No effects on other anthropometric variables were seen at the 6 month visit.

Length-for-age 'Z' score was significantly lower in the iron dextran group at the 12 month visit. On 2 way analysis of variance this effect was explained by malaria slide positivity and the treatment effect was no longer significant. 6 to 12 month weight velocity was higher in the iron dextran group than in the placebo (221 gm/month vs 181 gm/month; $F = 7.38$; d.f. 1,371; $p=0.007$). However, this was offset by the weight deficit at 6 months in the iron dextran group and the mean 12 month weight was not significantly different between groups (Table 1).

No other effects on growth associated with treatment were detected (Tables 1 and 2).

Malaria

No significant effects on growth associated with malaria slide positivity were detected at 6 months. At the one year visit malaria slide positivity was associated with significantly lower weight, height-for-age 'Z' scores, and head circumference (Table 3).

DISCUSSION

As with all other descriptive variables tested (Chapter 7), birth and two month anthropometric measurements were not significantly

TABLE 1

Weight (kg) and weight-for-age 'Z' score at 6 and 12 month visits by injection group. Means (SD)n

	Iron dextran	Placebo	p
6 months			
Weight	6.86 (1.16) 208	7.09 (0.93) 218	0.025
'Z' score	-0.726 (1.013)	-0.518 (0.964)	0.030
12 months			
Weight	8.17 (1.19) 189	8.12 (1.40) 199	N.S.
'Z' score	-1.613 (0.977)	-1.556 (0.921)	N.S.

Length (cm) and length-for-age 'Z' score at 6 and 12 month visits by injection group. Means (SD)n

	Iron dextran	Placebo	p
6 months			
Length	63.8 (6.97) 204	64.7 (2.87) 219	N.S.
'Z' score	-0.987 (1.017)	-0.849 (1.036)	N.S.
12 months			
Length	70.1 (9.50) 186	70.7 (9.24) 196	N.S.
'Z' score	-1.475 (1.091)	-1.257 (0.987)	0.042

TABLE 2

Weight-for-height 'Z' score at 6 and 12 month visits by injection group means (SD)n

	Iron dextran	Placebo	p
6 months	0.045 (0.974) 200	0.189 (1.092) 216	N.S.
12 months	-0.737 (0.888) 181	-0.841 (0.904) 191	N.S.

Head circumference (cm) at 6 and 12 month visits by injection group: means (SD)n

	Iron dextran	Placebo	p
6 months	41.1 (4.34) 204	41.7 (1.46) 217	0.08
12 months	42.9 (6.47) 189	43.5 (4.58) 198	N.S.

TABLE 3

12 month weights, weight-for-age 'Z' scores, lengths, length-for-age 'Z' scores, weight-for-height 'Z' scores and head circumferences by malarial slide positivity. Means (SD)n

	Malaria		
	Slide positive	Slide negative	p
Weight (kg)	7.98 (1.31) 96	8.24 (1.10) 269	0.057
Weight-for-age 'Z' score	-1.749 (0.974)	-1.547 (0.930)	0.07
Length (cm)	70.1 (7.95) 93	71.2 (6.81) 268	N.S.
Length-for-age 'Z' score	-1.609 (1.117)	-1.284 (1.024)	0.011
Weight for height 'Z' scores	-0.770 (0.900) 92	-0.796 (0.894) 262	N.S.
Head circumference (cm)	42.6 (6.39) 96	43.8 (3.00) 268	0.019

different between treatment groups in the study, thus differences detected at follow-up visits were likely to have been related to intervention. Iron dextran treatment was associated with significantly lower weights at 6 months and a lower mean length-for-age 'Z' score at one year. The latter effect disappeared when malaria was controlled for. Weight growth velocity between 6 months and one year was significantly greater in the iron dextran group returning the two groups to weight parity at one year.

In contrast to the equivocal results associated with iron treatment, malaria was associated at one year with significantly lower length for age and head circumference implying a stunting effect. Sharp and Harvey (1980) also noted growth stunting associated with splenic enlargement in Papua New Guinea.

Iron treatment was not clearly associated with better growth in this study. A possible explanation of the equivocal results observed was an interaction with the effects of malaria which was more prevalent in the iron treatment group, with catch-up growth in the latter half of the first year.

APPENDIX III.i

DYNAMICS OF HAEMATOLOGICAL CHANGES IN MADANG INFANTS IN THE FIRST 4 MONTHS OF LIFE; EVIDENCE FOR RIBOFLAVIN DEFICIENCY

SUMMARY

In a field survey, 89 healthy infants in Madang aged 0 - 18 weeks had a basic haematological screen of haemoglobin, packed cell volume, reticulocyte count, serum iron, transferrin saturation and serum ferritin in order to analyse the timing and rate of post-natal haemoglobin fall and the start of haemopoesis. The main fall in haemoglobin was complete by the sixth week of life corresponding with a rapid initial postnatal-weight gain. Reticulocyte counts rose after this age and transferrin saturation fell.

70/83 tested (84%) had evidence of riboflavin deficiency. It is suggested that pregnant and lactating women in this area may be deficient, and that riboflavin deficiency may protect infants against malaria.

INTRODUCTION

The pilot haematological studies (Chapter 5) suggested a more rapid post-natal haemoglobin fall in Madang than is generally found in western populations (see Fig. 1, Chapter 5) (Matoth et al., 1971).

This survey was carried out on a larger sample of infants aged under 18 weeks, primarily to determine more accurately the initial rate of fall of haemoglobin, relate this to weight gain and time the haemoglobin nadir and onset of haemopoesis.

During the course of the survey, specimens were also taken from the same infants to measure riboflavin status.

PATIENTS AND METHODS

89 infants aged 0-4 months (mean 7.6 weeks) were selected from those attending well-baby clinics in the North and South Ambenob

census divisions of Madang Province. After consent had been obtained for inclusion in the survey, mothers were interviewed on feeding practice, infant's health, and social background. Three questions were asked on feeding practice: firstly if the child was breast-fed (which all were); secondly if cow's milk supplements were used, and if so for how long; and thirdly if solids had been introduced and for how long. The mothers were asked if the baby was sick now and, if not, if it had been sick in the last two weeks. If the baby was reported as sick it was excluded from the study and appropriate treatment initiated. Only infants who were well and afebrile were included in the study.

Additional information obtained on each baby included the following:

date of birth

birth weight (76 recorded in health record book)

sex

domicile (urban/urban settlement/rural village (indigenous)/ rural settlement (migrants from another area, and plantation workers)

housing type (high cost/low cost/self-build - permanent materials/self built - bush materials)

Each infant was then fully examined, with a specific record of spleen and liver size, and a sample of 3 ml of venous blood was obtained. Weight, length and head circumference were measured. EDTA and clotted samples of blood were used for estimation of Hb, PCV, MCHC, reticulocyte count, serum iron and transferrin saturation, serum ferritin, and Erythrocyte Glutathione Reductase Activation Coefficient (EGRAC). Frozen red cell lysates were sent to the London School of Tropical Medicine and Hygiene for this latter assay, which was kindly performed by Dr. David Thurnham. EGRAC is the most sensitive method

of analysing riboflavin status (Thurnham & Prapimporn, 1982). Normal values give a coefficient of between 1 and 1.3. High values indicate riboflavin deficiency. Serum samples were used for estimation of serum iron, total iron binding capacity, and serum ferritin. A thin blood film was stained to observe red cell morphology and thick films were examined at the Papua New Guinea Institute of Medical Research for malarial parasites.

The Liverpool University Computer was used for analysis with the MINITAB statistics package. Analyses include only complete sets of variables for that analysis.

RESULTS

Haematological changes with age and weight

Mean ((SD);n) birth weight in the survey was 2.71 kg ((0.41);76). Proportional weight gain (present weight-birthweight) / (birthweight) correlated linearly with age over this age range ($r = 0.75$; $p<0.0001$) with birthweight doubling on average in 103 days indicating a rapid postnatal weight gain. Haemoglobin fell sharply in the first month of life but the nadir was reached by 6 weeks of age with no significant further fall (Fig. 1); mean Hb (SD) thereafter was 9.23 g/dl (1.42). The regression equation with age for the 0-6 week period was:

Hb (g/dl) = 15.7 - 1.09 (Age in weeks); $r = - 0.77$

Post-natal haemoglobin correlated inversely with age, weight, proportional weight gain and reticulocyte count (Table).

Using multiple regression analysis, the inverse correlation of Hb with age, weight and proportional weight gain showed marked colinearity. However, even when controlling for age as first term in sequential regression analysis, both weight and proportional weight gain still explained significant further inverse correlation (for weight: $F = 4.9$; d.f. 1,84; $p<0.05$) (for proportional weight-gain: $F = 4.0$; d.f. 1,72; $p<0.05$) indicating age independant effects.

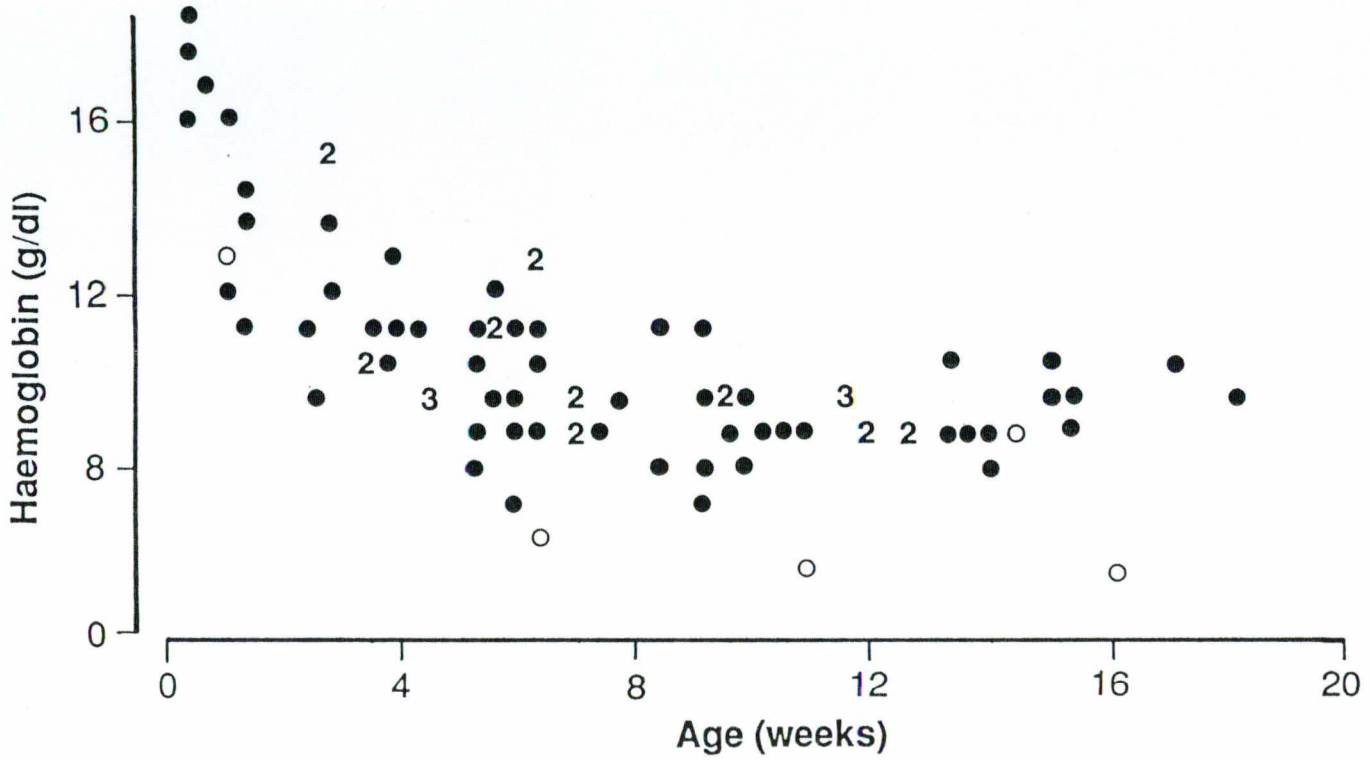

Figure 1 Haemoglobin levels plotted by age. Clear circles indicate positive malaria blood slides.

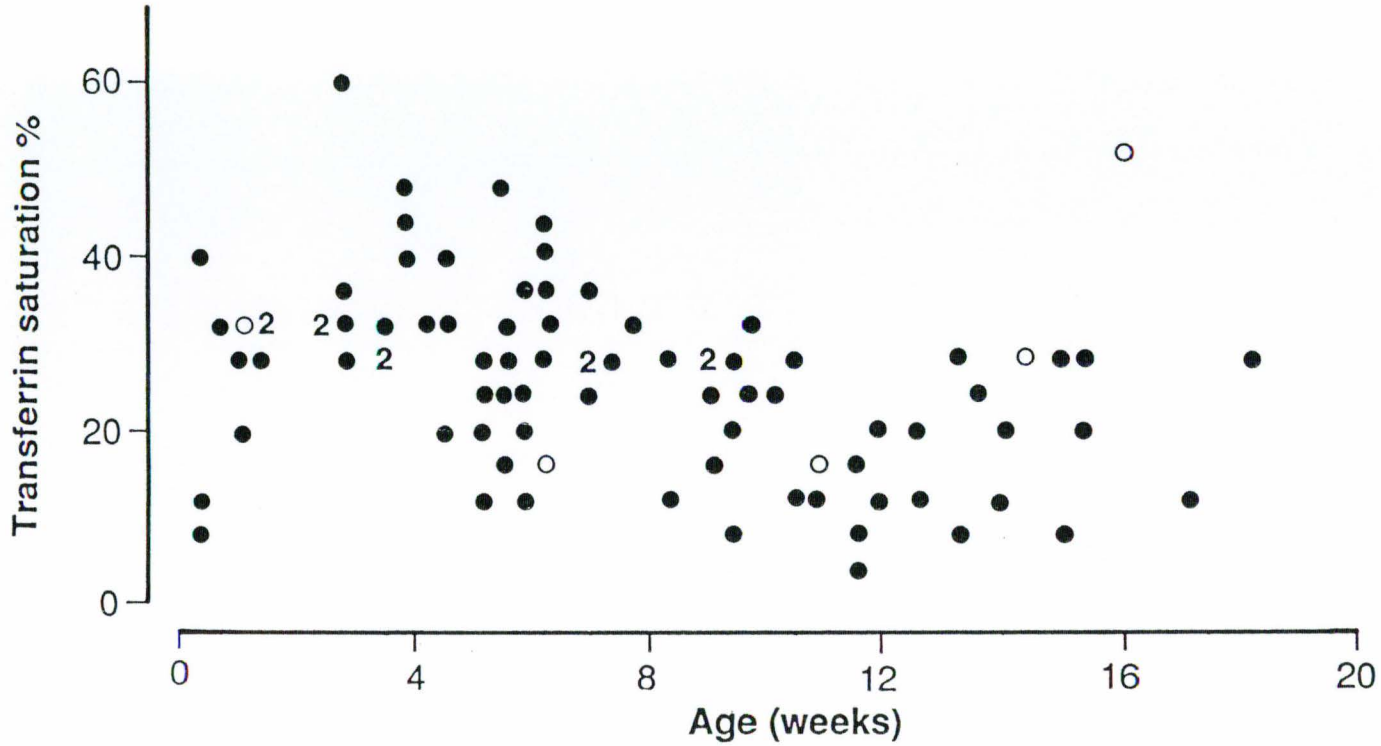

Figure 2 Transferrin saturation plotted by age. Clear circles indicate positive malaria blood slides.

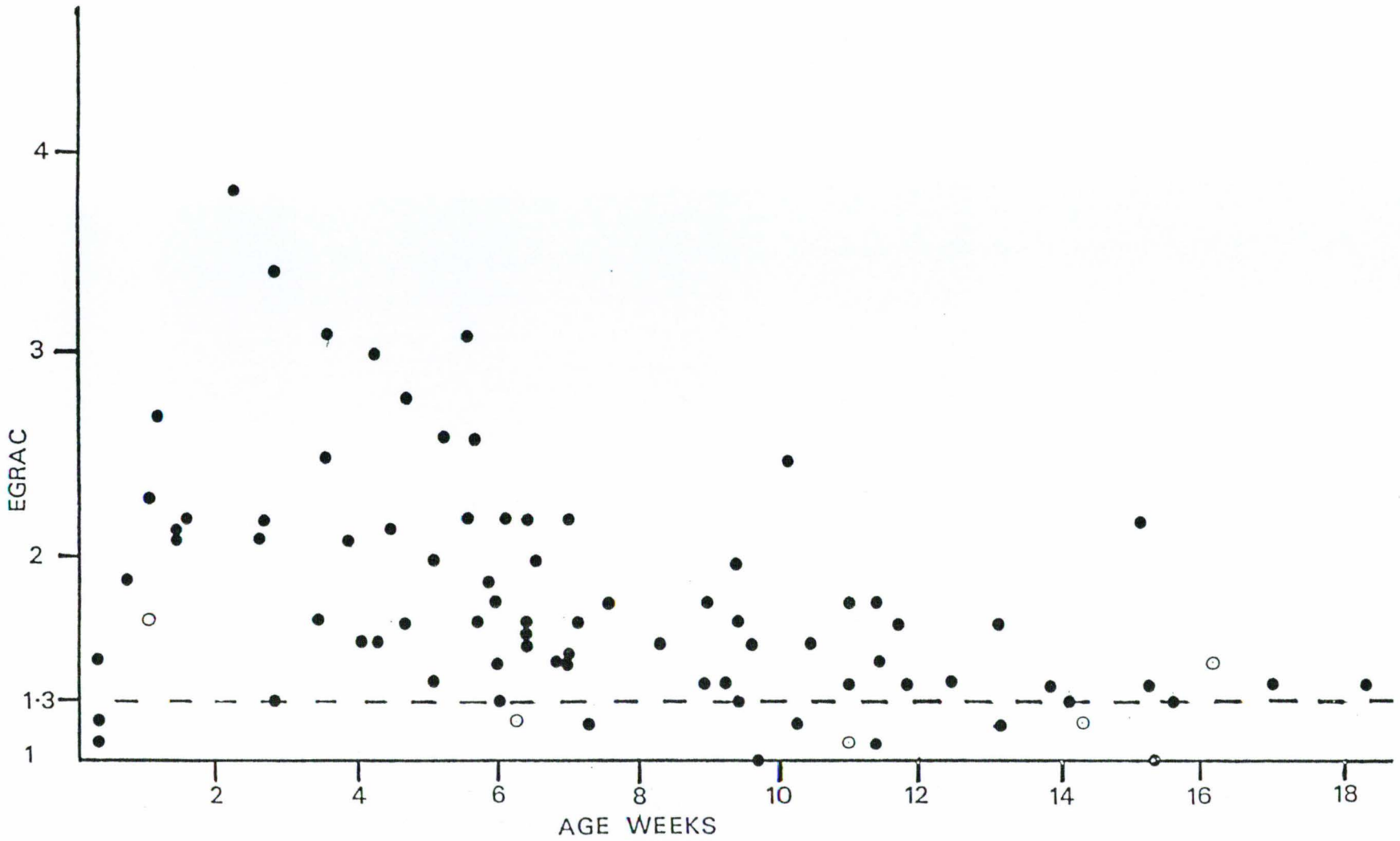

Figure 3 Erythrocyte Glutathione Reductase Activation Coefficient (EGRAC) plotted by age (weeks). Normal range 1-1.3. Open circles represent malaria slide positive cases.

Reticulocyte counts which averaged less than 1% (geometric mean 0.5%) in the first month rose thereafter with geometric mean values of 1.2% in the 2nd and 3rd and 4th months of life. 3 values over 3% were all associated with malaria positivity.

Transferrin saturation following an initial rise in the first 2 weeks of life fell progressively to a nadir of around 11% at 3 months of life, thereafter stabilising (Fig. 2). Levels below 16% were apparent by 6 weeks of age. The regression equation for the period 2 weeks-12 weeks was:

% Transferrin saturation = 42.5-2.33 (Age in weeks) r = - 0.59

Total serum ferritin showed no correlation with age or any other variable over this time period. Geometric mean serum ferritin for the whole group was 215.7 ng/ml (coeff. var. (in log transform) = 10.6%). Further analysis of ferritin binding to concanavalin A is discussed in the next section.

A correlation matrix for these variables is shown in the Table.

Erythrocyte Gluthatione Reductase Activation Coefficient (EGRAC) showed high (i.e. riboflavin deficient) values in 70 out of 83 cases tested (84%) (see Fig. 1). 11 cases had EGRAC's greater than 2.5, indicating severe deficiency. The mean EGRAC (SD) value for all 83 assays was 1.8 (0.568)(SD).

As can be seen from Figure 3, the elevation of EGRAC was age-related, showing higher (deficient) values in younger babies. Linear regression of EGRAC on age gave $r = -0.53$, ($p<0.0001$) for boys and $r = -0.45$ ($p<0.002$) for girls. When age had been controlled for, EGRAC did not correlate with any of the haematological variables. To investigate the age-related fall in EGRAC further, infants were ranked according to riboflavin status and analysed by presence or absence of solid food in the diet. Using the Mann-Whitney Rank Sum Test a test statistic of 1101.5 was obtained ($p=0.019$). This effect disappeared

TABLE

Correlation matrix of main haematological variables, weight and EGRAC (r values)

	Age	Weight	Hb	Reticulocyte count %	Transferrin % saturation	Serum ferritin (log)
Weight	0.754**					
Hb	-0.631**	-0.595**				
Reticulocyte count %	0.164	0.079	-0.348**			
Transferrin % saturation	-0.350**	-0.329*	0.261*	-0.201		
Serum ferritin (log)	-0.129	-0.097	0.143	0.121	0.167	
EGRAC	-0.464**	-0.280*	0.230*	-0.255*	0.257*	0.080

* $p < 0.02$

** $p < 0.001$

when age was controlled for. The effect of social background (domicile and housing type) on riboflavin status was also examined. The only group that stood out from the others were those infants living in high-cost housing where 5 out of 10 infants (50%) had EGRAC's in the normal range (1.0-1.3). The mean EGRAC (SD) in this group was 1.37 (0.47). Ranking cases by EGRAC and dividing housing by high-cost against all other housing gave a Mann-Whitney test statistic of 597.5 ($p=0.011$). This effect was independent of age.

Five out of 83 thick blood films were positive for malarial trophozooites: three were Plasmodium falciparum and two were Plasmodium vivax. Of the five with malaria, three had a normal EGRAC. Mean EGRAC (SD) for the malaria cases was 1.34 (0.258).

Using the Mann-Whitney 'U' test and ranking the value of EGRAC the association of malaria with normal riboflavin status was significant when controlling for age ($p<0.02$). The three cases with heavy density malaria were also the three with normal riboflavin status. Ranks by density were also the exact inverse of the ranks by EGRAC value.

DISCUSSION

The newborn is normally well endowed with iron (75 mg/kg) both in the form of haemoglobin (65%) and also in storage forms (25%) (Lanzkowsky, 1976).

Cord haemoglobins of term infants in the West average 17 gm/dl (O'Brien and Pearson, 1971; Lanzkowsky, 1976; Gill & Schwarz, 1972). In the first few hours of life these values increase to 19.6 gms/dl (14.7 gms/dl in Madang infants - see Chapter 7) due to haemoconcentration; the main determinants of this increase being the time of clamping of the cord and the position the baby is held prior to the clamping. Delayed clamping and holding the baby below the mother may

result in an increase of 20% of the total blood volume or 15% of the total body iron. (This is not practiced in Madang).

A profound redistribution of body iron takes place in the first four weeks of life. Erythropoesis ceases and haemolysis of excess haemoglobin drops the haemoglobin from 19.6 gms/dl to 14.7 gms/dl by the 5th week (Matoth et al., 1971). Most of the iron released by this haemolysis is taken up by tissue stores. 2 week old infants have as much as 27 mg/kg storage iron/kg body weight corresponding with a serum ferritin of 300 ng/ml (Saarinen & Siimes, 1978). At six weeks of age only 40% of the body iron may be present as haemoglobin. The haemoglobin reaches its lowest level at approximately eight weeks of age. At this point significant erythropoetin activity returns accompanied by resumption of active erythropoesis (Lanzkowsky, 1976). In European infants not receiving dietary iron there may be no measurable ferritin by six months of age (Lanzkowsky, 1976).

The timing and extent of these dynamic changes in iron distribution depend on the population studied. In Rabaul and Kieta in PNG, haemoglobins have fallen to 10.5 and 9.9 gms/dl respectively by the age of 1 month (Karicks, 1969). As erythropoesis in the neonate (as in the adult) is controlled by arterial saturation (Gairdner et al., 1952), one would expect that the New Guinean child should restart blood formation at about 1 month of age.

This small haematological and nutritional survey of healthy Madang infants aged 0-18 weeks indicated a rapid post-natal weight gain accompanied by a fall in haemoglobin steeper and to lower levels than seen in European infants (see Fig. 1; Chapter 6). The post-natal haemoglobin decline was complete by 6 weeks by which time a modest increase in reticulocyte count was apparent in some infants. Transferrin saturation fell progressively to subnormal levels in the

first 3 months of life although serum ferritin levels remained high (see Discussion, Chapter 5).

It is reasonable to suppose that part of the reason for the steep post-natal fall in haemoglobin to subnormal levels was a combination of the low mean birthweight and rapid post-natal proportional weight gain seen in these infants compounded by low initial birth haemoglobins (see Chapter 7). This is supported by the regression analysis of Hb showing significant inverse effects associated with weight and proportional weight gain even after controlling for age. A similar phenomenon is seen in low birthweight infants in the West (Dallman et al., 1980).

The EGRAC results indicate that there is an apparently high prevalence (84%) of riboflavin deficiency in Madang infants under 4 months. The deficiency measured by EGRAC is most severe in the younger infants and is significantly associated with full breast feeding as opposed to mixed breast milk and solids.

Interpretation of the relationship of EGRAC to the introduction of solids should be guarded because the volumes and type of solids introduced vary widely and were not recorded in these infants and there is strong colinearity with age. Common weaning supplements are paw-paw and pumpkin. There are several possible explanations for these findings. Firstly, the EGRAC, being an indirect measure of riboflavin deficiency, may be affected by other unknown factors, or age, and not be a true reflection of the riboflavin status. There is no clear evidence at present that a raised EGRAC is not specific for riboflavin deficiency, nor that it is altered in the neonate (Thurnham, 1981; Cooperman et al., 1973). However, Anderson et al., 1986) working in an Italian population previously exposed to malaria, have suggested that a high frequency (50%) of raised EGRAC values not obviously related to dietary riboflavin deficiency may be the result

of genetic selection for slow intracellular FAD production. The alternative explanation, since the infants in this study were almost entirely nutritionally dependent on the mother, is that the mothers were riboflavin deficient. This is supported by observations that (a) the younger babies had high EGRAC's and (b) severe deficiency was associated with total breast feeding. A recent report from the Medical Research Council unit in the Gambia (Bates et al., 1981) noted riboflavin deficiency in pregnant and lactating women. The deficiency reached its peak at parturition, with a mean maternal EGRAC of 1.95. The same workers have more recently shown riboflavin deficiency in infants in the same areas which did not improve when weaning supplements were introduced (Bates et al., 1982). EGRAC elevation was most marked at birth and improved in the first 4 months of life.

One potential relevance of these findings in this population is that riboflavin deficiency is associated with a block of iron release from stores (Sirivech et al., 1974, 1977; Zaman & Verwilghen, 1977), which may produce an apparent iron-deficient picture with normal or raised iron stores.

The second possible relevance of riboflavin deficiency in this population is in relation to protection against malaria. It has been shown experimentally that riboflavin deficiency may protect against malaria (Kaikai & Thurnham, 1983; Seeler & Ott, 1944). This would be supported in the present study by the observation that low EGRAC was significantly associated with slide positivity, although the numbers were obviously very small. The protection shown in both experimental studies cited consisted of a reduction in parasitaemia.

Riboflavin deficiency lowers the potential activity of erythrocyte glutathione reductase (which is FAD dependent) and, in the face of oxidant stress which is increased in malarial infection (Etkin

& Eaton, 1975), may impair the synthesis of reduced glutathione and survival of the red cell (Powers & Thurnham, 1981), and hence of the parasite.

APPENDIX III.ii.

SOURCE OF SERUM FERRITIN IN MALARIA

In Chapter 9 an association between malarial slide positivity and raised serum ferritins was demonstrated.

It has been suggested that the increase in serum ferritin occurring during acute infections may be a result of increased reticulo-endothelial release rather than non-specific release from damaged tissue (Birgegard, 1980). This argument is based on the observation that concanavalin A absorption of ferritin occurs to the same extent in sera from infected patients as in normals thus indicating that the bulk of ferritin in these sera is glycosylated (Birgegard, 1980) and consequently, probably reticulothelial in origin.

Preliminary evidence is presented here suggesting that in malarial infection, this may not be the case.

A cross-sectional haemataological survey of 89 apparently well and afebrile infants aged 0-17 weeks was conducted in Madang, a coastal town in Papua New Guinea, with endemic malaria. The study is described in the first part of this Appendix. Five infants from the study had asymptomatic malaria noted on thick blood film. Three cases were P.falciparum infections and two were P.vivax. Their ages were 1, 6, 11, 14 and 16 weeks respectively. Sera were obtained from venous blood samples from 63 cases for the following assay: Serum ferritin was measured before and after absorption on concanavalin A-sepharose by the method of Worwood et al., (1979). Geometric mean total serum ferritin in the five malaria cases was 331 ng/ml while that in the non-malarious cases was 201 ng/ml ($t = 1.57$ $p>0.05$). However, mean (SD) per cent binding of ferritin to concanavalin A was 35% (8.6) in the malarious cases which was lower than that observed in the

non-malarious cases: 53% (8.7) (Figure). This difference was significant at the 0.1% level using the Mann-Whitney 'U' test. Geometric mean non-binding ferritin in the malarious cases was 209 ng/ml while that in the non-malarious cases was 95 ng/ml ($t = 2.18$: $p<0.05$).

These findings suggest that most of the ferritin in sera from the malarious cases was non-glycosylated and therefore may have been derived from damaged tissues e.g. liver or spleen. Derived from small numbers in a narrow age bracket, the results clearly need further confirmation. More recently Phillips et al. (1986) have also demonstrated high serum ferritin levels in malaria.

The relevance of raised serum ferritin associated with malaria to the main cohort study is that malaria becomes a confounding factor in the assessment of iron status throughout the study (see Chapter 9) and must be appropriately controlled for in analysis. Cruder independant measures of initial total iron status such as birthweight and birth haemoglobin (see Chapters 9 and 10) are less likely to be confounded or biased.

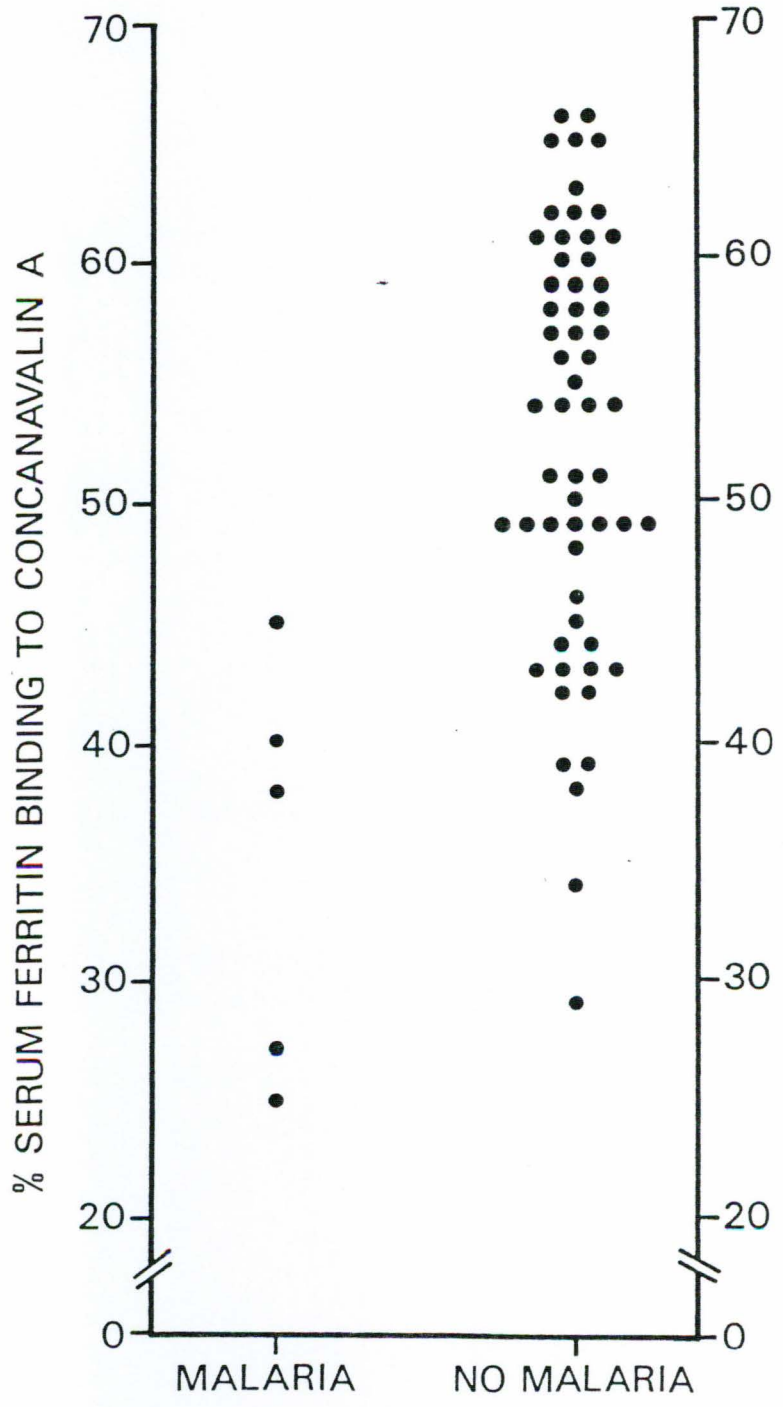

Figure % of serum ferritin binding to concanavalin A in malaria slide positive and negative cases.

CHILDHOOD ANAEMIA PROJECT - PO 378 MADANG (IMR/TP:LSTM 1)

BIRTH FORM

NAME _____ PRESENT ADDRESS _____

MADANG REG. NO. _____ NEAREST MCH CLINIC _____

STUDY NO. [ ][ ][ ][ ] (1 2 3 4) FORM NO. [1] (5) DATE OF BIRTH DAY [ ][ ] MTH [ ][ ] YEAR [ ][ ] (6 7 8 9 10 11) AREA CODE [ ][ ][ ] (12 13 14)

DETAILS OF MOTHER

MARITAL STATUS (15)
- Single [1]
- Married [2]
- Widowed [3]
- Divorced [4]
- NK [9]

EDUCATION (16)
- No formal [1]
- Primary [2]
- Secondary [3]
- Tertiary [4]
- NK [9]

HOUSING (17)
- High Cost [1]
- Low Cost [2]
- Bush [3]
- NK [9]

ANTENATAL CARE (18)
- Yes/Not Xeroxed [1]
- Yes/Xeroxed [2]
- No [3]
- NK [9]

MALARIA COUNT ON ADMISSION (19)
- Positive [1]
- Negative [2]
- Not Done [8]
- NK [9]

If 'POSITIVE' Specify
Numerator [ ][ ][ ][ ] (20 21 22 23)
Denominator [ ][ ][ ] (24 25 26)

WEIGHT (After) [ ][ ].[ ] kgs (27 28 29 30)

Hb (After) [ ][ ].[ ] g/dl (31 32 33 34)

PARITY [ ][ ] (35 36)

IMFERON (if given before birth) [ ][ ] ccs (37 38)

BLOOD GROUP (Mother)
- O
- A
- B
- AB
- NK

TYPE OF DELIVERY (39)
- Spont. vertex [1]
- Caesar [2]
- Other [3]
- NK [9]

(40)
- [1]
- [2]
- [3]
- [9]

COMPLICATIONS OF PREGNANCY, DELIVERY AND PUERPERIUM

DETAILS OF BABY

SEX
- Male [1] (41)
- Female [2]

MULTIPLE BIRTH
- Single [1] (42)
- Twin [2]

BLOOD GROUP (Baby)
- O [1] (43)
- A [2]
- B [3]
- AB [4]
- NK [9]

BIRTH WEIGHT [ ].[ ][ ] kgs (44 45 46)

LENGTH [ ][ ].[ ] cms (47 48 49)

HEAD CIRCUM. [ ][ ].[ ] cms (50 51 52)

GESTATIONAL AGE [ ][ ] wks (53 54)

JAUNDICE (55)
- Yes [1]
- No [2]
- NK [9]

If 'YES':
Bilirubin (max. level) [ ][ ][ ] mg/100ml (56 57 58)
Duration over 15mg/100ml [ ][ ] days (59 60)

DIAGNOSES OF PERINATAL INFECTIONS (IF ANY)

DIAGNOSIS	ACCURACY	DIAGNOSIS	ACCURACY
1.	[ ][ ][ ][ ][ ] (61 62 63 64 65)	2.	[ ][ ][ ][ ][ ] (66 67 68 69 70)
3.	[ ][ ][ ][ ][ ] (71 72 73 74 75)	4.	[ ][ ][ ][ ][ ] (76 77 78 79 80)

CHILDHOOD ANAEMIA PROJECT - PO 378 MADANG (IMR/TP:LSTM 2)

BIRTH LABORATORY FORM

NAME _____ MADANG REG. NO. _____

STUDY NO. [ ][ ][ ][ ] (1 2 3 4) FORM NO. [2] (5)

A. CORD BLOOD Cross Box if NOT DONE [ ]

Hb.	PCV	Reticulocytes
[ ][ ][ ] g/dl	[ ][ ][ ] %	[ ][ ][ ] %
6 7 8	9 10 11	12 13 14

B. 24-HOUR BLOOD Cross Box if NOT DONE [ ] BLOOD SAMPLE 17

TIME AFTER DELIVERY [ ][ ] hrs (15 16)

Capillary [1]
Venous [2]

Hb	PCV	WBC	Reticulocytes
[ ][ ][ ] g/dl	[ ][ ][ ] %	[ ][ ][ ][ ] 10^3/cu.mm.	[ ][ ][ ] %
18 19 20	21 22 23	24 25 26 27	28 29 30

DIFFERENTIAL

Neut.	Lymph.	Eosin.	Mono.	Baso.	Early Myeloids and Banc
[ ][ ] %	[ ][ ] %	[ ][ ] %	[ ][ ] %	[ ][ ] %	[ ][ ] % OF NEUTROPHI
31 32	33 34	35 36	37 38	39 40	41 42

BLOOD FILM

HYPOCHROMASIA 43 [1 2 3 4 5 6 9]

ANISOCYTOSIS 44 [1 2 3 4 5 6 9]

0 ± + 2+ 3+ 4+ NK

POLYCHROMASIA 45 [1 2 3 4 5 6 9]

POIKILOCYTOSIS 46 [1 2 3 4 5 6 9]

0 ± + 2+ 3+ 4+ NK

MICROCYTOSIS 47 [1 2 3 4 5 6 9]

TOXIC GRANULATIONS NEUTROPHILS 48 [1 2 9] (Y N NK)

G6PD 49

Completely deficient	1
Partially deficient	2
Present normally	3
Not done	8
NK	9

CHILDHOOD ANAEMIA PROJECT - PO 378 MADANG P.N.G. (IMR/TP:LSTM 3.1)

FIELD CHECK FORM (PART 1)

NAME _____ ADDRESS _____

STUDY NO. [ ][ ][ ][ ] (1 2 3 4) FORM NO [3][1] (5 6) DAY [ ][ ] MTH [ ][ ] YEAR [ ][ ] (7 8 9 10 11 12) AREA CODE [ ][ ][ ] (13 14 15)

If baby is dead complete a DEATH FORM instead of this form and tick in: BABY DEAD [ ] 16

RECENT SICKNESS Ask mother:

Is baby sick now 17 [Y] [N] If 'NO' baby sick since black shut 32 [Y] [N]

If baby is not sick now and has not been sick in the last week GO STRAIGHT TO EXAMINATION without completing the rest of this page.

HISTORY OF RECENT SICKNESS

Mother's own description

Length of sickness (days) [ ][ ] 33 34

Ask mother if baby has shown any of the following symptoms during this sickness:

1.	Fever	18 [Y] [N]	8.	Diarrhoea	35 [Y] [N]
2.	Cough nothing	19 [Y] [N]	9.	Bloody stools	36 [Y] [N]
3.	Paroxysmal cough	20 [Y] [N]	10.	Ear discharge	37 [Y] [N]
4.	Cough with blood	21 [Y] [N]	11.	Nasal discharge	38 [Y] [N]
5.	Vomiting after cough	22 [Y] [N]	12.	Convulsions	39 [Y] [N]
6.	Vomiting nothing	23 [Y] [N]	13.	Coma	40 [Y] [N]
7.	Short wind	24 [Y] [N]	14.	Jaundice	41 [Y] [N]
			15.	Stopped feeding	42 [Y] [N]

TREATMENT FOR THIS SICKNESS Ask mother:

Baby received treatment 25 [Y] [N]

If 'YES'

Source of Treatment

Hospital inpatient	26 [Y] [N]
Hospital outpatient	27 [Y] [N]
Town clinic	28 [Y] [N]
Health centre	29 [Y] [N]
Aide-post orderly	30 [Y] [N]
Village aide	31 [Y] [N]

Type of Treatment

Medicine	43 [Y] [N]
Black shut	44 [Y] [N]
Other shut	45 [Y] [N]

NOW DO EXAMINATION

CHILDHOOD ANAEMIA PROJECT - PO 378 MADANG P:N.G: (IMR/TP:LSTM 3.2)

FIELD CHECK FORM (PART 2)

STUDY NO. [ ][ ][ ][ ] (1 2 3 4) FORM NO. [3][2] (5 6)

EXAMINATION Put thermometer under baby's arm, then continue to examine:

1. HEAD
   - Fontanelle sunken 7 [Y] [N]
   - Fontanelle raised 8 [Y] [N]
   - Fontanelle pulsating 9 [Y] [N]
   - Signs of jaundice 10 [Y] [N]
2. EYES
   - Conjunctivitis 11 [Y] [N]
   - Other ..... 12 [Y] [N]
3. MOUTH
   - Tongue:Cyanosis 13 [Y] [N]
   - Koplic spots 14 [Y] [N]
   - Sores around the mouth 15 [Y] [N]
   - Other ..... 16 [Y] [N]
4. NOSE
   - Flaring 17 [Y] [N]
   - Discharge 18 [Y] [N]
5. CHEST
   - Short of breath 19 [Y] [N]
   - Indrawing 20 [Y] [N]
   - Typical whoop 21 [Y] [N]
   - Grunting 22 [Y] [N]
   - Crepitations 23 [Y] [N]
   - Bronchial breathing 24 [Y] [N]
   - Other ..... 25 [Y] [N]

   Pulse rate [ ][ ][ ] (26 27 28)
6. EARS
   - Discharging 29 [Y] [N]
   - Drum inflamed 30 [Y] [N]
7. NECK
   - Specify ..... 31 [Y] [N]
8. ABDOMEN
   - Distended 32 [Y] [N]
   - Skin dehydrated 33 [Y] [N]
   - Other ..... 34 [Y] [N]
   - Liver (fbs) 35 [ ]
   - Spleen (fbs) 36 [ ]
9. REMOVE THERMOMETER (°C) [ ][ ].[ ] (37 38 39)
10. LEGS
    - Oedema 40 [Y] [N]
    - Paralysis 41 [Y] [N]
    - Other ..... 42 [Y] [N]
11. SKIN
    - Uninfected scabies 43 [Y] [N]
    - Infected scabies 44 [Y] [N]
    - Measles-like rash 45 [Y] [N]
    - Other ..... 46 [Y] [N]

COMMENTS Include your own diagnosis when relevant

DIAGNOSES

	DIAGNOSIS	ACCURACY		DIAGNOSIS	ACCURACY
1.	.....	[ ][ ][ ] (47 48 49) [ ] (50)	4.	.....	[ ][ ][ ] (51 52 53) [ ] (54)
2.	.....	[ ][ ][ ] (55 56 57) [ ] (58)	5.	.....	[ ][ ][ ] (59 60 61) [ ] (62)
3.	.....	[ ][ ][ ] (63 64 65) [ ] (66)	6.	.....	[ ][ ][ ] (67 68 69) [ ] (70)

CHILDHOOD ANAEMIA PROJECT - PO 378 MADANG P.N.G. (IMR/TP:LSTM 4.1)

FIELD CLINICAL FORM (PART 1)

NAME _____ ADDRESS _____

STUDY NO. [ ][ ][ ][ ] (1 2 3 4) FORM NO [4][1] (5 6) VISIT NO [ ] (7) DATE [ ][ ] [ ][ ] [ ][ ] (8 9 10 11 12 13) AREA CODE [ ][ ][ ] (14 15 16)

If baby is dead complete DEATH FORM instead of this form and tick: BABY DEAD 17 [ ]

CHANGE OF GUARDIAN 18 [Y] [N]

FEEDING Ask mother if baby is

Breast fed	19 [Y] [N]		
Bottle fed	20 [Y] [N]	If 'YES' How long (wks)	[ ][ ] (38 39)
Eating solids	21 [Y] [N]	If 'YES' How long (wks)	[ ][ ] (40 41)
Feeding well	22 [Y] [N]	If 'NO' Mother no milk	42 [Y] [N]

RECENT SICKNESS Ask mother if baby

Is baby sick now 23 [Y] [N] If 'NO' was baby sick in last 2 weeks 43 [Y] [N]

If baby is not sick now and has not been sick in last 2 weeks GO STRAIGHT TO EXAMINATION without completing the rest of this page.

HISTORY OF RECENT SICKNESS

Mother's own description

Length of sickness (days) [ ][ ] (44 45)

Ask mother if baby has shown any of the following symptoms during this sickness

		How long (days)			How long (days)
1. Fever	24 [Y] [N]	_____	8. Diarrhoea	46 [Y] [N]	_____
2. Cough nothing	25 [Y] [N]	_____	9. Bloody stools	47 [Y] [N]	
3. Paroxysmal cough	26 [Y] [N]	_____	10. Ear discharge	48 [Y] [N]	_____
4. Cough with blood	27 [Y] [N]	_____	11. Nasal discharge	49 [Y] [N]	
5. Vomiting after cough	28 [Y] [N]		12. Convulsions	50 [Y] [N]	
6. Vomiting nothing	29 [Y] [N]		13. Coma	51 [Y] [N]	
7. Short wind	30 [Y] [N]	_____	14. Jaundice	52 [Y] [N]	
			15. Stopped feeding	53 [Y] [N]	

TREATMENT FOR THIS SICKNESS Ask mother

Baby received treatment 31 [Y] [N]

If 'YES'

Source of Treatment

Hospital inpatient	32 [Y] [N]
Hospital outpatient	33 [Y] [N]
Town clinic	34 [Y] [N]
Health centre	35 [Y] [N]
Aide-post orderly	36 [Y] [N]
Village aide	37 [Y] [N]

Type of Treatment

Medicine	54 [Y] [N]
Black shut	55 [Y] [N]
Other shut	56 [Y] [N]

NOW DO EXAMINATION

CHILDHOOD ANAEMIA PROJECT - PO 378 MADANG P.N.G. (IMR/TP:LSTM 4.2)

FIELD CLINICAL FORM (PART 2)

STUDY NO. [ ][ ][ ][ ] (1 2 3 4) FORM NO. [4][2] (5 6) VISIT NO. [ ] (7) INJECTION NO. [ ][ ][ ][ ] (8 9 10 11)

(2 month visit only)

EXAMINATION Put thermometer under baby's arm, then continue to examine:

1. HEAD
   - Fontanelle sunken 12 [Y][N]
   - Fontanelle raised 13 [Y][N]
   - Fontanelle pulsating 14 [Y][N]
   - Signs of jaundice 15 [Y][N]
2. EYES
   - Conjunctivitis 16 [Y][N]
   - Other ..... 17 [Y][N]
3. MOUTH
   - Tongue:Cyanosis 18 [Y][N]
   - Koplic spots 19 [Y][N]
   - Sores around mouth 20 [Y][N]
   - Other ..... 21 [Y][N]
4. NOSE
   - Flaring 22 [Y][N]
   - Discharge 23 [Y][N]
5. CHEST
   - Short of breath 24 [Y][N]
   - Indrawing 25 [Y][N]
   - Typical whoop 26 [Y][N]
   - Grunting 27 [Y][N]
   - Crepitations 28 [Y][N]
   - Bronchial breathing 29 [Y][N]
   - Other ..... 30 [Y][N]
   - Pulse rate [ ][ ][ ] (31 32 33)
6. EARS
   - Discharging 41 [Y][N]
   - Drum inflamed 42 [Y][N]
7. NECK
   - Specify ..... 43 [Y][N]
8. ABDOMEN
   - Distended 44 [Y][N]
   - Skin dehydrated 45 [Y][N]
   - Other ..... 46 [Y][N]
   - Liver (fbs) 47 [ ]
   - Spleen (fbs) 48 [ ]
9. REMOVE THERMOMETER (°C) [ ][ ].[ ] (49 50 51)
10. LEGS
    - Oedema 52 [Y][N]
    - Paralysis 53 [Y][N]
    - Other ..... 54 [Y][N]
11. SKIN
    - Uninfected scabies 55 [Y][N]
    - Infected scabies 56 [Y][N]
    - Measles-like rash 57 [Y][N]
    - Other ..... 58 [Y][N]

Ask mother if baby has received inferon from another health worker 59 [Y][N]

COMMENTS Include your own diagnosis when relevant

WEIGHT (kgs) [ ][ ].[ ] (60 61 62)

LENGTH (cms) [ ][ ].[ ] (63 64 65)

HEAD CIRCUMFERENCE (cms) [ ][ ].[ ] (66 67 68)

If venepuncture not done

Hb (g/dl) [ ][ ].[ ] (34 35 36)

PCV (%) [ ][ ].[ ] (37 38 39)

Was blood film done 40 [Y][N]

DIAGNOSES STUDY NO. [ ][ ][ ][ ] (1 2 3 4) FORM NO. [4][3] (5 6) VISIT NO. [ ] (7)

	DIAGNOSIS	ACCURACY		DIAGNOSIS	ACCURACY
1.	..... [ ][ ][ ] (8 9 10)	[ ] (11)	4.	..... [ ][ ][ ] (12 13 14)	[ ] (15)
2.	..... [ ][ ][ ] (16 17 18)	[ ] (19)	5.	..... [ ][ ][ ] (20 21 22)	[ ] (23)
3.	..... [ ][ ][ ] (24 25 26)	[ ] (27)	6.	..... [ ][ ][ ] (28 29 30)	[ ] (31)

CHILDHOOD ANAEMIA PROJECT - PO 378 MADANG P.N.G. (IMR/TP:LSTM 5

FIELD LABORATORY FORM

NAME _____

STUDY NO [ ][ ][ ][ ] (1 2 3 4) FORM NO [5] (5) DATE BLOOD TAKEN DAY [ ][ ] (6 7) MTH [ ][ ] (8 9) YEAR [ ][ ] (10 11) VISIT NO [ ] (12)

VENEPUNCTURE CROSS BOX IF NOT DONE [ ] BLOOD FILM CROSS BOX IF NOT DONE [ ]

Hb [ ][ ].[ ] g/dl (13 14 15) PCV [ ][ ].[ ] % (16 17 18) WBC [ ][ ].[ ][ ] 10^3/cu.mm. (19 20 21 22) Reticulocytes [ ].[ ] % (23 24 25)

DIFFERENTIAL Neut. [ ][ ] % (26 27) Lymph. [ ][ ] % (28 29) Eosin. [ ][ ] % (30 31) Mono. [ ][ ] % (32 33) Baso. [ ][ ] % (34 35) Early Myeloids and Band [ ][ ] % OF NEUTROPHILS (36 37)

BLOOD FILM

HYPOCHROMASIA 38 [1 2 3 4 5 6 9] (0 ± + 2+ 3+ 4+ NK)

ANISOCYTOSIS 39 [1 2 3 4 5 6 9] (0 ± + 2+ 3+ 4+ NK)

POLYCHROMASIA 40 [1 2 3 4 5 6 9] (0 ± + 2+ 3+ 4+ NK)

POIKILOCYTOSIS 41 [1 2 3 4 5 6 9]

MICROCYTOSIS 42 [1 2 3 4 5 6 9]

TOXIC GRANULATION NEUTROPHILS 43 [1 2 9] (Y N NK)

MALARIAL PIGMENT IN MONOCYTES 44 [1 2 9] (Y N NK)

COMMENTS

CHILDHOOD ANAEMIA PROJECT - PO 378 MADANG P.N.G. (IMR/TP:LSTM 5

FIELD LABORATORY FORM

NAME _____

STUDY NO [ ][ ][ ][ ] (1 2 3 4) FORM NO [5] (5) DATE BLOOD TAKEN DAY [ ][ ] (6 7) MTH [ ][ ] (8 9) YEAR [ ][ ] (10 11) VISIT NO [ ] (12)

VENEPUNCTURE CROSS BOX IF NOT DONE [ ] BLOOD FILM CROSS BOX IF NOT DONE [ ]

Hb [ ][ ].[ ] g/dl (13 14 15) PCV [ ][ ].[ ] % (16 17 18) WBC [ ][ ].[ ][ ] 10^3/cu.mm. (19 20 21 22) Reticulocytes [ ].[ ] % (23 24 25)

DIFFERENTIAL Neut. [ ][ ] % (26 27) Lymph. [ ][ ] % (28 29) Eosin. [ ][ ] % (30 31) Mono. [ ][ ] % (32 33) Baso. [ ][ ] % (34 35) Early Myeloids and Bands [ ][ ] % OF NEUTROPHILS (36 37)

BLOOD FILM

HYPOCHROMASIA 38 [1 2 3 4 5 6 9] (0 ± + 2+3+4+NK)

ANISOCYTOSIS 39 [1 2 3 4 5 6 9] (0 ± + 2+3+4+NK.)

POLYCHROMASIA 40 [1 2 3 4 5 6 9] (0 ± +2+3+4+ NK)

POIKILOCYTOSIS 41 [1 2 3 4 5 6 9]

MICROCYTOSIS 42 [1 2 3 4 5 6 9]

TOXIC GRANULATION NEUTROPHILS 43 [1 2 9] (Y N NK)

MALARIAL PIGMENT IN MONOCYTES 44 [1 2 9] (Y N NK)

COMMENTS

CHILDHOOD ANAEMIA PROJECT - PO 378 MADANG P.N.G. (IMR/TP:LSTM 6.1)

HOSPITAL ADMISSION FORM (PART I)

NAME _____ MADANG REG. NO. _____ ADDRESS _____

STUDY NO. [ ][ ][ ][ ] (1 2 3 4) FORM NO [6][1] (5 6) DATE OF ADMISSION DAY [ ][ ] (7 8) MTH [ ][ ] (9 10) YEAR [ ][ ] (11 12) AREA CODE [ ][ ][ ] (13 14 15)

DATE OF BIRTH DAY [ ][ ] (16 17) MTH [ ][ ] (18 19) YEAR [ ][ ] (20 21)

FEEDING Ask mother if:

Breast fed	22 [Y][N]		
Bottle fed	23 [Y][N]	If 'YES' How long (wks)	[ ][ ] (40 41)
Eating solids	24 [Y][N]	If 'YES' How long (wks)	[ ][ ] (42 43)
Feeding well	25 [Y][N]	If 'NO' Mother no milk	44 [Y][N]

HISTORY OF ILLNESS

Mother's own description

Length of sickness (days) [ ][ ] (44 45)

Ask mother if baby has shown any of the following symptoms during this sickness

		How long (days)			How long (days)
1. Fever	26 [Y][N]	_____	8. Diarrhoea	47 [Y][N]	_____
2. Cough nothing	27 [Y][N]	_____	9. Bloody stools	48 [Y][N]	
3. Paroxysmal cough	28 [Y][N]	_____	10. Ear discharge	49 [Y][N]	_____
4. Cough with blood	29 [Y][N]	_____	11. Nasal discharge	50 [Y][N]	
5. Vomiting after cough	30 [Y][N]		12. Convulsions	51 [Y][N]	
6. Vomiting nothing	31 [Y][N]		13. Coma	52 [Y][N]	
7. Short wind	32 [Y][N]	_____	14. Jaundice	53 [Y][N]	
			15. Stopped feeding	54 [Y][N]	

TREATMENT FOR THIS ILLNESS Ask mother:

Baby received treatment 33 [Y][N]

If 'YES'

Source of treatment

Hospital inpatient	34 [Y][N]
Hospital outpatient	35 [Y][N]
Town clinic	36 [Y][N]
Health centre	37 [Y][N]
Aide-post orderly	38 [Y][N]
Village aide	39 [Y][N]

Type of Treatment

Medicine	55 [Y][N]
Black shut	56 [Y][N]
Other shut	57 [Y][N]

NOW DO EXAMINATION

CROSS SECTIONAL STUDY ONLY (Do not complete for babies in Cohort Study)

DETAILS OF MOTHER

MARITAL STATUS (58)

Single	1
Married	2
Widowed	3
Divorced	4
NK	9

EDUCATION (59)

No formal	1
Primary	2
Secondary	3
Tertiary	4
NK	9

HOUSING (60)

High cost	1
Low cost	2
Bush	3
NK	9

PARITY [ ][ ] (61 62)

SEX OF BABY (63)

Male	1
Female	2

CHILDHOOD ANAEMIA PROJECT - PO 378 MADANG P.N.G. (IMR/TP:LSTM 6.2

HOSPITAL ADMISSION FORM (PART 2)

STUDY NO. [ ][ ][ ][ ] (1 2 3 4) FORM NO. [6][2] (5 6)

EXAMINATION

1. HEAD
- Fontanelle sunken 7 [Y][N]
- Fontanelle raised 8 [Y][N]
- Fontanelle pulsating 9 [Y][N]
- Signs of jaundice 10 [Y][N]

2. EYES
- Conjunctivitis 11 [Y][N]
- Other ..... 12 [Y][N]

3. MOUTH
- Tongue:Cyanosis 13 [Y][N]
- Koplic Spots 14 [Y][N]
- Sores around mouth 15 [Y][N]
- Throat inflamed 16 [Y][N]
- Other ..... 17 [Y][N]

4. NOSE
- Flaring 18 [Y][N]
- Discharge 19 [Y][N]
- Stridor 20 [Y][N]

5. CHEST
- Accessory muscles 21 [Y][N]
- Chest recession 22 [Y][N]
- Typical whoop 23 [Y][N]
- Grunting 24 [Y][N]
- Crepitations 25 [Y][N]
- Bronchial breathing 26 [Y][N]
- Percussion dull 27 [Y][N]
- Respiration rate rapid 28 [Y][N]
- Other ..... 29 [Y][N]
- Pulse rate [ ][ ][ ] (30 31 32)

COMMENTS Include your own diagnosis

6. EARS
- Discharging 34 [Y][N]
- Drum inflamed 35 [Y][N]

7. NECK
- Specify ..... 36 [Y][N]

8. ABDOMEN
- Distended 37 [Y][N]
- Skin dehydrated 38 [Y][N]
- Other ..... 39 [Y][N]
- Liver (fbs) 40 [ ]
- Spleen (fbs) 41 [ ]

9. REMOVE THERMOMETER (°C) [ ][ ].[ ] (42 43 44)

10. LEGS
- Oedema 45 [Y][N]
- Paralysis 46 [Y][N]
- Other ..... 47 [Y][N]

11. SKIN
- Uninfected scabies 48 [Y][N]
- Infected Scabies 49 [Y][N]
- Measles-like rash 50 [Y][N]
- Other ..... 51 [Y][N]

X-RAY 52
- Not done [0]
- Not pneumonia [1]
- Doubtful pneumonia [2]
- Bronchopneumonia [3]
- Unisegmental pneumonia [4]
- Multisegmental pneumonia [5]

- Evidence of Bronchiolitis 53 [Y][N]
- Cysts 54 [Y][N]
- Empyema 55 [Y][N]
- Pneumothorax 56 [Y][N]
- Effusion 57 [Y][N]

WEIGHT (kgs) [ ][ ].[ ] (58 59 60)

LENGTH (cms) [ ][ ].[ ] (61 62 63)

HEAD CIRCUMFERENCE (cms) [ ][ ].[ ] (64 65 66)

REASON FOR DISCHARGE 33
- Discharged [1]
- Absconded [2]
- Discharged to die [3]
- Died [4]
- NK [9]

DIAGNOSES STUDY NO. [ ][ ][ ][ ] (1 2 3 4) FORM NO. [6][3] (5 6)

DIAGNOSIS		ACCURACY	DIAGNOSIS		ACCURACY
1.	[ ][ ][ ][ ] (7 8 9 10)	[ ] (11)	4.	[ ][ ][ ][ ] (12 13 14 15)	[ ] (16)
2.	[ ][ ][ ][ ] (17 18 19 20)	[ ] (21)	5.	[ ][ ][ ][ ] (22 23 24 25)	[ ] (26)
3.	[ ][ ][ ][ ] (27 28 29 30)	[ ] (31)	6.	[ ][ ][ ][ ] (32 33 34 35)	[ ] (36)

CHILDHOOD ANAEMIA PROJECT - PO 378 MADANG P.N.G. (IMR/TP:LSTM

HOSPITAL ADMISSION LABORATORY FORM

NAME _____ MADANG REG. NO. _____

STUDY NO. [ ][ ][ ][ ] (1 2 3 4) FORM NO. [7] (5) ADMISSION DATE DAY [ ][ ] MTH [ ][ ] YEAR [ ][ ] (6 7 8 9 10 11)

A. ADMISSION BLOOD CROSS BOX IF NOT DONE [ ]

Hb. [ ][ ][ ] g/dl (12 13 14) PCV [ ][ ][ ] % (15 16 17) WBC [ ][ ][ ][ ] 10^3/cu.mm. (18 19 20 21) RETICULOCYTES [ ][ ][ ] % (22 23 24) REFRACTOMETI [ ][ ] (25 26)

DIFFERENTIAL Neut. [ ][ ] % (27 28) Lymph. [ ][ ] % (28 30) Eosin. [ ][ ] % (31 32) Mono. [ ][ ] % (33 34) Baso. [ ][ ] % (35 36)

Early Myeloids and Banc [ ][ ] % OF NEUTROPHIL (37 38)

BLOOD FILM

HYPOCHROMASIA 39 [1|2|3|4|5|6|9] (0 ± + 2+3+4+NK)

ANISOCYTOSIS 40 [1|2|3|4|5|5|9] (0 ± + 2+3+ 4+NK)

POLYCHROMASIA 41 [1|2|3|4|5|6|9] (0 ± +2+3+4+NK)

POIKILOCYTOSIS 42 [1|2|3|4|5|6|9]

MICROCYTOSIS 43 [1|2|3|4|5|6|9]

TOXIC GRANULATION NEUTROPHILS 44 [1|2|9] (Y N NK)

B. DISCHARGE BLOOD CROSS BOX IF NOT DONE [ ]

Hb. [ ][ ][ ] g/dl (45 46 47) PCV [ ][ ][ ] % (48 49 50) REFRACTOMETER [ ][ ] (51 52)

C. BONE MARROW 53

Stainable Iron	1
No Stainable Iron	2
Reduced	3
Not Done	8
NK	9

D. CSF 54

Only complete rest of section if CSF is not normal

Normal	1
Other	2
Not done	8
NK	9

RBC [ ][ ][ ][ ][ ][ ] cu.mm. (55 56 57 58 59 60) WBC [ ][ ][ ][ ][ ] (61 62 63 64 65) Poly. [ ][ ] % (66 67) Lymph. [ ][ ] % (68 69)

PREVIOUS ANTIBIOTIC 70

Yes	1
No	2
NK	9

SUGAR 71

Present	1
Absent	2
Reduced	3
Not done	8
NK	9

PROTEIN 72

±	1
+	2
++	3
+++	4
NK	9

E. ANY BACTERIOLOGY 73

Positive	1
Negative	2
Not done	8

F. MALARIA COUNT

Positive	1
Negative	2
Not Done	8
NK	9

If 'POSITIVE' specify:

Numerator [ ][ ][ ][ ] (74 75 76 77)

Denominator [ ][ ][ ] (78 79 80)

CHILDHOOD ANAEMIA PROJECT - PO 378 MADANG P.N.G. (IMR/TP:LSTM

DEATH FORM

NAME _____ ADDRESS _____

STUDY NO. [ ][ ][ ][ ] (1 2 3 4) FORM NO [9][1] (5 6) DAY MTH YEAR [ ][ ][ ][ ][ ][ ] (7 8 9 10 11 12) AREA CO [ ][ ][ ] (13 14 1

DATE OF DEATH DAY MTH [ ][ ][ ][ ] (16 17 18 19) OR How long ago [ ][ ] (20 21)

HISTORY

Mother's own description

Was cause of death an accident 22 [Y] [N] If 'YES' do not complete rest of form

Was baby sick before death 23 [Y] [N] If 'NO' do not complete rest of form

If 'YES' How long [ ][ ] (24 25) wks

Ask mother if baby showed any of the following symptoms during the period before death

			How long (days)				How long (days)
1.	Fever	26 [Y] [N]	[ ][ ] (27 28)	10.	Abdominal distension	53 [Y] [N]	[ ][ ] (54 55)
2.	Cough Nothing	29 [Y] [N]	[ ][ ] (30 31)	11.	Paralysis	56 [Y] [N]	[ ][ ] (57 58)
3.	Paroxysmal cough	32 [Y] [N]	[ ][ ] (33 34)	12.	Convulsions	59 [Y] [N]	[ ][ ] (60 61)
4.	Cough with blood	35 [Y] [N]	[ ][ ] (36 37)	13.	Coma	62 [Y] [N]	[ ][ ] (63 64)
5.	Vomiting after cough	38 [Y] [N]	[ ][ ] (39 40)	14.	Jaundice	65 [Y] [N]	[ ][ ] (66 67)
6.	Vomiting nothing	41 [Y] [N]	[ ][ ] (42 43)	15.	Stopped feeding	68 [Y] [N]	[ ][ ] (69 70)
7.	Short wind	44 [Y] [N]	[ ][ ] (45 46)	16.	Mother no milk	71 [Y] [N]	[ ][ ] (72 73)
8.	Diarrhoea nothing	47 [Y] [N]	[ ][ ] (48 49)	17.	Weight loss	74 [Y] [N]	[ ][ ] (75 76)
9.	Bloody stools	50 [Y] [N]	[ ][ ] (51 52)	18.	Oedema/swelling	77 [Y] [N]	[ ][ ] (78 79)

NOW COMPLETE HISTORY ON NEXT PAGE

CHILDHOOD ANAEMIA PROJECT - PO 378 MADANG P.N.G. (IMR/TP:LSTM 9.2)

DEATH FORM

STUDY NO [ ][ ][ ][ ] (1 2 3 4)

FORM NO [9][2] (5 6)

Ask mother if baby received any treatment for the sickness

If 'YES' give:

Source of Treatment			Type of Treatment		
Hospital inpatient	8	Y N	Medicine	14	Y N
Hospital outpatient	9	Y N	Black shut	15	Y N
Town clinic	10	Y N	Other shut	16	Y N
Health Centre	11	Y N	Did baby die at home	17	Y N
Aide post orderly	12	Y N	Other	18	Y N
Village aide	13	Y N			

If death occurred at Health Centre repeat form with HEO or OIC

To be completed by Dr. Oppenheimer only:

CAUSE OF DEATH

			ACCURACY
1	1(a)	[ ][ ][ ][ ] (19 20 21 22)	[ ] (23)
2	1(b)	[ ][ ][ ][ ] (24 25 26 27)	[ ] (28)
3	1(c)	[ ][ ][ ][ ] (29 30 31 32)	[ ] (33)
4	2(a)	[ ][ ][ ][ ] (34 35 36 37)	[ ] (38)
5	2(b)	[ ][ ][ ][ ] (39 40 41 42)	[ ] (43)

Underlying cause [ ] (44)

APPENDIX V

PUBLICATIONS ARISING FROM AND ASSOCIATED WITH WORK ON THIS THESIS

Appendix V.i "Material included in text of Thesis: original reprints not included"

Oppenheimer, S.J. (1979). Laboratory evaluation of miniature portable apparatus for estimation of red cell indices. Ann. Trop. Med. Parasit. 73(6), 593-595.

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Oppenheimer, S.J., Hendrickse, R.G., Macfarlane, S.B.J., Moody, J.B., Harrison, C., Alpers, M., Heywood, P. & Vrbova, H. (1984). Iron and infection in infancy. Report of field studies in Papua New Guinea. 2. Protocol and description of study cohort. Ann. Trop. Paed. 4, 145-153.

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Appendix V.ii "Work arising from or referred to in Thesis: papers bound in"

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Bowden, D.K., Hill, A.V.S., Higgs, D.R., Oppenheimer, S.J., Weatherall, D.J. & Clegg, J.B. (1986). Different hematologic phenotypes are associated with the leftward ($\alpha^{4.2}$) and rightward ($\alpha^{3.7}$) α^{+} thalassemia deletions. J. Clin. Invest. 79, 39-43.*

* Reprints bound in this Appendix

DECLARATION

I designed the whole study reported in this thesis. I decided on methods to be used and personally tested their application.

During the period of preparation for the field work (in 1979), I personally tested the Compur equipment (Appendix I.i), the means of prolonged frozen storage (transtemp) in transit between Madang and Liverpool and was directly involved in the development of the micro iron assay and ferritin ELISA (Appendices I.ii and I.iii).

With the help of a nurse fieldworker (Mrs Olivia Bunari) I carried out the pilot studies reported in Chapter 6 and at the same time designed and had built a field haematology laboratory.

During the main study I personally examined, admitted, bled and looked after the bulk (>95%) of admissions from the cohort and performed a daily round. When I was on leave this was kindly carried out by the paediatrician (Dr John Stace). Over 2,500 patient interactions were involved in recruiting and follow-up of the cohort over 3 years. More than half of the visits were in the field. This would have been impossible for one person. I therefore recruited and trained State Registered Nurse/midwives as fieldworkers and continued supervision throughout the study, in addition to carrying out a significant proportion of the fieldwork personally. The bulk of the lab haematology in Madang was carried out by a Medical Technician recruited for the project, Mrs. Ann Spencer. I was conversant with all techniques used and was able to cover for the technician when unavailable.

I designed the field study described in Appendix III which was carried out by Dr Richard Bull (on medical elective) who also performed the basic haematology. Erythrocyte Glutathione Reductase

Activation Co-efficient tests in this survey were kindly carried out by Dr. David Thurnham at the London School of Tropical Medicine and Hygiene. Ferritin binding to concanavalin A was kindly performed by Dr. Mark Worwood.

The malaria microscopy and maternal haematology described in Chapter 8 was performed in the Madang Provincial Hospital laboratories (malaria and general). The thick film malaria microscopy described in the rest of the study was performed in the PNG Institute of Medical Research laboratory at Yagaum under supervision of Mr. David Gibson. The bulk of the microscopy results were also corroborated independantly on thin films by the project medical technician, Mrs. Ann Spencer.

All the ferritin and iron assays were carried out in Liverpool by Ms. Cathy Harrison in the Department of Tropical Paediatrics. Mr. John Pringle carried out the estimations of HbA_2 and HbF in the same Department. Mr. Phil Cashin in the Liverpool University Department of Haematology performed all the folate and B_{12} assays. All the Hb Barts estimations and alpha-thalassaemia genotyping were kindly performed in the Nuffield Department of Medicine in Oxford and results and reprints are used with permission of Professor Sir David Weatherall and Dr. John Clegg.

I was personally responsible for the analysis and presentation of results. I am indebted to Miss Sarah Macfarlane (Senior Lecturer Statistics) who advised and taught me. Mr. Barry Moody (computer programmer) was responsible for the programming side of data management and SPSS analysis. Mrs. Wanda Russell helped with data vetting and map drawing.

I wrote all the papers in Appendix V in which my name appears as first author.

The study was initiated and carried out from a base in the Department of Tropical Paediatrics at the Liverpool School of Tropical Medicine (Head: Professor R.G. Hendrickse) in full collaboration with the Papua New Guinea Institute of Medical Research (Director: Dr. M. Alpers; Deputy Director i.c. Madang Branch: Dr. Peter Heywood).

I am particularly indebted to Professor Hendrickse without whose continuous support the study would not have taken place.

ACKNOWLEDGEMENTS

I would like to express my gratitude to the following people, without whose help and direct involvement the study would not have been possible.

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FISONS clinical trials section supplied masked, coded, batched trial injections.

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