

Iron and immunity

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There are many ways in which iron can affect immune responsiveness and resistance to infection. On the one hand, metabolic events associated with the mounting of a specific immune response may require Fe, while at the same time invading micro-organisms may utilize Fe within the host's body for their own multiplication. Moreover, the limited solubility and potentially toxic nature of ionic Fe means that an excess of this element, or an abnormal distribution, may also predispose to disease. It is therefore not surprising to find that both Fe deficiency and Fe overload may be associated with increased susceptibility to infectious disease and impaired immune mechanisms. However, while Fe deficiency may very frequently have a nutritional cause it is extremely unusual for severe Fe overload to be induced by purely dietary means, it being much more commonly associated with inherited disorders of Fe metabolism such as haemochromatosis, or the excessive blood transfusions required in diseases such as thalassaemia. This review will therefore concentrate mainly on the effects of Fe deficiency on immunity.

Fe and microbial growth

The ability of proteins of the transferrin class (serum transferrin, lactoferrin and ovotransferrin) to inhibit microbial growth by sequestering Fe has been known for over 40 years, and has been the subject of much experimental work. This has been extensively reviewed elsewhere (Finkelstein *et al.* 1983; Weinberg, 1984), and hence only the *in vitro* significance of this activity and its possible perturbation by nutritional means will be discussed here.

It has been shown that many species of bacteria multiply more rapidly in serum if sufficient Fe is added to saturate the transferrin present, and animals can be made more susceptible to certain bacterial infections if the challenge dose is administered together with sufficient parenteral Fe to saturate serum transferrin (Weinberg, 1984). However, it is very doubtful if saturation of transferrin can ever be achieved by oral administration of Fe, and in those cases where Fe overload is sufficiently severe to approach this situation it is probable that other factors such as tissue damage and dysfunction of cells of the immune system would already have caused increased susceptibility to infection. Conversely, in man the normal level of saturation of serum transferrin (about 30%) is quite low enough to prevent

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rapid multiplication of Fe-requiring micro-organisms, and any further reduction resulting from Fe deficiency is unlikely to produce a significant enhancement in antimicrobial activity. In experimental animals such as mice, in which serum transferrin saturation is normally as high as 70–85% (Elin & Wolff, 1974; Mainou-Fowler & Brock, 1985), it is, however, possible that moderate Fe deficiency can actually enhance resistance to infection (Puschmann & Ganzoni, 1977). It has also been reported that Fe-deficient mice were more resistant to experimental infection with *Trypanosoma cruzi* than normal controls (Lalonde & Holbein, 1984), but it is not known by what mechanism trypanosomes acquire Fe. It has been suggested that the reduction in serum transferrin saturation associated with inflammatory and infectious disease (the so-called hypoferraemia of inflammation) may also serve to enhance the effectiveness of the transferrin-mediated antimicrobial system (Weinberg, 1984). Reduced susceptibility to experimental *Candida* infection following induction of hypoferraemia by endotoxin (Elin & Wolff, 1974) has been reported, but again this study was conducted with mice.

In situations where Fe is not readily available, bacteria normally acquire this metal by synthesizing high-affinity, low-molecular-weight Fe chelators (siderophores; Neilands, 1981). Although many of these are capable of removing Fe from host Fe-binding proteins, including transferrin, it does not necessarily follow that transferrin-bound Fe is the most accessible to siderophores *in vivo*. Virtually the only studies in this area have been conducted using the siderophore desferrioxamine, which is used clinically to relieve Fe overload. Desferrioxamine can remove Fe from transferrin *in vitro* only very slowly unless other chelators with more ready access to the transferrin Fe-binding site are present (Pollack *et al.* 1977) and, although this siderophore can acquire sufficient Fe from transferrin in serum to enhance microbial growth, *in vitro* the rate of acquisition of Fe is very slow (Brock & Ng, 1983). It is well recognized that desferrioxamine preferentially chelates intracellular Fe *in vitro* (Hoffbrand, 1980), perhaps from the so-called labile pool (Jacobs, 1977), and consequently reduced availability of intracellular Fe may be more important than the lower transferrin saturation in impeding microbial acquisition of Fe during inflammation, if indeed this is an important function of the hypoferraemic response. It may therefore be concluded that while the Fe-binding properties of transferrin constitute an important part of non-specific immunity, it seems unlikely that the efficacy of this system can be greatly affected either by nutritional factors or inflammation. However, one allied mechanism which may be affected by dietary Fe is the potential ability of lactoferrin in maternal milk to prevent growth of Fe-requiring organisms such as coliforms in the neonatal gut. Again, there are numerous studies testifying to the *in vitro* bacteriostatic properties of lactoferrin (e.g. Reiter *et al.* 1975; Bishop *et al.* 1976; Mickelsen *et al.* 1982; Brock *et al.* 1983), but there are some *in vivo* studies which also suggest that orally administered Fe favours a coliform flora in the gut of the newborn. Bullen *et al.* (1972) showed that the numbers of *Escherichia coli* in the gut of newborn sucking guinea-pigs increased if the animals were dosed with

haematin. Although haematin may exert this effect by a mechanism other than by saturating the lactoferrin in guinea-pig milk (Brock, 1980), a reduction in coliforms was also observed when ovotransferrin was added to an artificial milk given to young guinea-pigs (Antonini *et al.* 1977). Clinical studies have yielded conflicting results. Corda *et al.* (1983) were able to improve the rate of recovery from infantile gastroenteritis by feeding ovotransferrin, but Moreau *et al.* (1983) found that addition of bovine lactoferrin to a formula milk did not significantly change the number of coliforms in the faeces of bottle-fed babies. Further clinical studies in this area would be worthwhile to help resolve the question of whether Fe supplementation is harmful or beneficial to the newborn infant.

Fe status and the specific immune response

A specific immune response of any kind involves a complex series of events which include cellular activation and proliferation, and protein synthesis. Such events clearly presuppose an increase in the metabolic activity of certain cells and it is well known that major nutritional deficiencies such as protein-energy malnutrition can markedly affect cellular activity and proliferation. However, because Fe is required mainly for haemoglobin synthesis, it is not obvious that Fe deficiency may affect the function of non-erythroid cells, such as those involved in immune reactions. Nevertheless, there is evidence of impaired immune responses associated with Fe deficiency, and in this section the effects of Fe deficiency on the various arms of the immune response will be discussed, together with our somewhat limited knowledge of the underlying mechanisms.

Clinical and experimental studies

Antibody production. The evidence for impaired antibody production due to Fe deficiency is somewhat scanty. Several clinical studies have reported that serum and secretory immunoglobulin levels, and responses to immunization are normal in Fe deficiency (Chandra, 1975; MacDougall *et al.* 1975; Bagchi *et al.* 1980; Van Heerden *et al.* 1981; Krantman *et al.* 1982) even when other immune mechanisms such as cell-mediated immunity or neutrophil function are impaired. However, MacDougall & Jacobs (1978) did report that a small sample of Fe-deficient children failed to respond to diphtheria immunization, although pre-existing immunity was not impaired. In animal models, Nalder *et al.* (1972) reported that the response of rats to tetanus toxoid decreased in relation to the Fe content of the diet, and Kuvibidila *et al.* (1982) found a decreased number of plaque-forming cells in the spleens of Fe-deficient, anaemic mice immunized with sheep erythrocytes.

Clearly, clinical studies suffer from the difficulty of rigorously excluding additional nutritional deficiencies, infections, or other factors that may affect antibody levels. The paucity of supporting information from animal studies where such variables are much easier to control (and wholly negative results less likely to be reported) may be significant. Furthermore, no attempt has been made to

investigate mechanisms by which Fe might be involved in antibody production. Under these circumstances it must be concluded that evidence for an impairment of antibody production in Fe deficiency is rather unconvincing.

Cell-mediated immunity. The evidence for impaired cell-mediated immune responses resulting from Fe deficiency is rather stronger than that for an effect on antibody production. Reductions in delayed-type hypersensitivity skin responses in Fe deficiency have been reported in a number of studies (Joynson *et al.* 1972; Jacobs & Joynson, 1974; Chandra, 1975; MacDougall *et al.* 1975; Krantman *et al.* 1982), the defect often being corrected after Fe therapy. Reduced *in vitro* proliferative responses to mitogens have also been reported (Joynson *et al.* 1972; Fletcher *et al.* 1975; MacDougall *et al.* 1975; Sawitsky *et al.* 1976) although in some studies no difference was observed between Fe-deficient subjects and Fe-replete controls (Kulapongs *et al.* 1974; Gross *et al.* 1975; Gupta *et al.* 1982; Krantman *et al.* 1982). Apart from the difficulties associated with adequate control of other factors in clinical studies, the conditions under which the lymphocytes are cultured may be important, as discussed later. Studies with animal models have also shown decreased skin responses (Kuvibidila *et al.* 1981) and lymphocyte responses to mitogens (Soyano *et al.* 1982; Kuvibidila *et al.* 1983*b,c*;) which returned to normal after Fe repletion. Certain infections in which cell-mediated immunity is thought to play an important protective role may be associated with Fe deficiency, such as chronic mucocutaneous candidiasis (Higgs & Wells, 1972; Fletcher *et al.* 1975) and experimental infection with *Nippostrongylus brasiliensis* (Duncombe *et al.* 1979). However, susceptibility to infections with other gastrointestinal parasites such as *Giardia muris* in the mouse (Duncombe *et al.* 1980) or *Trichomonas* in children (Greenberg & Cline, 1979) was not affected and it is worth noting that there is little evidence of an association between increased susceptibility to tuberculosis and Fe deficiency.

Thus there appears to be better evidence for impairment of cell-mediated immunity in Fe deficiency than for impaired antibody responses, although there are still some conflicting results.

Phagocytic and cytolytic defences. There is some evidence that neutrophil function may be impaired in Fe deficiency. Decreased bactericidal activity and reduction of nitro-blue tetrazolium (NBT) have been reported (Chandra, 1975; MacDougall *et al.* 1975; Srikantia *et al.* 1976; Celada *et al.* 1979; Prasad, 1979; Yetgin *et al.* 1979) and Baggs & Miller (1973, 1974) found that reduced resistance to experimental *Salmonella typhimurium* infection in Fe-deficient rats correlated with lower numbers of phagocytic cells in the mucosal tissue. However, there have been reports of normal neutrophil function in Fe-deficient subjects (Kulapongs *et al.* 1974; Van Heerden *et al.* 1981). There is very little information on the effect of Fe deficiency on macrophage function although Kuvibidila *et al.* (1983*a*) reported that the cytolytic activity of spleen and peritoneal cells from Fe-deficient mice against allogeneic tumour cells was reduced. Thus there may be some impairment of *in vitro* phagocytic activity in Fe deficiency, but a correlation with increased susceptibility to bacterial infection still remains to be established, and

more information is required before the effect of Fe deficiency on macrophage function can be assessed.

Mechanisms of impairment of immune function in Fe deficiency

From the previous section it can be seen that despite a certain amount of conflicting evidence, certain parts of the immune response, and in particular cell-mediated immunity, may be impaired in Fe deficiency. To some extent the controversial nature of these findings could be resolved as more becomes known about the involvement of Fe in cellular and molecular events in immune mechanisms. There are three broad categories into which such mechanisms can be divided. First, Fe deficiency may give rise to intrinsic defects in cells such as lymphocytes or macrophages involved in immunological priming, with the result that they can no longer function normally in response to foreign antigens. Second, cells may be normal in function, but reduced in number. Third, cells may be normal in both number and in function, but Fe deficiency impairs the ability of these normal cells to respond to antigenic challenge. It is difficult to make such investigations in clinical studies, and consequently investigations using experimental models have proved more useful in this area. There is some evidence for all three types of mechanism.

Lymphocyte function. Evidence for intrinsic defects in lymphocytes associated with Fe deficiency comes from electron-microscopic studies in which degenerative changes in mitochondria have been found (Jarvis & Jacobs, 1972; Jimenez *et al.* 1982), the latter group noting a correlation between the degree of abnormality and the severity of Fe deficiency. These findings suggest that Fe deficiency could affect protein synthesis by lymphocytes, but evidence for this is not very convincing, as described later. The mechanism by which Fe deficiency causes mitochondrial abnormalities is unknown.

Reports of reduced numbers of lymphocytes, or alterations in the relative proportion of subpopulations, have to be considered along with the possibility of abnormal distribution of these cells within the body. Some clinical studies have reported that Fe-deficient or anaemic children had reduced numbers of circulating T-cells (Srikantia *et al.* 1976; Bagchi *et al.* 1980) even though B-cell numbers were normal (Bagchi *et al.* 1980). However, normal T-cell levels were reported in other studies (Van Heerden *et al.* 1981; Krantman *et al.* 1982). Information from experimental studies are also conflicting. Rothenbacher & Sherman (1980) reported fewer T-cells in the thymus, and fewer B- and T-cells in the spleens of Fe-deficient sucking rats. Soyano *et al.* (1982) found that in Fe-deficient rats the response to mitogens was decreased in thymus cells but increased in spleen cells, a result which suggests that the distribution of lymphocytes in the different organs may have been altered. However, studies in the authors' laboratory (Mainou-Fowler & Brock, 1985) have shown that in Fe-deficient (but not anaemic) mice the proportion of T-cells and of the Ly1⁺ and Ly2⁺ subsets in lymph nodes were the same as in Fe-replete animals. Although differing degrees of Fe deficiency or the presence or absence of anaemia, or both, might account for these conflicting

results, it is difficult to draw any firm conclusions until more is known about the way in which Fe may be involved in lymphocyte production, maturation and recirculation.

The events occurring when lymphocytes respond to an antigen are complex, consisting first of an activation stage involving interaction with antigen-presenting cells and soluble factors such as interleukins, followed by proliferation and effector activities such as antibody and lymphokine production, or cytotoxic activity. The activation stage appears to be unaffected by Fe deficiency. Kuvibidila *et al.* (1981) showed that sensitization to dinitrofluorobenzene was unaffected in Fe-deficient anaemic mice even though the inflammatory response was reduced, while Cummins *et al.* (1978) used lymphocyte transfer experiments to show that sensitization to *Nippostrongylus brasiliensis* was unaffected in Fe-deficient rats. In contrast, there is considerable evidence to suggest that the proliferation stage is critically affected by Fe deficiency. Hoffbrand *et al.* (1976) have shown that the enzyme ribonucleotide reductase is Fe-dependent, and that DNA synthesis by lymphocytes is impaired if insufficient Fe is available. At the same time, it has been shown that lymphocyte proliferation in vitro requires the presence of transferrin-bound Fe (Phillips & Azari, 1974; Dillner-Centerlind *et al.* 1979; Brock, 1981) and that activated lymphocytes express receptors for transferrin (Galbraith *et al.* 1980).

A large number of investigations in the past decade have established that proliferating cells acquire transferrin-bound Fe by a process of receptor-mediated endocytosis, and this extensive literature has been recently reviewed elsewhere (Brock & Mainou-Fowler, 1983; Seligman, 1983; Brock, 1985). In the case of lymphocytes, it is now known that expression of transferrin receptors occurs only after binding of interleukin-2 to the Tac receptor (Hamilton, 1982; Neckers & Cossman, 1983), which is consistent with Fe being required for the proliferative step rather than for activation or sensitization.

Studies in the authors' laboratory (Mainou-Fowler & Brock, 1985) have shown that lymphocytes from Fe-deficient and normal mice proliferate to an equal extent when stimulated by concanavlin A in vitro, but that proliferation is reduced, for both groups, if there is an inadequate supply of transferrin-bound Fe in the culture medium. In particular, serum from the Fe-deficient mice was less effective at supporting proliferation than normal mouse serum, irrespective of whether the cells came from normal or Fe-deficient animals. Proliferation improved if sufficient Fe was added to the Fe-deficient serum to bring the transferrin saturation to the same level as that in normal serum. This result might seem surprising, given that lymphocytes have only a modest Fe requirement (Brock & Rankin, 1981), and that even Fe-deficient mouse serum contained at least 0.5 mg Fe/l. However, it must be remembered that the transferrin saturation will be much lower than in normal serum, so that a relatively large amount of Fe-free (apo) transferrin will be present which, despite its lower affinity for the transferrin receptor, may interfere with binding of Fe-containing molecules. From the findings of Young *et al.* (1984), giving the affinity of the different forms of transferrin (apo, monoferric and

diferric) for the transferrin receptor, and that of Williams *et al.* (1978) for the relative proportions of each transferrin species present at different saturations, the theoretical proportions of each species binding to the cell receptors can be calculated. At 30% saturation, 37% of the receptors will be occupied by diferric transferrin, 55% by monoferric transferrin and 8% by apotransferrin, but at 10% saturation the corresponding values are 28, 41 and 31%. Thus at 10% saturation nearly one-third of the receptors will be occupied by apotransferrin, which will significantly reduce the rate at which the cells acquire Fe, whereas at 30% saturation the proportion drops to less than one-tenth. Since the total physiological transferrin concentration is almost certainly high enough to saturate all receptors effectively, it is evident that Fe delivery to the cells *in vivo* will be affected more by transferrin saturation than by the total amount of Fe available. Although these are theoretical calculations, they do provide a basis for explaining reduced lymphocyte proliferation and cell-mediated immune responses observed in clinical and experimental studies. Moreover, it may be possible to reconcile some of the contradictory findings regarding the responses of lymphocytes from patients with Fe deficiency. In three of the studies which reported normal proliferation responses (Gross *et al.* 1975; Van Heerden *et al.* 1981; Krantman *et al.* 1982) and in which concomitant infection or protein malnutrition were excluded, cells were cultured in medium containing normal human serum rather than serum from the Fe-deficient patients.

There is little information as to whether effector functions of lymphocytes are affected by Fe deficiency. Production of migration inhibition factor (MIF) was found by Joynson *et al.* (1972) to be reduced in subjects with Fe deficiency, and the limited evidence for reduced immunoglobulin production might also be taken as evidence of impaired effector functions. However, it is not clear whether these effects were due to an intrinsic loss of effector function by the transformed cells, or whether they simply reflected a lower number of active cells due to decreased proliferation. In the authors' laboratory a recent study (Mainou-Fowler & Brock, 1985) showed that total protein synthesis by transformed lymphocytes from Fe-deficient mice, cultured in medium containing autologous serum, was normal if allowance was made for the reduced number of transformed cells. While this does not rule out the possibility that production of certain specific proteins might be impaired it does suggest that further investigation is needed to distinguish between impaired lymphokine or immunoglobulin production and impaired proliferation.

In conclusion, it seems likely that Fe deficiency can affect lymphocyte function by preventing the activated cells from proliferating at an optimal rate. The electron-microscopy studies referred to earlier suggest that Fe deficiency may, if prolonged, also produce intrinsic defects in lymphocytes, although the effect of such defects on function remains to be established. The fact that cell-mediated responses tend to be more affected than antibody production may indicate that the proliferation step is of more critical importance in cell-mediated than humoral immunity. A recent report showing that secretion of immunoglobulin by B-cells

does not necessarily require previous expression of transferrin receptors or proliferation (Neckers *et al.* 1984) supports this suggestion.

Fe requisites of phagocytic cells

Since neither neutrophils nor mononuclear phagocytes proliferate to any extent once they leave the bone marrow any impairment of their function associated with Fe deficiency is likely to be due to either an intrinsic defect in enzymic or other activities, or to depressed responses to chemotactic or macrophage-activating factors. The limited evidence available suggests that neutrophils from Fe-deficient subjects respond normally to chemotactic factors (MacDougall *et al.* 1975; Van Heerden *et al.* 1981) but it is not known whether Fe can influence macrophage activation.

Decreased bactericidal activity of neutrophils seems to be due primarily to defects in intracellular killing mechanisms, as ingestion was found to be normal in those studies which employed a method allowing the two stages to be distinguished (Kulapongs *et al.* 1974; Chandra, 1975; Van Heerden *et al.* 1981). Defects in intracellular killing may be related to reduced production of reactive oxygen compounds such as superoxide, as shown by reduced NBT reduction (Chandra, 1975; MacDougall *et al.* 1975; Celada *et al.* 1979). However, Prasad (1979) found that neutrophils from Fe-deficient patients had a normal capacity to produce hydrogen peroxide, but that peroxidase (EC 1.11.1.7)-mediated iodination of *E. coli* was reduced, and Yetgin *et al.* (1979) found that NBT reduction was normal in neutrophils from Fe-deficient children even though bactericidal activity was impaired.

The role of Fe in O₂ metabolism in phagocytic cells is still unclear. On the one hand, Fe is present in cytochrome b₅₅₉ which is required for the production of superoxide (Segal & Jones, 1978). Fe deficiency could impair synthesis of this cytochrome and thus explain the reduced NBT reduction and iodination capacities mentioned previously. On the other hand, certain forms of Fe can catalyse the reaction of superoxide with H₂O₂ to form the short-lived but highly reactive hydroxyl radical (Baldwin *et al.* 1984; Graf *et al.* 1984), which many believe to be the most toxic of the reactive O₂ species. In theory, Fe deficiency could therefore either impair superoxide and H₂O₂ production or decrease their rate of breakdown to hydroxyl radicals. Studies of the effect of Fe on the function of neutrophils from normal and Fe-deficient subjects may help to improve our knowledge of how O₂ metabolism is affected.

Virtually all the studies on phagocyte function in Fe deficiency have dealt with neutrophils, or uncharacterized cell populations, and almost nothing is known about the effect on macrophages. Addition of Fe did not affect H₂O₂ production by cultured mouse macrophages (Thompson *et al.* 1986), although the haemoglobin content of erythrocytes reduced both H₂O₂ production (L. S. Stewart and J. H. Brock, unpublished results) and tumouricidal activity (Weinberg & Hibbs, 1977). However, none of these observations related to cells from Fe-deficient animals.

Conclusions

The present review has attempted to assess critically the evidence for involvement of Fe in immune mechanisms and, in particular, the evidence for altered immune responses associated with Fe deficiency. It is clear that the most urgent need is for further investigations aimed at elucidating the role of Fe in the cellular and molecular events occurring during immune responses. Such information may help to explain the frequently conflicting results obtained in studies with patients, and allow the clinician to decide whether Fe deficiency or other factors are more likely to be the cause of reduced immune responsiveness in malnourished patients.

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