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REVIEW

Immune cell functions in iron overload

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SUMMARY

A number of studies done in the last 10 years demonstrate the importance of iron in regulating the expression of T lymphoid cell surface markers, in influencing the expansion of different T cell subsets and in affecting different immune cell functions *in vitro*. It has been argued that some of the results obtained could be explained by the formation of iron polymers in the experimental conditions used *in vitro* (Soyano Fernandez & Romano, 1985). In this review the results of studies of immunological function in clinical situations of iron overload are analysed. From this analysis, it is concluded that the majority of the observations made *in vitro* have a counterpart *in vivo*, thus providing additional compelling evidence for the importance of iron as an immunoregulator.

Keywords iron T lymphocytes neutrophils β-thalassaemia haemochromatosis AIDS

INTRODUCTION

Studies of immune cell function in clinical situations of iron overload seem to have emerged from two major interests: an interest in the greater susceptibility to infections found in splenectomised patients with iron overload due to β-thalassaemia major (Caroline *et al.*, 1969) and an interest in the modulating interactions of iron with cells of the immune system (de Sousa, 1978; de Sousa, Smithyman & Tan, 1978; de Sousa & Nishiya, 1978; Keown & Descamps, 1978). Interest in infection led to studies of phagocytic and killing functions of polymorphonuclear neutrophils (PMN) and monocytes (Khan *et al.*, 1977; van Asbeck *et al.*, 1982; Khalifa *et al.*, 1983, van Asbeck *et al.*, 1984; Martino et al., 1984; Ballart *et al.*, 1986; Flament *et al.*, 1986). Studies motivated by the interest in the interactions of iron and cells of the immune system concentrated on the quantification of lymphoid cell sets (Kapadia *et al.*, 1980; Guglielmo *et al.*, 1984; Bryan *et al.*, 1984; Grady *et al.*, 1985; Akbar *et al.*, 1985; Dwyer *et al.*, 1987; Pardalos *et al.*, 1987) and on the analysis of different immunological functions, namely, mitogen responses (Munn *et al.*, 1981; Dywer *et al.*, 1987), B cell differentiation (Akbar *et al.*, 1985; Nualart *et al.*, 1987), NK activity (Neri *et al.*, 1984, Goicoa *et al.*, 1986; Akbar *et al.*, 1986, Akbar *et al.*, 1987) and the mixed lymphocyte reaction (MLR) (Dywer *et al.*, 1987).

PHAGOCYTIC CELLS (Table 1)

Phagocytosis is accompanied by an activation of oxidative metabolism resulting in the production of the superoxide anion (O_2^-) and hydrogen peroxide. In a study of O_2^- release by PMN from iron overloaded haemodialysis patients Flament *et al.* (1986) found that without stimulation there were no significant differences observed between patient and control cells. After stimulation with opsonized zymosan however, PMN from patients with ferritin levels higher than 1 000 ng/ml, produced significantly less superoxide anion than patients with lower levels of circulating serum ferritin (Flament *et al.*, 1986). Diminished superoxide anion production was also reported in patients with β-thalassaemia major when the PMN were stimulated with zymosan (Martino *et al.*, 1984). Resting neutrophils or neutrophils stimulated with phorbol myristate acetate (PMA) from the same patients had a significantly higher superoxide anion generation than controls, however. Further differences between resting neutrophils and neutrophils stimulated with PMA or zymosan were noted when the correlations between serum ferritin levels and O_2^- production were analysed. A positive correlation between serum ferritin levels and O_2^- production was only observed with resting PMN (Martino *et al.*, 1984).

In another study of PMN function in β-thalassaemia major, Cantinieaux *et al.* (1987) examined nitroblue tetrazoleum (NBT) reduction, yeast phagocytosis, *E. coli* phagocytosis, *E. coli* killing and intracellular PMN iron by the Perl's reaction. On average 13% of PMN were Perl's positive in the patient group. All phagocytosis tests were impaired in comparison with the control PMN; there were no significant differences, however, between the reduction of NBT and the killing rate in the two groups. In this study the effect of incubation of normal PMN with thalassaemic serum was also investigated. After incubation of normal PMN with thalassaemic serum, 9% of the cells became Perl's positive and an impairment of phagocytosis was observed (Cantinieaux *et al.*, 1987). An earlier study of PMN phagocytosis of *S. typhi* and staphylococcus album had also demonstrated impaired phagocytic indices in Egyptian thalas-

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Table 1. Immune cell functions in iron overload: phagocytic cells

Function	Cell	Conditions of Study	Iron overload	Result	References
Superoxide anion (O_2^-) generation	PMN	resting	haemodialysis	normal	Flament *et al.* (1986)
			β-thal. major	increased	Martino *et al.* (1984)
		zymosan activated	haemodyalisis with serum ferritin (1 000 ng/ml)	impaired	Flament *et al.* (1986)
			β-thal. major	impaired	Martino *et al.* (1984)
		PMA activated	β-thal. major	increased	Martino *et al.* (1984)
Phagocytosis	PMN	*S. typhi*	β-thal. major	impaired	Khalifa *et al.* (1983)
		Staphylococcus	β-thal. major	impaired	Khalifa *et al.* (1983)
		Yeast	β-thal. major	impaired	Cantinieaux *et al.* (1987)
		E. coli	β-thal. major	impaired	Cantinieaux *et al.* (1987)
		S. aureus	Idiopathic haemochromatosis (IH)	normal	van Asbeck *et al.* (1982)
					van Asbeck *et al.* (1982)
	Monocytes	*S. aureus*	Idiopathic haemochromatosis	impaired	
		C. pseudotropicalis	β-thal. major	normal	Ballart *et al.* (1986)
Killing	PMN	*E. coli*	β-thal. major	normal	Cantinieaux *et al.* (1987)
	Monocytes	*C. pseudotropicalis*	β-thal. major	impaired	Ballart *et al.* (1986)

saemia children (Khalifa *et al.*, 1983). This impairment was most marked for *S. typhi* in splenectomised patients.

In a separate study of the ability of peripheral blood monocytes from thalassaemia major patients to phagocytose and kill *C. pseudotropicalis*, no abnormality of the phagocytic activity was found. The patients' monocytes showed, however, a decreased lytic ability which was significantly lower than that observed with control monocytes and independent of splenectomy (Ballart *et al.*, 1986). In this study a significant inverse correlation was noted between lytic activity and serum ferritin levels (Ballart *et al.*, 1986).

In a patient with iron overload due to idiopathic haemochromatosis, van Asbeck *et al.* (1982), using *S. aureus* as the test microorganism, found an impaired phagocytic function of monocytes, but not of PMN.

LYMPHOID CELL SETS (Table 2)

Diminished proportions of CD4⁺ lymphocytes

Our early study of T lymphoid cell subsets in thalassaemia intermedia done at a time when monoclonal antibodies against T cell surface differentiation antigens were not yet widely available reported for the first time the finding of abnormal proportions of T cell subsets associated with iron overload (Kapadia *et al.*, 1980).

Since that study, the most consistent observation relating iron overload and lymphoid cell subsets concerns the finding of abnormally low numbers and functionally defective $CD4^+$ cells. This finding has been reported in thalassaemia intermedia (Guglielmo *et al.*, 1984) in thalassaemia major (Grady *et al.*, 1965; Dwyer *et al.*, 1987; Pardalos *et al.*, 1987) and *in vitro* (Bryan *et al.*, 1986). In addition, ferric citrate has been shown to diminish the cloning efficiency of human memory $CD4^+$ lymphocytes *in vitro* (Good *et al.*, 1986); spleen cells from iron overloaded mice seem to lack precursor $CD4^+$ cells and fail to generate an allo-specific cytotoxic response in the absence of interleukin-2 (Good *et al.*, 1987).

Further evidence for the presence of a defective T helper cell population in thalassaemia major, derives from a study of the cellular components involved in the defective generation of immunoglobulin-producing cells in culture in response to stimulation by pokeweed mitogen (PWM). Comparing co-cultures of patients' T cells and control non-T cells. Nualart *et al.* (1987) provided evidence indicating that the impaired B cell response was attributable to a defective T helper cell population and not to the non-T cell population.

In an immunocytochemistry and ultrastructural study of the rat synovium following a transient increase in the transferrin saturation above 100% after a single i.v. injection of ferric citrate, we found an increase in the numbers of T cells reaching the subsynovial tissue and the tendon sheath at 24 h with a higher proportion of $W3/25^+$ than $OX8^+$ cells (de Sousa *et al.*, 1988). The transient overload provoked in the rat contrasts with the tissue overload in Good's experiments in which the animals received three intraperitoneal injections of iron dextran over 2 weeks or had 0·5% carbonyl iron added to their diet for a period of 3–4 weeks (Good *et al.*, 1987). Nevertheless, the transient overload experiments illustrate and reinforce the apparent 'specific' response of the helper/inducer T cell set to changes in serum iron concentration. The mechanism whereby this could occur remains unclear. The demonstration by Lum *et al.*, (1986) that activated $CD4^+$ and not $CD8^+$ cells synthesize transferrin may provide a speculative basis for designing further experiments to clarify the apparent particular sensitivity of the $CD4^+$ subset to iron.

Increased proportions of CD8⁺ cells

Increases in the numbers of $CD8^+$ cells have been reported in patients with thalassaemia major, whether splenectomised or not (Grady *et al.*, 1985; Dwyer *et al.*, 1987). Statistically significant correlations were observed between the increasing number of blood transfusions received and the proportions of $CD8^+$ cells (Grady *et al.*, 1985). It is therefore difficult to implicate iron overload alone in this result. Dwyer *et al.*, (1987), however, in a careful statistical analysis of all the variables involved, i.e. age, splenectomy, number of transfusions and amount of desferrioxamine received, observed a negative correlation between the amount of desferrioxamine received and the

Table 2. Immune cell functions in iron overload: lymphoid cells

Function	Cell	Conditions of Study	Iron overload	Result	References
—	$CD4^+$	immunofluorescence microscopy	β-thal. intermedia	diminished	Gugliemo *et al.* (1984)
		immunofluorescence microscopy	β-thal. major	diminished	Grady *et al.* (1985)
		immunofluorescence microscopy	β-thal. major	diminished	Dwyer *et al.* (1987)
		immunofluorescence microscopy	β-thal. major	diminished	Pardalos *et al.* (1987)
		cloning efficiency of human memory cells	*in vitro*	diminished	Good *et al.*, (1986)
—	$CD8^+$	immunofluorescence microscopy	β-thal. major	increased	Grady *et al.* (1985)
			β-thal. major	increased	Dwyer *et al.* (1987)
			β-thal. major	increased	Pardalos *et al.* (1987)
—	$CD1^+$	immunofluorescence microscopy	β-thal. intermedia	increased	Guglielmo *et al.* (1984)
—	Thermostable E-rosettes	rosette formation at 37°C	IH	increased	Bryan *et al.* (1984)
			IH	within the control range	Reimão and de Sousa (unpublished data)
Mitogen responses		PHA, Con A	β-thal. intermedia	diminished	Munn *et al.* (1981)
		PWM	β-thal. intermedia	unaltered	Munn *et al.* (1981)
		PHA, *C. albicans*	β-thal. major	diminished	Dwyer *et al.* (1987)
		MLR	β-thal. major	diminished	Dwyer *et al.* (1987)
Spontaneous supressor activity			β-thal. major	augmented	Dwyer *et al.* (1987)
NK activity			β-thal. major	diminished	Neri *et al.* (1984)
			β-thal. major	diminished	Akbar *et al.* (1986)
			β-thal. major	diminished	Goicoa *et al.* (1986)
			IH	unaltered	Good *et al.*, (1988)

numbers of $CD8^+$ cells present (Dwyer *et al.*, 1987). This observation thus constitutes an indirect piece of evidence in favour of iron overload influencing the expansion of the $CD8^+$ cell population *in vivo*.

Circulating $CD1^+$ cells in patients with thalassaemia intermedia
One other abnormality observed in patients with iron overload uncomplicated by multiple blood transfusions was reported by Guglielmo *et al.* (1984) in a study of the 14 patients with thalassaemia intermedia. In addition to the finding of the 'consensus abnormality' of low percentages of $CD4^+$ cells, they have noted significantly higher proportions of circulating $CD1^+$ cells (5–15%) in the patient group than in the control group, where $CD1^+$ cells were almost absent. In-vitro incubation of peripheral blood mononuclear cells from the patients with a crude thymic extract for 48 h resulted in a return to the normal representation of the different T cell differentiation antigens, i.e. increase in the percentage of $CD4^+$ cells and absence of $CD1^+$ lymphocytes. These observations led the authors to conclude that in thalassaemia intermedia there may be a thymus-dependent anomaly of T cell maturation. This conclusion and the interesting observations upon which it is based have not been followed up by other groups.

Thermostable E-rosette forming lymphocytes in idiopathic hemochromatosis (IH)
In a study of IH patients Bryan *et al.* (1984) reported that the numbers of peripheral blood lymphocytes making thermostable erythrocyte rosettes was significantly higher than that seen in controls. A value was considered significantly increased if it was greater than two standard deviations above the mean percentage control value (22%). Applying this criterion, 81% of the patients studied had abnormally high numbers of thermostable E-rosettes. Analysis of other cell surface markers, immunoglobulin levels and mitogen responses to PHA, conA and PWM all seemed to be normal. We have recently re-examined the question of the thermostable E-rosette forming cell in IH patients and have failed to confirm the results of Bryan *et al.* (1984). In the test conditions used by Bryan *et al.* (1984) no differences were found between the patient and the control population studied (Reimão and de Sousa, unpublished data).

LYMPHOID CELL FUNCTIONS (Table 2)

NK activity
The observation of diminished natural killer (NK) activity has been reported in patients with β-thalassaemia major (Neri *et al.*, 1984; Goicoa *et al.*, 1986; Akbar *et al.*, 1986; Akbar *et al.*, 1987). This observation has been attributed to an effect of iron overload because in one study (Akbar *et al.*, 1987) pretreatment of effector but not of target cells with desferrioxamine resulted in the recovery of NK activity of the patients' cells. Moreover, unpublished data of Cunningham-Rundles and her co-workers, following-up the changes in NK activity of peripheral blood cells from patients receiving an i.v. infusion of desferrioxamine at various times before and after the infusion, also indicate that in some cases the iron chelating therapy results in recovery of NK activity *in vivo* (Cunningham-Rundles, Hilgartner and Giardina, personal communication).

In a study of NK activity in patients with idiopathic haemochromatosis Good, Powell & Halliday (1988) could not find any alteration of that function, nor could they reproduce *in*

vitro an inhibitory action of iron, concluding that the diminished NK activity reported by others in β-thalassaemia is probably a result of the effect of the multiple blood transfusions received by these patients (Kaplan *et al.*, 1984).

The mixed lymphocyte reaction (MLR)

Studies of the MLR in iron overload *in vivo* have been done in homozygote β-thalassaemia patients (Dwyer *et al.*, 1987). Earlier, Keown & Descamps (1978) and Bryan *et al.* (1981) examined the effect of addition of ferric salts to MLR cultures of normal peripheral blood mononuclear cells. Both in thalassaemia major patients and *in vitro*, a diminished MLR was observed. In the studies *in vitro* we were able to demonstrate that pre-treatment of the effector but not of the target cells, was necessary for the inhibitory effect to be observed and that there was an individual variation which could be related to the HLA-A locus. Thus, HLA-A2 donors appeared to be less sensitive to the inhibitory action of iron than non-HLA-A2 donors (Bryan *et al.*, 1981).

Ferritin secretion by PHA-stimulated peripheral blood mononuclear cells

The observation that the individual variation in sensitivity to the inhibitory action of iron on the MLR could be related to HLA phenotype led us to consider whether a variation in ferritin secretion, observed with PHA-activated peripheral blood mononuclear cells in the presence of ferric citrate, could also be associated with HLA phenotype (de Sousa *et al.*, 1982). Studies of ferritin secretion measured by a modified indirect haemolytic plaque assay (Gronowicz, Coutinho & Melchers, 1976) of PHA-stimulated peripheral blood mononuclear cells in the presence of ferric citrate, from HLA-phenotyped normal blood donors, led to the demonstration that HLA-A-3 subjects produced a smaller number of haemolytic plaques than non-HLA-A3 subjects (Martins da Silva, *et al.*, 1982; Pollack *et al.*, 1983), signifying that control of ferritin secretion by activated cells seemed to be associated to the major histocompatibility complex (MHC). The fact that low ferritin secretion after activation seemed to be controlled by the HLA-A locus and related particularly to the antigen A-3, known to be the antigen most frequently represented in idiopathic haemochromatosis (Simon *et al.*, 1987), raises the question of whether these observations may have some relevance to the understanding of the behaviour of the macrophage system in idiopathic haemochromatosis (IH).

The macrophage in idiopathic haemochromatosis (IH)

Idiopathic haemochromatosis is an autosomal hereditary disease transmitted recessively, characterized by a failure of the still unknown mechanism(s) regulating the absorption of iron. As a consequence of this failure, a serum and tissue iron overload develops, reflected biochemically in high serum iron and ferritin levels, transferrin saturation in some cases exceeding 100%, with demonstrable non-transferrin bound serum iron and circulating low molecular weight iron complexes (Batey *et al.*, 1980; Gutteridge *et al.*, 1985). Clinically, the manifestations of the disease reflect the abnormal accumulation of iron in the liver (cirrhosis), pancreas (diabetes), heart, joints (arthropathies), skin (abnormal pigmentation), etc. (Sheldon, 1935; Jacobs, 1977, Powell *et al.*, 1980). An important advance leading to the early detection of family cases came with the demonstration that the frequency of the HLA antigen A3 is significantly higher in IH patients than in controls (Simon *et al.*, 1976) and that certain HLA haplotypes are more frequent in the disease group, namely, A3B7, A3B14, A11B35 and A11B5 (see Simon *et al.*, 1987 for review). Idiopathic haemochromatosis provides an almost exclusive 'experimental' model of iron overload, uncomplicated, from the immunological point of view, by the effects of multiple blood transfusion or splenectomy seen in β-thalassaemia. This raised the expectation that the observations made in transfusional iron overload, thought to be attributable to iron, would also be found in IH. Although sporadically there have been case reports of infection associated with IH iron overload (Abbott *et al.*, 1985; van Asbeck *et al.*, 1982), in general, infections do not constitute a major clinical problem in this disease. As mentioned earlier the association of infection with iron overload seems to pass through the demonstrable presence of intracellular iron in polymorphonuclear neutrophils (PMN) and macrophages. Rather surprisingly and in marked contrast to the heavy parenchymal iron overload, the amount of demonstrable intracellular iron in the macrophages in IH is minimal until the late stages of the disease (Ross *et al.*, 1975; Valberg *et al.*, 1975; Brink *et al.*, 1976).

This dichotomy between parenchymal iron overload and iron 'sparing' of the reticulo-endothelial system is perhaps most striking in the liver, where heavy iron deposits are seen in the parenchymal cells and only 'inconspicuous' amounts are seen in the Kupffer cells (Ross *et al.*, 1975). The evidence indicating that the absence of demonstrable iron in macrophages is in itself abnormal can be derived from comparative studies of IH patients and patients subjected to a diet containing excessive quantities of absorbable iron (Brink *et al.*, 1976). In a comparative study of South African negro subjects and idiopathic haemochromatosis patients, Brink *et al.* (1976) examined non-haeme iron concentrations in bone marrow and liver. In the subjects with a dietary iron overload, statistically significant correlations were observed between the amounts of non-haeme iron in both organs ($r = +0{\cdot}84$, $P = 0{\cdot}001$, $n = 66$); in the eight IH cases, however, higher amounts of iron were consistently measured in the liver than in the bone marrow or spleen (Brink *et al.*, 1976). One other disease in which tissue iron overload due to high dietary iron intake has been documented is Kaschin–Beck's disease. This disease was described in remote communities of nine provinces of Manchoukuo (Hiyeda, 1939) in patients found among farmers, hunters and woodcutters, who drank well-water unusually rich in iron (0·3 mg/l). The description of the pathology of this disease and its illustration provide clear evidence that there were substantial amounts of iron present in the splenic macrophages (Fig. 5 from Hiyeda, 1939). Thus the absence of demonstrable iron in IH macrophages can be viewed as evidence of some underlying metabolic abnormality. Studies of ferritin synthesis by IH macrophages have failed, however, to demonstrate any abnormality in the synthesis of this protein (Jacobs & Summers, 1981; Bassett *et al.*, 1982).

Mitogen responses

Studies of the peripheral blood mononuclear cell responses to non-specific mitogens have been done in patients with β-thalassaemia intermedia (Munn *et al.*, 1981) and β-thalassaemia major (Dwyer *et al.*, 1987). Decreased responses to PHA and conA were reported in the thalassaemia intermedia patients with serum iron levels higher than 200 μg/dl and serum ferritin higher

than 600 ng/ml. Pre-treatment of responding cells from normal donors with ferric citrate led to variable results depending on the dose of mitogen used. Inhibition was observed with the lowest doses of PHA (1·5 μg/ml and 3 μg/ml) enhancement with the highest doses of conA (29 μg/ml and 57 μg/ml), no effect with all doses of PWM (Munn *et al.*, 1981).

Unchanged responses to PWM were also seen in the β-thalassaemia intermedia patients regardless of serum iron or serum ferritin levels (Munn *et al.*, 1981). The response to *C. albicans* antigens was tested in patients with β-thalassaemia major (Dwyer *et al.*, 1987) and found to be negatively correlated to serum ferritin levels.

Implications

Dwyer *et al.* (1987) have wondered whether 'the challenge of understanding the basic immunological abnormalities in β-thalassaemia may be of greater importance than the consequences of the disease itself'. Their comment was prompted by the small number of AIDS cases reported among these patients. Indeed the finding of a diminished ability of macrophages to kill *C. pseudotropicalis* reported by Ballart *et al.* (1986) and the failure of PBM to respond to *C. albicans* reported by Dwyer *et al.* (1987) might lead to the expectation of severe progression of infections in the HIV positive cases. This does not appear to be the case (Dwyer *et al.*, 1987; Pardalos *et al.*, 1987; Hillgartner and Giardina, personal communication, 1988).

A great deal of work has illustrated the importance of iron in the development of bacterial infections (Weinberg, 1984; Griffiths & Bullen, 1987). To my knowledge, there is no comparable work illustrating the role of iron in viral infections. I have suggested that a possible explanation for the observation of HIV^+, clinically asymptomatic cases among the β-thalassaemia patients might reside on the impairing effects of iron on the expansion of the $CD4^+$ cell population (de Sousa, 1988). The challenge of the experience with the HIV^+, β-thalassaemia children, in my view, is to determine whether the reasons why they have not developed fulminating forms of AIDS, may be of relevance to the control of progression of the disease in other risk groups.

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