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# Functional defects in phagocytic cells from patients with iron overload

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## Summary

Phagocytic functions were studied in patients with iron overload. Phagocytosis of radiolabelled opsonised *Staphylococcus aureus* by mononuclear (MN) leucocytes and polymorphonuclear (PMN) leucocytes was measured in 15 and 16 patients, respectively. The intracellular killing capacity of MN and PMN leucocytes of seven and nine patients, respectively, and chemotaxis of PMN leucocytes of eight patients, were assessed also. These cellular functions were compared with phagocytic functions of controls tested on the same day, and with the normal ranges of phagocytic cell functions obtained with MN and PMN leucocytes from 48 and 59 healthy donors, respectively. One or more phagocytic functions were impaired in 62·5 per cent of the patients. Comparison of the various phagocytic functions in patients and simultaneously tested controls showed a significant decrease of the mean phagocytic capacity of the patients' MN and PMN leucocytes ($P < 0{\cdot}015$ and $P < 0{\cdot}03$, respectively), as well as the mean bactericidal activity of the MN leucocytes ($P < 0{\cdot}05$) and the mean chemotactic responsiveness of the PMN leucocytes ($P < 0{\cdot}025$). Patients with excess iron must be regarded as compromised hosts, not only because of the increased availability of iron for bacterial growth, but also because of the associated functional impairment of monocytes and granulocytes.

## Introduction

Phagocytic cells play a crucial role in the host defence against invading micro-organisms. Since it became known that the underlying defect in patients with chronic granulomatous disease is a diminished bactericidal activity of the phagocytic cells,[1] an increasing number of functional disorders of MN and PMN leucocytes in patients with various diseases has been described.[2–9] All such patients are more susceptible to infection.

Recently we described a diminished phagocytic activity of the MN leucocytes in a patient with haemochromatosis who suffered from *Listeria monocytogenes* meningitis.[10] After phlebotomies, this defective phagocytosis became completely normal. It was suggested that not only the increased availability of iron for bacterial growth, but also a deleterious effect of iron on the phagocytic cells favoured susceptibility of the iron-overloaded host to infection with *L. monocytogenes*. Other studies of the role of iron in host defence have also suggested that iron predisposes to infection.[11] Iron stimulates bacterial

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Table I *Clinical characteristics of patients, and phagocytic function of mononuclear (MN) leucocytes and polymorphonuclear (PMN) leucocytes of patients and healthy controls*

| Patient number | Diagnosis | Age (years) | Percentage serum iron saturation[a] | Serum ferritin[b] (μg/l) | Liver iron* | Phagocytosis[c] percentage MN leucocytes | Phagocytosis[c] percentage PMN leucocytes | Remarks |
|---|---|---|---|---|---|---|---|---|
| 1 | Idiopathic haemochromatosis | 58 | 81 | 2050 | +++ | 45 | 80 | Diabetes mellitus, history of *L. monocytogenes* meningitis |
| 2 | Idiopathic haemochromatosis | 60 | 77 | 2570 | +++ | 86 | 99 | |
| 3 | Idiopathic haemochromatosis | 67 | 88 | 1300 | +++ | 67 | 77 | Diabetes mellitus, recurrent respiratory-tract infections |
| 4 | Idiopathic haemochromatosis | 44 | 91 | 930 | +++ | 29 | 89 | |
| 5 | Idiopathic haemochromatosis | 68 | 69 | 240 | +++ | 78 | 88 | Phlebotomy |
| 6 | Congenital sideroblastic anaemia | 26 | 89 | 1800 | +++ | 97 | 91 | Deferoxamine subcutaneously |
| 7 | Congenital sideroblastic anaemia | 53 | 74 | 2800 | +++ | 11 | 35 | |
| 8 | Congenital sideroblastic anaemia | 58 | 80 | 3315 | +++ | ND | 88 | History of tuberculosis |
| 9 | Congenital sideroblastic anaemia | 51 | 94 | 2845 | +++ | 63 | 56 | Diabetes mellitus, recurrent respiratory-tract infections |
| 10 | Congenital sideroblastic anaemia | 33 | 88 | 3500 | +++ | 67 | 64 | |
| 11 | Acquired sideroblastic anaemia | 79 | 96 | 2500 | ND | 95 | 93 | Blood transfusions |
| 12 | Congenital dyserythropoietic anaemia type II | 45 | 60 | 2130 | ++ | 29 | 82 | Recurrent respiratory-tract infections and sinusitis |
| 13 | Refractory anaemia | 59 | 88 | 3360 | +++ | 29 | 61 | Blood transfusions |
| 14 | Homozygous sickle-cell anaemia | 18 | 57 | 960 | ND | 91 | 88 | Blood transfusions |
| 15 | Chronic alcoholism | 52 | 94 | 570 | ND | 50 | 80 | |
| 16 | Porphyria cutanea tarda | 42 | 70 | 360 | ++ | 58 | 81 | Diabetes mellitus |

Normal ranges [a] 25–60 per cent, and [b] 10–150 μg/l, respectively. Per cent phagocytosis [c] represents the percentage of the total number of bacteria in the medium taken up by MN and PMN leucocytes after 12 minutes incubation. Phagocytic function of PMN and MN leucocytes of healthy donors was tested in a separate study.

Mean uptake (±SD) of Staphylococci by PMN and MN leucocytes was $84 \pm 15$ ($n = 48$) and $71 \pm 24$ ($n = 59$) respectively.

* Graded according to Ploem (35); Normal ±/+.

ND, Not done.

growth,[12] and *in vitro* studies have shown that iron impairs leucocyte antibacterial activity.[10, 13–15] These findings led us to investigate MN and PMN leucocyte function in patients with iron overload. Phagocytosis, intracellular killing, and chemotaxis of the phagocytes were assessed. Several significant defects in various MN and PMN leucocyte functions were observed.

## Patients and methods

### Patients

Sixteen patients aged 18–79 years, with iron overload and 16 healthy controls, aged 18–69 years, all without any clinical evidence of infection, were included in the study. The iron status of the patients was assessed by measuring the concentrations of iron, transferrin and ferritin in the serum and in the urine (after intramuscular injection of deferoxamine) as well as in bone marrow and liver biopsy specimens. The controls were healthy members of the laboratory staff who were regularly subjected to routine blood tests.

Relevant clinical characteristics of the patients are summarised in Table I. Five patients had idiopathic haemochromatosis and 11 had secondary iron overload. The patients' serum ferritin concentration, which offers a useful clinical index of the size of body iron stores in iron overload,[16] ranged from 240 to 3500 μg/l (normal range 10–150 μg/l). During assessment of the leucocyte functions, one patient was being treated with subcutaneous deferoxamine, and one with weekly phlebotomies. Three of the patients regularly received blood transfusions, four had diabetes mellitus with well-controlled blood sugar, and three suffered from recurrent respiratory-tract infections. One patient had a history of *L. monocytogenes* meningitis and another of tuberculosis.

Phagocytic functions of patients and controls were assessed simultaneously in order to exclude experimental errors. All results were compared with the normal ranges (mean ± 2 SD) for phagocytosis, killing capacity, and chemotaxis of MN and PMN leucocytes as obtained in a separate study with 48 and 59 healthy donors, respectively, aged 16–74 years.

### Leucocytes

Leucocytes were harvested from 30 ml heparinised venous blood samples according to a modification of Böyum's method.[17] Briefly, erythrocytes were allowed to settle for one hour in 6 per cent Dextran '7' (Fluka AG, Buchs, Switzerland). The leucocyte-rich plasma was centrifuged at 160 **g** for 5 minutes, and the pellet was resuspended in Eagle's minimum essential medium (MEM) and layered on a Ficoll-Isopaque gradient (Ficoll, Nyegaard, Pharmacia, Uppsala, Sweden; Isopaque, Oslo, Norway). Ater centrifugation at 200 **g** for 35 minutes, the MN and PMN leucocytes were removed separately and washed twice in MEM. Total and differential leucocyte counts were performed. The leucocyte pellets finally obtained were resuspended to a concentration of $5 \times 10^6$ MN/ml and $5 \times 10^6$ PMN/ml in Hanks' balanced salt solution containing 0·1 per cent gelatin (GHBSS). Viability was evaluated by trypan blue exclusion and exceeded 95 per cent.

**Culturing and radiolabelling of bacteria**

*Staphylococcus aureus* Ev., a clinical isolate, was grown for 18 hours in Mueller-Hinton broth (Difco, Detroit, MI, U.S.A.) containing 0·02 mCi [$^3$H]methyl-thymidine (specific activity 5 Ci/mmol; Amersham, Bucks, U.K.), washed three times in phosphate-buffered saline (PBS), pH 7·4, and suspended to a concentration of $2{\cdot}5 \times 10^8$ colony-forming units (cfu)/ml PBS. These bacteria were incubated (37 °C) in pooled normal human serum (5 per cent in GHBSS) from 10 healthy donors. After 30 minutes the serum was removed by centrifugation, and the opsonised bacteria were resuspended in GHBSS to a concentration of $5 \times 10^7$ cfu/ml.

**Phagocytosis and killing assay**

Phagocytosis and killing were quantitatively measured in duplicate as described elsewhere.[18] Briefly, 0·2 ml of the suspension of opsonised bacteria was added to 0·2 ml of the MN or PMN leucocyte suspension in four polypropylene vials (Biovials, Beckman, Chicago, Il., U.S.A.). The final bacteria-to-phagocyte ratio was 10:1. The mixtures were incubated in a shaking waterbath at 37 °C. After 2, 6 and 12 minutes, phagocytosis was stopped in one of the vials by adding 3 ml ice-cold PBS. Leucocyte-associated bacteria, i.e. bacteria ingested by and attached to the MN or PMN leucocytes, were then separated from non-leucocyte-associated bacteria by three cycles of centrifugation (160 **g** at 4 °C). The leucocyte pellets finally obtained were dispersed in 2·5 ml scintillation liquid (toluene containing fluoralloy TLA, Beckman, and 20% Biosolve, Beckman). Leucocyte-associated radioactivity was measured in a liquid scintillation counter (Mark II, Nuclear Chicago, Chicago, Il., U.S.A.) and expressed as a percentage of the total added radioactivity, i.e. per cent uptake of added *S. aureus*. Total added radioactivity (representing both non-leucocyte-associated and leucocyte-associated bacteria) was determined in the pellet of the fourth vial, obtained by centrifugation at 1600 **g** for 15 minutes.

The percentage of viable leucocyte-associated bacteria at 12 minutes was determined in a sample taken from the washed cell suspension, by means of a standard pour-plate technique. The results are expressed as per cent intracellular killing of *S. aureus* by MN and PMN leucocytes after 12 minutes of incubation.

**Chemotaxis**

Chemotaxis of PMN leucocytes was determined under agarose according to the method of Nelson and colleagues.[19] Seven µl of a suspension containing $5 \times 10^7$ PMN leucocytes/ml was brought into the centre well. The outer well contained zymosan-activated serum (ZAS) as attractant, and the inner well was filled with GHBSS. The plates were incubated for 18 hours at 37 °C in a humidified atmosphere containing 5 per cent carbon dioxide in air. Migration distances were measured microscopically and the results expressed as mm migrated towards ZAS minus the mm of spontaneous migration towards GHBSS. The results represent the mean values of directed migration of samples incubated in triplicate on two plates.

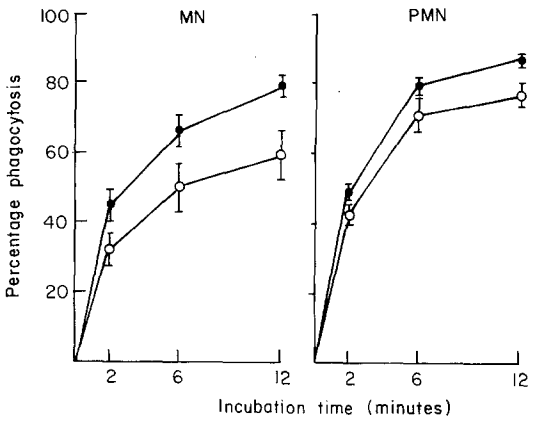


Fig. 1. Mean phagocytosis values for mononuclear (MN) leucocytes and polymorphonuclear (PMN) leucocytes derived from 15 and 16 patients with iron overload (○), respectively, and from controls (●). Data represent the mean uptake of *S. aureus* by MN and PMN leucocytes after 2, 6 and 12 minutes of incubation. Percentage phagocytosis represents the percentage of the total number of bacteria taken up from the medium by MN and PMN leucocytes. Bars indicate SEM.

### Statistical analysis

Two-way analysis of variance[20] was used to compare the phagocytic capacity of MN and PMN leucocytes from patients with iron overload and controls after the various times of incubation. To compare the intracellular killing scores and migration distances of MN and PMN leucocytes of the two populations, we used the one-sided Wilcoxon rank-sum test.[21]

## Results

### Phagocytosis

Uptake of *S. aureus* by MN and PMN leucocytes of 15 and 16 controls, respectively, and of patients with iron overload, is shown in Fig. 1. The mean phagocytic capacity of the patients' MN and PMN leucocytes assessed after 2, 6 and 12 minutes of incubation was significantly lower than that of the control MN and PMN leucocytes ($P < 0{\cdot}015$ and $P < 0{\cdot}03$), respectively. As can be seen in Table 1, 5 of 15 patients had MN leucocytes and 4 of 16 patients had PMN leucocytes with a phagocytic capacity below the normal range obtained with MN and PMN leucocytes from 48 and 59 donors, respectively (only the values for phagocytosis after 12 minutes of incubation are shown). In two patients with defective MN phagocytosis, bacterial uptake by PMN leucocytes was also impaired.

### Bactericidal activity

The mean killing capacity of MN leucocytes, which was studied in seven patients, was significantly lower than intracellular killing by control MN leucocytes ($P < 0{\cdot}05$) (Fig. 2). Also in Fig. 2 is shown the normal range of bactericidal activity of MN and PMN leucocytes of 52 donors. As may be seen, the killing capacity of MN leucocytes of three patients was below the normal

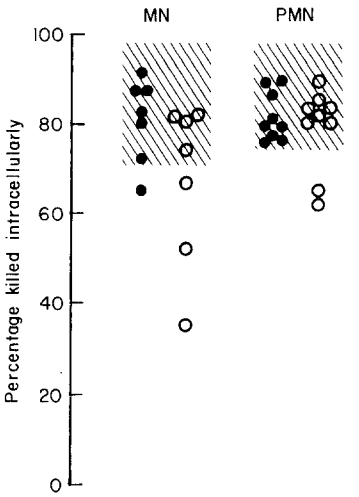


Fig. 2. Intracellular killing of *S. aureus* by mononuclear (MN) leucocytes and polymorphonuclear (PMN) leucocytes from 7 and 9 patients with iron overload (○), respectively, and from controls (●). Percentage killed bacteria represents the percentage of leucocyte-associated bacteria killed after 12 minutes of incubation. The shaded areas indicate the normal range (mean ± SD) of the killing capacity of MN and PMN leucocytes of 52 donors.

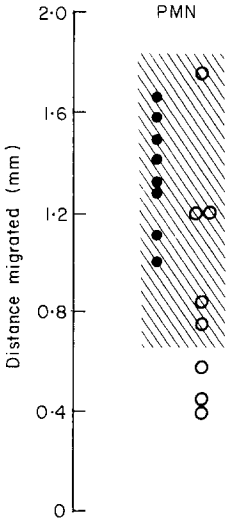


Fig. 3. Chemotaxis of polymorphonuclear (PMN) leucocytes from 8 patients with iron overload (○), and from controls (●). Migration distances represent the distance migrated in 18 hours by phagocytes under agarose. The shaded area represents the normal range (mean ± SD) of chemotaxis by PMN leucocytes of 23 donors.

range, whereas only one control had MN leucotyes with a killing capacity below normal. In two out of nine patients, the PMN leucocytes had a killing capacity below the normal range, and the MN leucocytes of both of these patients showed similar impairment. In only one patient were the phagocytosis and killing capacity of both MN and PMN leucocytes below the normal range.

### Chemotaxis

Compared with control PMN leucocytes, migration of PMN leucocytes from eight iron-overloaded patients was significantly decreased ($P < 0{\cdot}025$) (Fig. 3). In three patients, PMN leucocyte chemotaxis was below the normal range obtained from 23 donors. Both chemotaxis and phagocytosis were impaired in two patients.

## Discussion

The results show that in 10 of the 16 patients (62·5 per cent) with iron overload in whom phagocytosis, intracellular killing, and/or chemotaxis of MN or PMN leucocytes were assayed, one or more of these functions were below the normal range. When the phagocytic functions of patients were compared with those of simultaneously tested controls, a significant reduction was found in the phagocytic capacity, the MN leucocyte bactericidal activity, and in the leucocyte chemotactic responsiveness of the patient's MN and PMN leucocytes.

Although our patients had various underlying diseases, they all suffered from iron overload; this was established in several ways (see Table I). Certain diseases such as diabetes[5, 22–24] and acute infections[6] are sometimes associated with functional defects in phagocytic cells. Functional abnormalities in diabetic PMN leucocytes disappear when the blood sugar concentration is controlled.[22–24] During assessment of the various phagocytic functions, patients as well as controls were free of infection, and the blood sugar of the four patients who suffered from diabetes mellitus was well controlled. It is therefore unlikely that the reduced phagocytic functions observed in the present study were related to one of these conditions. The assumption that the iron overload was responsible for our observations is supported by our recent report concerning a patient with haemochromatosis and impaired phagocytic function of the MN leucocytes, the function of which became normal after several phlebotomies.[10]

It was not possible to establish significant correlation between one of the measurements used to assess iron overload and any of the phagocytic cell functions tested. This makes the results not easy to explain. It is possible that our assay systems were not optimum for detecting every defect in the function of the phagocytic cells. In our standard assay we used a PMN leucocyte : bacteria ratio of 1 : 10. It is possible that with different ratios defects in more patients might have been seen. Our assay, however, has been used to detect defects in many different patients. We were able to show a diminished phagocytosis with this assay in patients with recurrent staphylococcal infections.[6] It is also possible that our group of patients was too heterogeneous. On the other hand toxicity of iron is non-specific, and many features such as the concentration of iron in a particular system or the sensitivity of the target to iron-induced alterations may play a role. Furthermore, since the noxious effect of iron seems to be the result of oxidation reactions,[25] factors involved in antioxidant protection such as the concentrations of ascorbic acid and vitamin E in the patients might also have influenced the results of the assays.

Iron plays a crucial role in the host defence against micro-organisms.[11, 12] It is used by bacteria, and a decrease in available iron – due, for example, to

binding by lactoferrin – results in bacteriostasis.[26] Iron, however, may also affect the phagocytic system. There are several reports concerning the influence of iron on phagocytic cells *in vitro*.[10,11,13–15] Excess iron decreases chemotactic responsiveness[15] and enhances the ability of ingested micro-organisms to proliferate within PMN leucocytes by lowering bactericidal capacity.[13] We found a dose-dependent inhibitory effect of iron on the phagocytic capacity of MN and PMN leucocytes[19] and, in the same study, ferritin isolated from the plasma of a patient with idiopathic haemochromatosis did not suppress phagocytic cell function. Our results, and the findings of other investigators, suggest that functional defects of phagocytes in patients with iron overload may be due to iron. Nevertheless, how iron affects the phagocytes is not clearly understood. Impairment of the microbicidal activity of PMN leucocytes in the presence of ferrous ions has been attributed to inactivation of cationic proteins[13] such as myeloperoxidase,[27] and to the reduction of hydrogen peroxide to water,[14] so that this compound is not available for bacterial killing. Reduction of hydrogen peroxide induced by ferrous ions, however, leads in the first instance to the formation of a hydroxyl radical[28,29] which can destroy almost all known biomolecules.[30,31] Although a hydroxyl radical can contribute to the bacterial activity of phagocytes,[32] it has also been held responsible for damage to the white blood cell itself.[33] Thus, it is possible that the impaired phagocyte in patients with iron overload is due to enhanced production of toxic oxygen species. This hypothesis is supported by the recent report of increased generation of hydroxyl radicals by human neutrophil leucocytes during exposure to iron.[34]

In summary, excess of iron may enhance bacterial virulence[11,12] and may impair the host's phagocytic cellular defences against micro-organisms. It is important that the phagocytic cells of patients with iron overload are tested. Further study may lead to the conclusion that patients with iron overload should be regarded as immunocompromised hosts especially vulnerable to infection.

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