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A POSSIBLE ROLE FOR FERRITIN DURING INFLAMMATION

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Inflammation induces the hepatic synthesis of the iron storage protein (apo)ferritin, which is released into the circulation, and behaves as an acute phase protein. The biological significance of the extracellular rise in serum (apo)ferritin is unknown.

We have observed that (apo)ferritin will stimulate superoxide production from neutrophils in the presence of cytochalasin B across a physiologically appropriate concentration range. We therefore propose that extracellular ferritin has an important role in host defence against bacteraemia by stimulating oxidative metabolism.

Key words: Ferritin; inflammation; superoxide production; neutrophils; infection.

INTRODUCTION

Iron and iron binding proteins have a complex role to play in human metabolism, particularly during both the acute and chronic phases of inflammation[1,2,3]. A significant reduction in serum iron occurs within hours of the initiating event and precedes the development of fever[4]. It has been widely speculated that the metabolic adjustment which results in this deprivation of iron is part of the body's defence mechanism against invading bacteria (for review — see Ref. 5).

Normally, iron in body fluids is very strongly attached to iron binding proteins, such as transferrin in the blood and lymph, and lactoferrin in secretions and in neutrophils[6]. How iron influences immune mechanisms remains the subject of speculation, but it appears to be an essential requirement for an effective inflammatory response[7]. The anaemia of chronic inflammation is characterised by a decreased plasma iron, decreased total iron binding capacity of the plasma and decreased saturation of transferrin with iron[4,8,9]. In one study of inflammation in the rat, increased liver synthesis of the intracellular iron storage protein ferritin preceded a reduction in serum iron levels[10]. This suggests that the inflammatory process itself is directly responsible for

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the induction of ferritin synthesis. Ferritin can therefore be considered to be an acute phase protein; its hepatic synthesis like that of other acute phase proteins being induced and maintained by the secretion of interleukin 1 (1L — 1 also known as leucocyte endogenous mediator, LEM)[2].

The rise in serum ferritin during chronic inflammation may reach levels that are in excess of 250% of initial values[11,12]. However, the biological advantage of the reticuloendothelial ferritin synthesis and the secondary rise of serum ferritin, as an acute phase reactant, is not known, although again it has been speculated that this may also play some indirect role in host defence[13].

The bactericidal properties of phagocytic cells are dependent, at least in part, on the release of oxygen free radicals into the phagocytic vacuole[14,15]. The release of free radicals can be initiated by membrane stimulation of the cell independently of phagocytosis by macromolecules such as aggregated IgG and complement[16]. Our aim in this paper was to investigate the possible influence of the ferritin molecule on the release of these toxic radical species from activated neutrophils.

MATERIALS

Phorbol myristate acetate (PMA), Cytochrome c (Type III, from horse heart), cytochalasin B, horse-radish peroxidase, superoxide dismutase, ferritin (Type I from horse spleen), transferrin (Substantially iron free), lactoferrin (from human milk 0.14 bound Fe/mole) and human albumin were all obtained from Sigma Chemical Co. Ltd, Poole, Dorset, U.K. Cytochalasin B and PMA were dissolved in DMSO and stored in aliquots at −70°C.

Apoferritin was prepared from ferritin by dialysis against 0.1 M thioglycollic acid in 0.1 M sodium acetate buffer pH 4.3, followed by exhaustive dialysis against 20 mM phosphate saline buffer.

Human ferritin and the iron loaded ferritin fractions (horse spleen) were a gift from the Department of Biochemistry, University of Sheffield. The fractions were prepared according to the method of Gutteridge *et al.*[17].

Medium RPMI 1640 was obtained from FLOW Laboratories. Ficoll was purchased from Pharmacia and Hypaque from Sterling Laboratories. All other reagents were of the highest quality available from BDH Ltd.

Synovial fluids were obtained for diagnostic or therapeutic purposes from patients with classical or definite RA presenting at the Department of Rheumatology, the Medical School, Birmingham.

METHODS

PMN Separation

Normal human neutrophils were separated from fresh heparinised whole blood using Ficoll-Hypaque density centrifugation followed by hypotonic lysis of erythrocytes. The blood was layered onto Ficoll-Hypaque (S.G. 1.114) and centrifuged at 200 g for 30 min. The neutrophil layer was removed and the cells washed with PBS. Erythrocytes contaminating the preparation were lysed with NH_4Cl buffer and after a final wash with PBS the cells were resuspended in RPMI 1640 containing 2 mM EDTA (to prevent cell aggregation). The neutrophils were then counted and resuspended in

medium to give a concentration of 2×10^6 cells/ml. Cell viability was tested by Trypan Blue exclusion and differential counts were made on smears stained with Leishmann's stain. All preparations were >95% viable and contained >95% PMN's.

Measurement of Superoxide and Hydrogen Peroxide

Superoxide radical ($O_2^{\bar{\cdot}}$) and hydrogen peroxide produced by neutrophils in culture were measured using a modification of the method developed for macrophages by Pick and Mizel (1981)[18]. This method depends on

a) the reduction of cytochrome c by the $O_2^{\bar{\cdot}}$ radical and

b) the horse radish peroxidase (HRPO) dependent conversion of phenol red by hydrogen peroxide (H_2O_2).

a) Superoxide Measurement Cultures of prepared human neutrophils were set up in 96 well microtitre plates. Row 1 contained medium only (to blank the Multiskan). 100 μl of cell suspension was pipetted into the remaining wells giving a final concentration of 2×10^5 cells/well. 10 μl of cytochalasin B (2 μg/ml) was added to half the wells which were incubated at 37°C for 5–10 mins. (Cytochalasin B is added to inhibit vacuole formation and thereby phagocytosis [As ferritin may aggregate, we wished to exclude any non specific effect of a phagocytosis induced increase in oxidative metabolism]). Cytochrome c solution (320 μM) was made up in phenol-red-free balanced salt solution and contained the appropriate test protein concentration. 100 μl of cytochrome c solution was added to each well. Half the wells in each row contained superoxide dismutase (300 U/ml [1 unit of SOD activity is defined as the amount of SOD required to inhibit the rate of reduction of cytochrome c by 50%]). The plate was then covered and placed in an incubator for 1 hour. (37°C, 95% air, 5% CO_2). After 1 hour the plate was removed and read at 550 nm on a Multiskan MC photometer (Flow Laboratories) interfaced to a microcomputer.

The amount of $O_2^{\bar{\cdot}}$ produced was calculated from the superoxide dismutase inhibitable reduction of cytochrome c, using the extinction coefficient of reduced cytochrome c (21.1×10^3 m^{-1} cm^{-1}). The results were expressed as nmoles of $O_2^{\bar{\cdot}}$ produced/ 60 mins/10^6 cells.

b) Hydrogen Peroxide Measurement For the measurement of H_2O_2 the cells were treated in the same way as for $O_2^{\bar{\cdot}}$ but after plating out and treatment with cytochalasin B, 100 μl of a phenol-red solution containing HRPO (8 Units/ml [1 unit will form 1 mg purpurogallin in 20 seconds from pyrogallol at pH 6 at 20°C]) was added to the cells. After 30 mins incubation the plate was removed from the incubator and 10 μl 1 M NaOH was added to each well to kill the cells and to produce the colour reaction which was detectable on the Multiskan MC at 610 nm.

Sucrose density gradient analysis of synovial fluid

Sucrose density gradient centrifugation was carried out as described by Worwood *et al.*[19]. Synovial fluid was diluted with an equal volume of 0.05 M sodium acetate, adjusted to pH 4.8 with 1 M acetic acid, heated rapidly to 70°C and maintained at

70–75°C for 10 mins. After cooling and centrifugation (10,000 g for 15 min) the supernatant was filtered and pH adjusted to 6.8 by stirring and adding 0.1 M NaOH dropwise. The human spleen ferritin and synovial fluid extracts were layered on sucrose gradients (approx 200–1000 ng ferritin in each case). Ferritin concentrations were determined by immunoradiometric assay.

RESULTS

When normal human neutrophils were mixed with apoferritin (horse spleen) in the presence of cytochalasin B, the cells were stimulated to produce $O_2^{\bar{\cdot}}$ radical in a dose dependent manner (Figure 1) across the serum concentration range of ferritin (0–10 μg/ml) found in health and disease. The highest activity was approximately

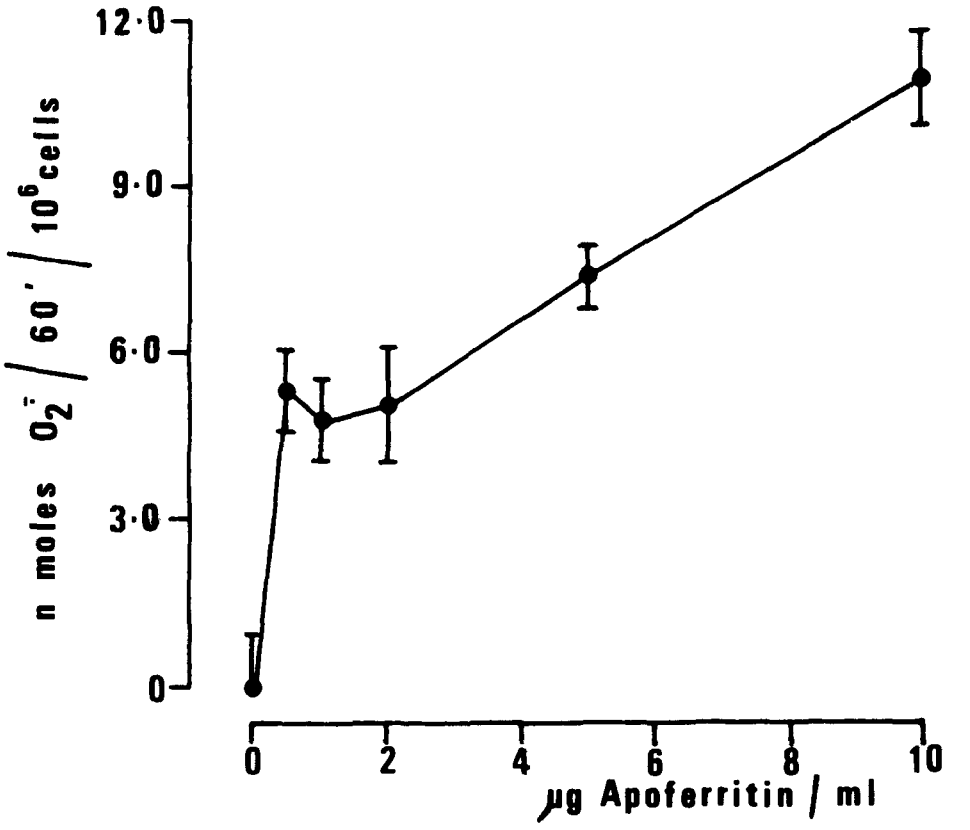

FIGURE 1 Stimulation of superoxide production from normal human neutrophils in the presence of cytochalasin B by varying doses of horse spleen apoferritin. The cells were incubated at 37°C with cytochrome c in the presence and absence of superoxide dismutase and the amount of superoxide produced in one hour was calculated using the extinction coefficient of reduced cytochrome c at 550 nm (21.1 × 10^3 M^{-1} cm^{-1}). Hydrogen peroxide production was not stimulated by the presence of apoferritin.

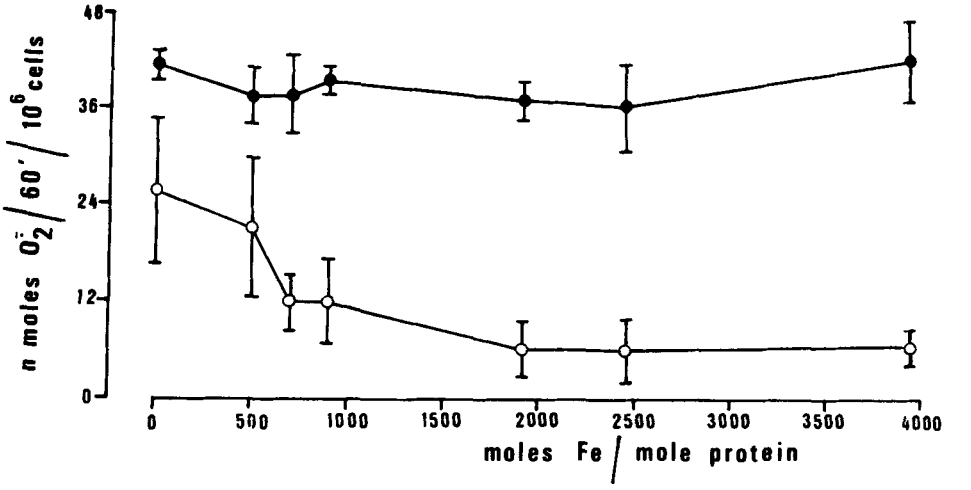

FIGURE 2 Effect of varying levels of iron saturation of the ferritin molecule on superoxide production from normal human neutrophils in the presence and absence of cytochalasin B. (—●—● no cytochalasin B, —○—○— + cytochalasin B). The bars indicate the SEM. The concentration of (apo)ferritin was 5 μg/ml. The iron content of ferritin was determined by the 2,2-bipyridyl method of Drysdale and Munro (see Ref. 17). The cells were incubated at 37°C with cytochrome c in the presence and absence of superoxide dismutase and the amount of superoxide produced in one hour was calculated using the extinction coefficient of reduced cytochrome c at 550 nm ($21.1 \times 10^3\ M^{-1}\ cm^{-1}$). Hydrogen peroxide production was unaffected by the presence of (apo)ferritin.

equivalent to 15% of the activity induced by 20 nM PMA in an average population of neutrophils.

When ferritin's iron loading was increased in stages as shown in Figure 2, its ability to stimulate neutrophil O_2^- production decreased with increased iron loading. In the absence of cytochalasin B, levels of superoxide production remained at a high level independent of the iron loading of the ferritin molecule. The membrane stimulatory component was the predominant cause of superoxide release from cells until saturation of ferritin with iron reached approximately 15%.

Figure 3 illustrates the response of peripheral blood neutrophils from several individuals after stimulation by both horse and human apoferritin. All individuals' neutrophils could be significantly stimulated to produce O_2^- by apoferritin in the presence of cytochalasin B. Human spleen apoferritin was more stimulatory toward neutrophils, increasing individual responses by approximately 50% at a concentration of 10 μg/ml.

A comparison of the effects of incubation of neutrophils with other major ferroproteins on O_2^- production was undertaken. The iron binding proteins transferrin and lactoferrin did not stimulate normal human neutrophils to produce O_2^- when added at normal physiological concentrations. No H_2O_2 was detectable from human neutrophils by the procedure used when the cells were mixed with any ferroprotein including

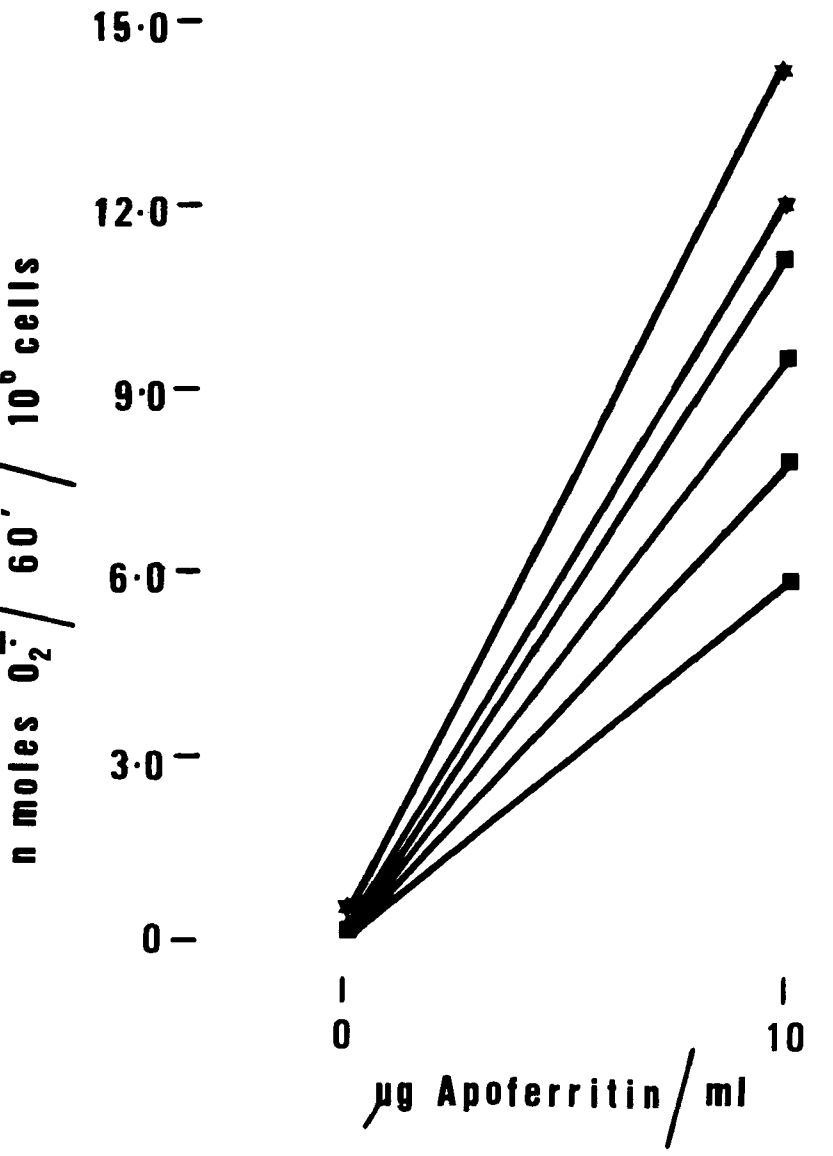

FIGURE 3 The production of superoxide from purified human neutrophils from several individuals in the presence and absence of horse (—■—■—) spleen and human (—★—★—) spleen apoferritin. The cells were incubated with cytochrome c for 1 hr at 37°C in the presence of cytochalasin B and the results are expressed as nmoles $O_2^{\cdot-}$/60 min/10^6 cells.

ferritin. Figure 4 roughly defines the degree of iron saturation of synovial fluid ferritin as assessed by sucrose density centrifugation. Normal human spleen ferritin, isolated from an iron loaded patient, containing approximately 0.3 µg Fe/pg protein,

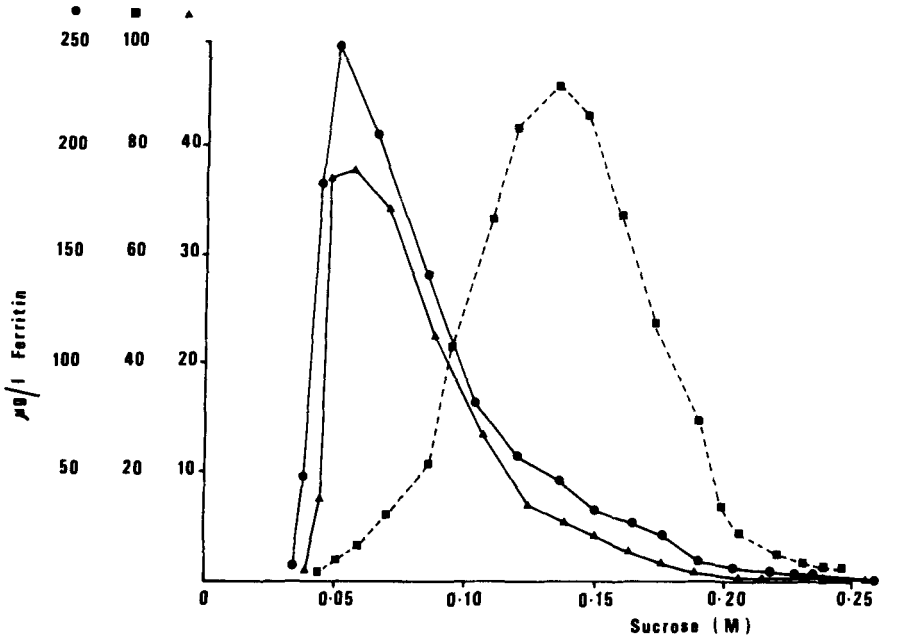

FIGURE 4 Sucrose density centrifugation of human spleen ferritin and synovial fluid ferritin. (—■—■—) human spleen ferritin — 600 g/l, (—●—●—). Synovial fluid ferritin extract 1.–920 g/l. (—▲—▲—) Synovial fluid ferritin extract 2.–190 g/l.

sedimented at a density of approximately 0.15 M sucrose. Ferritin isolated from 2 representative inflammatory joint fluids sedimented at a concentration of 0.05 M sucrose, indicating a far lower iron content than that of human spleen ferritin. The ferritin observed as shoulders on the sedimentation curve, indicate the relatively small amounts of ferritin containing iron within these fluids.

DISCUSSION

Bacterial killing involves a variety of different neutrophil mediated events, all of which are induced by either degranulation of the neutrophil, or the induction of the respiratory burst. The latter process involves an increase in oxygen uptake, $O_2^{\bar{\cdot}}$, H_2O_2 production and hexose mono-phosphate shunt activation. The $O_2^{\bar{\cdot}}$ alone appears relatively ineffective in killing bacteria, but in conjunction with H_2O_2 and an iron catalyst can generate the highly toxic hydroxyl radical ($OH^{\cdot}$) which has this capacity[20].

$O_2^{\bar{\cdot}}$ anion is produced by a membrane bound enzyme (NAD(P)H oxidase) which

can be activated independently of phagocytosis by non particulate stimuli (e.g. phorbol myristate acetate, FMLP, complement and denatured IgG)[21].

At concentrations comparable to those found in both acute and chronic inflammatory disease, both iron loaded ferritin and apoferritin have the capacity to stimulate the production of $O_2^{\bar{}}$ from normal human neutrophils. Apoferritin and ferritin which contained up to 700 atoms Fe/molecule maintained this effect in the presence of cytochalasin B which inhibits phagocytosis. The stimulation of $O_2^{\bar{}}$ production by apoferritin appears to be therefore a membrane mediated event. Lactoferrin, an iron binding protein of neutrophils appears to be released coincidentally following cell membrane stimulation of neutrophils with either FMLP or phorbol myristate acetate. It is argued, on the basis of injection of lactoferrin to rats[23], that lactoferrin will induce hypoferraemia by directly binding iron (behaving as a natural chelator) within the serum. Lactoferrin bound iron is then rapidly cleared by the reticuloendothelial cell[24], with the iron predominantly stored as ferritin. Depriving invading microorganisms of iron via these proteins, may be an important defence mechanism as the serum is rendered bacteriostatic. The secondary release of iron poor ferritin into the vascular compartment now forms an integrated part of the defence mechanism, by stimulating the respiratory burst and providing a bactericidal capacity.

In order to evaluate the in vivo relevance of our in vitro experimental findings, we determined the iron status of ferritin present in inflammatory joint fluid. As shown in our results, when ferritin from synovial fluid was examined by sucrose density centrifugation it was found to contain zero or minimal iron. Similar results have previously been obtained for serum ferritin[25,26].

These results would suggest that extracellular ferritin is essentially apoferritin, and though this protein has some capacity to incorporate exogenous iron this is poor in comparison with the major extracellular transport protein transferrin and probably of no physiological significance.

Iron deficient subjects may mount a modest rise in serum ferritin after an inflammatory insult but the level when measured in serum rarely exceeds 50 $\mu g/l$[27]. At this concentration superoxide production from neutrophils is low. Both children and rodents with an iron deficient anaemia exhibit a marked reduction in bactericidal efficiency of polymorphonuclear leucocytes, and also a diminished rate of nitroblue tetrazolium reduction by leucocytes. Opsonisation and phagocytosis are however unaffected[28,29].

In contrast in multitransfused thalassaemia patients spontaneous neutrophil superoxide production is increased and correlates with serum ferritin levels[30].

Our data suggests that the rise in extracellular serum (apo)ferritin induced by inflammation contributes to host defence against bacteraemia by augmenting oxidative metabolism of circulating neutrophils.

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