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Bleomycin-detectable iron in knee-joint synovial fluid from arthritic patients and its relationship to the extracellular antioxidant activities of caeruloplasmin, transferrin and lactoferrin

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Some 40% of knee-joint synovial fluids from arthritic patients show the presence of bleomycin-detectable iron. This is released from a protein component of the fluid to bleomycin at acidic pH values. Patients whose fluids release iron have lower contents of transferrin, lactoferrin and caeruloplasmin than do patients whose fluids do not release iron to bleomycin. These proteins are important extracellular antioxidants, and measured antioxidant activities are extremely low in the iron-releasing fluids. The propensity of some fluids to release iron at low pH values, characteristic of the microenvironment beneath adherent macrophages, coupled with their decreased antioxidant protection against iron-stimulated oxygen-radical damage, might explain previously reported correlations between clinical disease severity, lipid peroxide content and the presence of bleomycin-detectable iron [Rowley, Gutteridge, Blake, Farr & Halliwell (1984) Clin. Sci. **66**, 691–695].

INTRODUCTION

Contact between phagocytic cells and opsonized bacteria or immune complexes, leads to a 'respiratory burst' in which dioxygen is consumed and intermediate reduced forms of dioxygen are released (Babior, 1978*a*,*b*). The reduced oxygen products, superoxide ($O_2^{\cdot -}$) and H_2O_2, are not particularly reactive unless a suitable transition-metal catalyst is available to facilitate a further reductive step to form the aggressive hydroxyl radical ($OH^{\cdot}$). These 'active oxygen' species facilitate microbial killing by phagocytic cells.

In rheumatoid arthritis it has been suggested that an aggressive oxidant such as the $OH^{\cdot}$ radical may be responsible for membrane damage, hyaluronic acid degradation, inactivation of proteinase inhibitors and destruction of several antioxidants within the synovial membrane (McCord, 1974; Greenwald & Moy, 1980; Blake *et al.*, 1981; Gutteridge *et al.*, 1984*a*).

As mentioned above, the formation of a reactive species such as $OH^{\cdot}$ from $O_2^{\cdot -}$ and H_2O_2 requires a catalytic transition metal, and one of the most likely is iron. Iron, in a form able to catalyse $OH^{\cdot}$ formation in the above reactions, can be released from ferritin by ascorbate (Gutteridge *et al.*, 1983) or by $O_2^{\cdot -}$ (Biemond *et al.*, 1984; Thomas *et al.*, 1985), from transferrin at low pH values found in the microenvironment beneath adherent macrophages (Halliwell *et al.*, 1985), and from haem proteins by peroxides (Gutteridge, 1986*a*). An assay *in vitro* has been developed to detect iron complexable to the drug bleomycin. The principles of this technique are summarized in Fig. 1. When bound to bleomycin, iron in the presence of oxygen and ascorbate can degrade DNA, with the release of thiobarbituric acid-reactive products, which can be quantified and related to the amount of iron present (Gutteridge *et al.*, 1981). When this assay is applied to synovial fluids from arthritic patients, 'mal-placed' iron appears to be present in some of the fluids obtained from patients with both osteoarthritis and rheumatoid arthritis, (Gutteridge *et al.*, 1981; Rowley *et al.*, 1984). However, more recent studies have shown that this iron is present, although transferrin is not 100% saturated, demonstrated by calculation and by direct measurement of iron-binding capacity (Gutteridge *et al.*, 1986). The following studies were undertaken to understand the origin of the iron in synovial-fluid samples that is available to bleomycin under the assay conditions used.

MATERIALS AND METHODS

Materials

Caeruloplasmin (human), DNA (calf thymus), γ-globulins (rabbit), apotransferrin (human), albumin (human, fatty acid-free), diethyldithiocarbamate, bipyridyl and *o*-phenanthroline were from Sigma Chemical Co., Poole, Dorset, U.K. Immunodiffusion plates for albumin, transferrin, caeruloplasmin and lactoferrin were obtained from Behring Hoechst (Hounslow, Middx., U.K.). All other chemicals were of the highest purity available from BDH, Poole, Dorset, U.K.

Synovial-fluid samples were taken for authentic medical purposes into clear sterile plastic tubes and centrifuged (2000 *g*) to remove particulate matter. The supernatant was transferred to new plastic tubes and stored frozen at −20 °C until the time of analysis; storage times ranged from 76 h to 3 months. This procedure was shown not to affect the results of the bleomycin assay for iron. Samples were taken from three different rheumatology centres and originated from 15 rheumatoid and 9 osteo-arthritic patients.

Abbreviation used: BLM-iron, bleomycin-detectable iron.

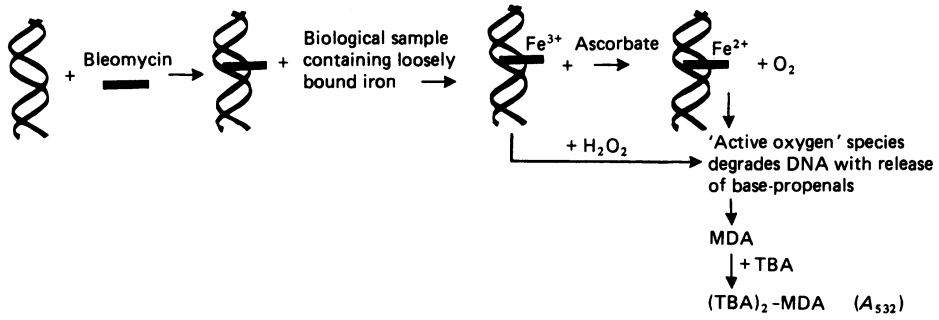

Fig. 1. Outline of the assay for measuring bleomycin-detectable iron

Addition of H_2O_2 can lead to DNA degradation through the formation of an 'active' Fe^{3+}–peroxide complex. Abbreviations: MDA, malondialdehyde; TBA, thiobarbituric acid.

Preparation of reagents

Caeruloplasmin was dialysed for 12 h at 4 °C against a 5% (w/v) solution of conalbumin, to remove contaminating iron and salts. Apotransferrin was loaded to 40% iron saturation with a solution of ferric nitrilotriacetate, and the iron-binding capacity was measured to check the percentage iron saturation. Buffers used (1 M) were acetate, imidazole/HCl and Tris/HCl. The imidazole and Tris buffers, covering the pH range 6.2–7.6, were dialysed with a membrane sac containing 5% conalbumin to remove complexable iron ions (Gutteridge & Hou, 1986). The sodium acetate solution was similarly treated before pH values covering the range 4.5–6.0 were titrated with 1 M-acetic acid. In addition, a sodium cacodylate/HCl buffer covering the critical pH range 5.0–7.4 was used to confirm the dependence of iron release from transferrin on the pH value and not on a component of the buffer system.

Measurement of the pH-dependent release of iron from transferrin or synovial fluids

***(a)* The bleomycin assay**. The assay described by Gutteridge *et al.* (1981) and subsequently modified for greater precision and versatility (Gutteridge & Hou, 1986) was used in these studies. Briefly, 0.5 ml of DNA (1 mg/ml), 0.1 ml of bleomycin (0.1 unit/ml, or as shown in Figures and Tables), 0.1 ml of $MgCl_2$ (50 mM), 0.1 ml of sample, 0.1 ml of buffer (1 M, as appropriate to the assay system) and 0.1 ml of ascorbic acid (7.5 mM) were added in the order stated to new clean plastic tubes and incubated at 37 °C for 1 h in a shaking water bath. After incubation, 0.1 ml of EDTA (1 M) was added, followed by 0.5 ml of thiobarbituric acid (1%, w/v, in 50 mM-NaOH) and 0.5 ml of HCl (25%, v/v). The tube heated contents were transferred to glass tubes and heated for 10 min at 100 °C, then cooled, and the resulting chromogen was extracted into 1.5 ml of butan-1-ol and the A_{532} was read. Appropriate blanks were prepared and subtracted from the A_{532} values. Reagent concentrations shown are those added to the reaction mixture, and iron values are those present in the standard or sample.

***(b)* The bipyridyl reaction**. Bipyridyl (0.1 mM) was dissolved in 1 ml of ethanol and made to 10 ml with metal-ion-free water (Chelex-resin-treated); 0.5 ml of transferrin (40% iron-loaded), 0.4 ml of buffer, 0.1 ml of ascorbic acid (7.5 mM) and 0.1 ml of bipyridyl (0.1 mM) were incubated at 37 °C for 1 h and the increase in A_{530} was measured.

When iron release from synovial fluids was measured in the above bipyridyl reaction, 0.5 ml of synovial fluid was substituted for transferrin and the solution was ultrafiltered after incubation to allow accurate spectrophotometric measurement.

***(c)* Change in the absorbance of the transferrin–Fe^{3+} complex**. When Fe^{3+} ions are complexed to transferrin, they produce a pink-coloured complex absorbing at 460 nm. Changes in A_{460} can be used to monitor changes in iron binding. The same reaction as described above for bipyridyl was used, but with the omission of bipyridyl, and with measurement of A_{460} changes.

Copper release from synovial fluids

The phenanthroline assay for loosely bound copper (Gutteridge, 1984) was performed at pH 5.3 in the presence of ascorbate (0.75 mM final concn.), which replaced mercaptoethanol.

Synovial-fluid antioxidant activities

It has previously been shown that the antioxidant activities of fluids dependent on (i) iron-binding capacity and (ii) ferroxidase activity can be separated by careful control of the reaction conditions (Gutteridge, 1986*b*). The mixture for reaction (i), measuring the iron-binding activity, contained 0.2 ml of phospholipid (bovine brain, 5 mg/ml), 0.2 ml of phosphate buffer (0.1 M; pH 7.4), 10 μl of fluid and 0.02 ml of ascorbic acid (6 mM). The mixture for reaction (ii), measuring ferroxidase activity, contained 0.2 ml of phospholipid (5 mg/ml), 0.2 ml of phosphate buffer (0.1 M; pH 6.5), 20 μl of fluid, 50 μl of $FeCl_3$ (0.5 mM) and 30 μl of ascorbic acid (0.1 mM). Reaction mixtures were incubated at 37 °C for 1 h. Thiobarbituric acid reactivity was developed in reactions (i) and (ii) by adding 0.5 ml of thiobarbituric acid (1% in 50 mM-NaOH) and 0.5 ml of 25% HCl, then heating for 15 min at 100 °C. The chromogen was extracted into 1.5 ml of butan-1-ol, and the A_{532} was read against appropriate controls and blanks. The results shown in all experiments are the means of two or more experiments which differed by less than $\pm 5\%$.

Determination of proteins

Protein concentrations were determined by single radial immunodiffusion, based on the Mancini *et al.*,

(1965) technique. The sensitivity of detection for lactoferrin, with LC-Partigen plates, was 6.5–45 mg/l.

RESULTS

Examination of knee-joint synovial fluids from patients with osteo-arthritis and rheumatoid arthritis shows that about 40% of the fluids exhibit bleomycin-detectable iron (BLM-iron) when the assay is carried out at an acid pH value. This must mean that (i) iron is present in the fluid or (ii) iron is released from something under the assay conditions and nothing stops such iron from binding to bleomycin.

In the original studies (Gutteridge *et al.*, 1981; Rowley *et al.*, 1984), the pH of the reaction undergoing incubation was not established. It is here shown to depend on a critical pH value of 5.3 (Fig. 2), at which some fluids are positive and some negative to the appearance of iron in the bleomycin assay.

Determination of protein values for caeruloplasmin, transferrin, lactoferrin and albumin in the synovial fluids revealed wide variation. When these values were grouped under those patients showing the presence of BLM-iron (bleomycin-positive samples) and those not showing iron (bleomycin-negative samples), it can be seen that two distinct protein profiles arise, with the BLM-iron-positive patients having substantially lower caeruloplasmin and decreased transferrin values (Fig. 3). Assuming measurements in each 'group' to be normally or log-normally distributed with equal variances, the probability that these groups have the same mean is shown in the legends to Figs. 3 and 4. In addition, lactoferrin could not be detected in the synovial fluids of this group (results not shown). This association is further supported by the finding that the ability of the fluids to inhibit peroxidizing lipids, dependent on iron-binding or ferroxidase activity, closely parallels the protein changes, showing substantial differences between the two groups (Fig. 4). Preliminary studies (Gutteridge *et al.*, 1986) have shown that synovial-fluid transferrin is not 100% saturated with iron and that BLM-iron-positive fluids are saturated at around 37% with iron, whereas BLM-iron-negative fluids have a mean iron saturation of 22%. The effect of 40%-iron-loaded transferrin was

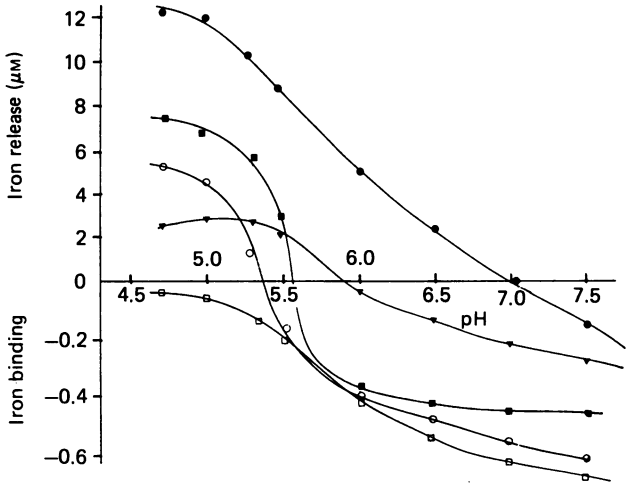

Fig. 2. pH-dependent release of iron detected in the bleomycin assays

●, Iron released from 40%-iron-loaded transferrin in the presence of 0.75 mM-ascorbate. ▲, Iron released from 40%-iron-loaded transferrin in the presence of 1 mM-H_2O_2. ■, Iron released from 40%-iron-loaded transferrin after the addition of caeruloplasmin (0.4 g/l) in the presence of 0.75 mM-ascorbate. ○, BLM-iron-positive synovial fluid. □, BLM-iron-negative synovial fluid. Iron-binding capacity is shown only as changes in A_{532} when values are below the blank.

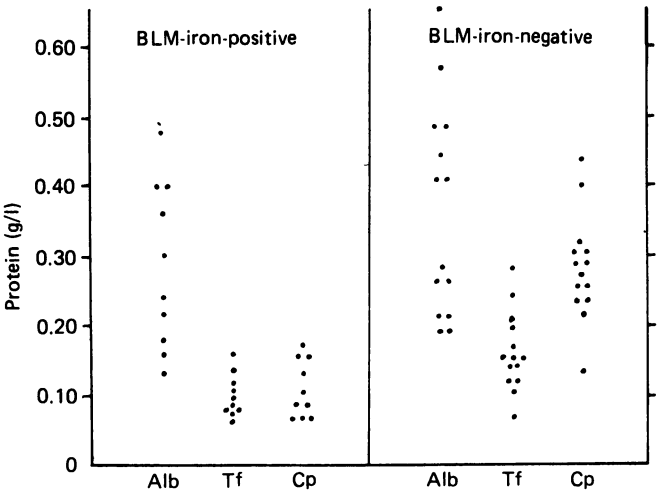

Fig. 3. Protein contents in arthritic-knee-joint synovial fluids

Values are to be multiplied by 100 for albumin (Alb), by 10 for transferrin (Tf), and as shown for caeruloplasmin (Cp). Proteins were measured by radial immunodiffusion. Bleomycin assays giving BLM-iron-positive and BLM-iron-negative results were performed with 0.7 unit of bleomycin/ml at pH 5.3. Significance values were: Cp, $P < 0.001$; Alb, $P > 0.05$; Tf, $0.01 < P < 0.05$.

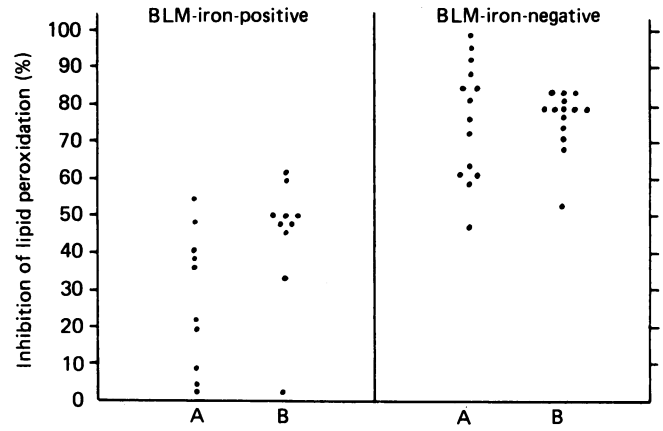

Fig. 4. Synovial-fluid antioxidant activities

Results shown are the percentage inhibition of a control (no synovial fluid) of peroxidizing phospholipid membranes. A, iron-binding antioxidant activity; B, ferroxidase antioxidant activity. Bleomycin-detectable iron was measured with 0.7 unit of bleomycin/ml at pH 5.3 to give BLM-iron-positive and BLM-iron-negative values. Significance values were: A, $P < 0.001$; B, $P < 0.001$.

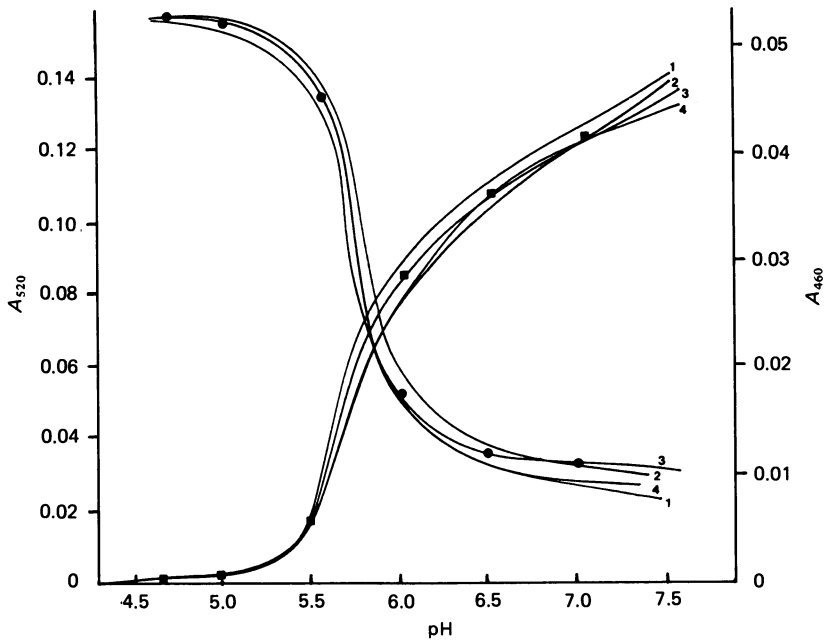

Fig. 5. Effect of proteins on the pH-dependent release of iron from 40%-iron-loaded transferrin

●, Iron released from transferrin to bipyridyl in the presence of 0.75 mM-ascorbate (A_{520}). ■, Iron released from 40%-iron-loaded transferrin in the presence of 0.75 mM-ascorbate; changes in A_{460} are shown. Proteins added: (3) control (no protein added to transferrin); (2) albumin (40 g/l); (4) γ-globulins (40 g/l); (1) caeruloplasmin (0.4 g/l).

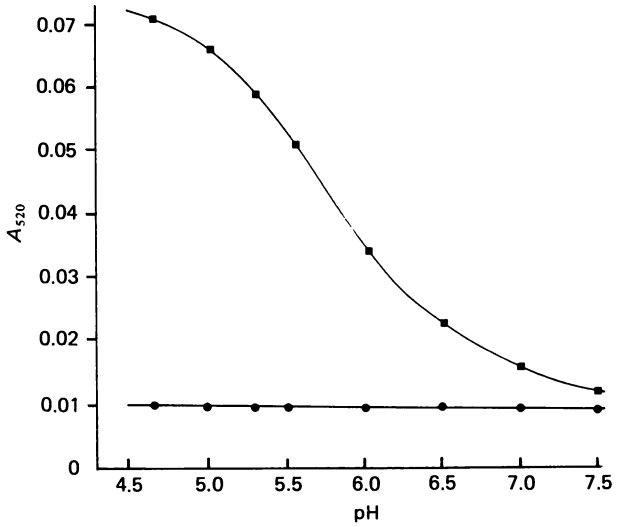

Fig. 6. Release of iron from synovial fluids to bipyridyl as a function of pH

■, BLM-iron-positive synovial fluid; ●, BLM-iron-negative synovial fluid.

therefore studied in the bleomycin assay and is shown in Fig. 2. In the presence of 0.75 mM-ascorbate, transferrin released iron to bleomycin at all acid pH values (Fig. 2). However, the release of iron in the presence of 0.75 mM-ascorbate was restricted to a pH range of 5.5–6.0 when 40%-iron-loaded transferrin was treated in the bipyridyl reaction (Fig. 5) or changes in absorbance of transferrin were measured at 460 nm (Fig. 5). A similar pattern of iron release was seen in the bleomycin assay when H_2O_2 was substituted for ascorbate (Fig. 2). The pH-dependent behaviour of synovial fluids, positive and negative to the bleomycin reaction, are shown in Figs. 2 and 6 and confirm that the release of a complexable form of iron does take place in the bleomycin-positive samples.

Addition of the proteins caeruloplasmin, albumin and γ-globulins to the bleomycin, bipyridyl and A_{460} assays for iron release showed that only caeruloplasmin substantially affected the bleomycin assay (Figs. 2 and 5), lowering the pH value at which iron was released to bleomycin (Fig. 2). Caeruloplasmin, a copper-containing protein, and copper ions can inhibit the bleomycin reaction when the copper ions are present in lower concentrations than iron ions and free metal-binding sites are still available on the bleomycin molecule (Gutteridge & Fu, 1981). Copper salt (5 μM) was sufficient to inhibit completely the detection of a simple iron salt (10 μM-$FeCl_3$) at a bleomycin concentration of 36 μM (Table 1). The amount of non-caeruloplasmin copper in the synovial fluids was therefore determined by the phenanthroline assay at pH 5.3 in the presence of 0.75 mM-ascorbate and found to be in the range 1.3–15.6 μM. These copper values, if equivalent to free copper ions, are theoretically sufficient (Table 1) to interfere substantially with the bleomycin assay. To test this possibility, the copper chelator diethyldithiocarbamate was used to inhibit copper ions reacting. Table 1 shows that diethyldithiocarbamate has only a small effect on the measurement of a simple iron salt in the bleomycin assay, but substantially diminishes the inhibitory effect of copper ions on the detection of iron. Caeruloplasmin was also found to have an inhibitory effect on the bleomycin reaction, which could not be related to release of copper ions from the protein, in view of the small effect of diethyldithiocarbamate in decreasing this inhibition (Table 1). Re-assay of BLM-ion-positive synovial fluids for BLM-iron in the presence of diethyldithiocarbamate did not reveal significantly changed concentrations of iron (Table 1). Hence the

Table 1. Effect of copper ions and the copper chelator diethyldithiocarbamate (DDC) on the bleomycin assay at pH 5.3

The concentrations of reagents shown are those added to the bleomycin reaction mixture. Transferrin was 3 g/l (40%-iron-saturated.)

	Iron (μM)	
	−DDC	+DDC
$FeCl_3$ (10 μM)	10	9.1
$FeCl_3$ + $CuCl_2$ (5 μM)	0	7.3
$FeCl_3$ + caeruloplasmin (0.4 g/l)	3.6	3.2
Transferrin	10	10.6
Transferrin + $CuCl_2$ (5 μM)	0	10.6
Transferrin + caeruloplasmin (0.4 g/l)	6.1	9.1
Synovial fluids		
1	0.1	0
2	3.6	3.0
3	0	0
4	3.1	3.2
5	0.4	0.3
6	1.2	1.5
7	0.4	0.6
8	1.5	1.4
9	0	0
10	4.9	4.6
11	0	0

non-caeruloplasmin phenanthroline-detectable copper does not act in the same way as a simple copper salt in inhibiting the bleomycin reaction.

Caeruloplasmin has both a ferroxidase activity and a weak ascorbate oxidase activity, and either or both of these could account for its inhibitory effect on the bleomycin assay (Table 1). A bleomycin–Fe^{3+} complex will also degrade DNA in the presence of H_2O_2, so by substituting H_2O_2 for ascorbate the ascorbate oxidase activity of caeruloplasmin can be eliminated. Further, since Fe^{2+} ions are not involved in the degradation of DNA after addition of H_2O_2 (Fig. 1), the ferroxidase activity of caeruloplasmin is also eliminated as an inhibitory mechanism, as well as the promoting effect of ascorbate on iron release from transferrin. The pH-dependent release of iron from pure transferrin (40%-iron-loaded) to bleomycin in the presence of H_2O_2 is shown in Fig. 2. Re-assays, in the presence of H_2O_2, of synovial fluids known to be either positive or negative for iron detection under the standard assay conditions are shown in Table 2. A similar pattern of iron release is seen with H_2O_2, although the iron concentrations are lower.

Pressure filtration of synovial fluids through a 10000-M_r exclusion membrane suggested that the inhibitor(s) of iron release in BLM-iron-negative synovial fluids at pH 5.3 is a high-M_r component (Table 3). Additions of apotransferrin, transferrin (40%-iron-loaded), albumin and caeruloplasmin to a BLM-iron-positive synovial fluid are shown in Table 4. Apotransferrin inhibits the detection of iron, whereas 40%-iron-loaded transferrin releases iron at pH 5.3 (Table 4). Partly iron-loaded lactoferrin (human milk) also decreased BLM-iron when added at a concentration of 0.3 g/l to a BLM-positive fluid (results not shown). This concentration of lactoferrin is some 10 times greater than normal values found in synovial fluid (Bennett *et al.*, 1973). When iron-loaded transferrin was added to a BLM-iron-negative synovial fluid (Table 4), there was a greater inhibitory effect on the release of iron from transferrin to bleomycin (Table 4). A feature of the BLM-iron-positive fluids is their lowered contents of transferrin (Fig. 2) and lactoferrin, which could not be detected on radial immunodiffusion plates. Lactoferrin could, however, be detected in all the BLM-iron-negative fluids (results not shown).

DISCUSSION

Previous studies (Gutteridge *et al.*, 1981, 1984*b*), one in detail (Rowley *et al.*, 1984), have shown that some synovial fluids from patients with either osteo-arthritis or rheumatoid arthritis show the presence of BLM-iron at micromolar concentrations. The concentration of this iron correlates with disease severity, lipid peroxide and C-reactive-protein content of synovial fluids (Rowley *et al.*, 1984). The pH value of the unbuffered reaction mixture used by Rowley *et al.* (1984) was not stated, but it may well have been acidic, since discrimination of synovial fluids into BLM-iron-positive or -negative values is here shown to be critically dependent on a pH value of 5.3.

Table 2. Release of iron to bleomycin from synovial fluids in the presence of H_2O_2

Values are shown minus blank readings.

	1 mM-H_2O_2		0.75 mM-Ascorbate	
	A_{532}	Iron (μM)	A_{532}	Iron (μM)
$FeCl_3$ (5 μM) control	0.100	5	0.520	5.0
Synovial fluids				
1	0	0	0	0
2	0.036	1.8	1.040	10.0
3	0.028	1.4	0.570	5.5
4	0	0	0	0
5	0	0	0	0
6	0.052	2.6	0.750	7.2
7	0	0	0	0
8	0	0	0.09	0.87

Table 3. Pressure filtration of synovial fluid through a 10000-M_r-exclusion membrane

In reaction (3) equal volumes of fluids 'A' and 'B' were pre-mixed, and a volume of 0.1 ml was sampled. In reactions (4) and (5) 0.1 ml of sample 'B' was included in the reaction together with 0.1 ml of ultrafiltrate or protein concentrate.

	Iron (μM)
1. Synovial fluid 'A' (before filtration)	0
2. Synovial fluid 'B' (before filtration)	6.1
3. Pre-mixing of equal volume of 'A'+'B'	3.2
4. Ultrafiltrate of 'A' added to sample 'B'	6.2
5. Protein concentrate of sample 'A' added to sample 'B'	1.0

Table 4. Effect of different proteins on iron release in synovial fluids to bleomycin at pH 5.3

Protein values shown are those added to the reaction.

	Iron (μM)
Synovial fluid positive to iron release	4.8
+Apotransferrin	
1 g/l	0
2 g/l	0
3 g/l	0
+Caeruloplasmin	
0.1 g/l	3.9
0.2 g/l	3.8
0.4 g/l	2.6
+Albumin	
10 g/l	4.8
20 g/l	4.8
40 g/l	4.8
+Transferrin (40% loaded)	
1 g/l	5.3
2 g/l	7.4
3 g/l	9.0
Transferrin (3 g/l) without synovial fluid	10.0
Synovial fluid negative to iron release	0
+Transferrin (40% loaded)	
1 g/l	0
2 g/l	0.5
3 g/l	2.0

All fluids showing the presence of BLM-iron have lowered concentrations of transferrin, lactoferrin and caeuloplasmin. These proteins are known to be important components of the extracellular antioxidant defences (Gutteridge, 1986*b*), and a significant difference was seen in the antioxidant activities of the BLM-iron-positive and -negative groups.

When rheumatoid-arthritis sera or synovial fluids, not grouped according to the appearance of BLM-iron, are compared with fluids from either normal healthy controls or patients with osteoarthritis, the rheumatoid patients show a wide scatter of values but generally higher degrees of antioxidant protection against iron-stimulated oxygen-radical damage, a feature ascribed to the higher contents of caeruloplasmin (Scudder *et al.*, 1978; Cranfield *et al.*, 1979; Gutteridge & Stocks, 1981) or the lower percentage iron saturation of the transferrin (Gutteridge, 1986*b*). Here we show that both the rheumatoid-arthritis and osteoarthritis patients can be sub-divided to give a group who show low degrees of synovial-fluid antioxidant protection against iron-stimulated radical damage. These patients are those who show the presence of BLM-iron, a finding which might explain the previous correlations of BLM-iron with increased amounts of lipid peroxide (Rowley *et al.*, 1984).

Previous studies have shown that copper ions inhibit the detection of iron in the bleomycin reaction when not all the metal-binding sites of bleomycin are occupied (Gutteridge & Fu, 1981) and that caeruloplasmin does not inhibit when the bleomycin reaction is initiated by the addition of a ferrous salt (Gutteridge & Shute, 1981). Here, using a bleomycin reaction dependent on the reduction of a ferric salt by ascorbate, I show that both copper ions and caeruloplasmin can influence the detection of iron. Caeruloplasmin has a ferroxidase (Osaki *et al.*, 1966) and a weak ascorbate oxidase activity (Osaki *et al.*, 1964), and both activities appear to decrease the sensitivity of detection of a simple iron salt in the bleomycin reaction, although this is less marked when iron is measured in synovial fluid. If H_2O_2 is used as an 'oxygen activator' in place of ascorbate for the bleomycin reaction, then caeruloplasmin cannot influence iron detection by enzymic activities, since there is no ascorbate present, and no reduction of iron(III) to iron(II) has been observed in the presence of H_2O_2 (Melnyk *et al.*, 1981). Ascorbate also influences the pH at which iron is released from pure iron-loaded transferrin, and its replacement by H_2O_2 probably accounts for the lower iron values found in synovial fluids as well as the different pH-dependent pattern of iron release from pure iron-loaded transferrin. Lower iron values with H_2O_2 may also be ascribed to the non-redox cycling of bleomycin-bound iron in the presence of H_2O_2.

The addition of diethyldithiocarbamate to the bleomycin reaction prevented simple copper ions from inhibiting the detection of iron, and confirmed that a complexable form of copper was not inhibiting the detection of iron in synovial fluids, although some form of non-caeuloplasmin copper was available to phenanthroline.

Pure iron-loaded (40%) transferrin in the presence of ascorbate released iron to bleomycin over the entire acid pH range, whereas BLM-iron-positive fluids released iron only at a pH value of 5.3 or less. By using two different assay systems, the pH-dependent iron release from pure iron-loaded transferrin was studied by measuring the formation of a Fe^{2+}–bipyridyl complex and the change in A_{460} as iron is released from transferrin. These two systems were little affected by addition of caeruloplasmin, in contrast with the bleomycin reaction, with ascorbate. The reason for this anomaly in the bleomycin–ascorbate reaction is probably related to the kinetics of iron redox cycling and the amount of reducing equivalents present in the final reaction mixture. In the bleomycin reaction the iron–bleomycin complex is acting catalytically, whereas in the bipyridyl reaction any Fe^{2+} ions formed are complexed in a fixed valence state to the bipyridyl molecule, where they are unavailable to transferrin. During the redox cycling of iron in the bleomycin reaction, superoxide radicals, H_2O_2 and organic peroxides are produced (Gutteridge & Hou, 1986), all of which may increase the pH-dependent release or iron from transferrin. Caeruloplasmin inhibited the release or iron above a pH value of 6.0, possibly by its ascorbate oxidase activity lowering the ascorbate concentration.

The BLM-iron-positive synovial fluids examined had lowered concentrations of transferrin and lactoferrin. Previous studies (Gutteridge *et al.*, 1986) have confirmed, however, that such fluids nevertheless still have an available iron-binding capacity.

During inflammation, the pH in the microenvironment beneath adherent macrophages can fall to 5.0 or less (Etherington *et al.*, 1981), and therefore the pH value of 5.3 used in these studies to determine release of iron is of physiological importance. At pH 5.3 it appears that iron is preferentially released from some synovial fluids, but not from others, and it is the iron-releasing fluids which contain extremely low concentrations of antioxidant protection against iron damage. Transferrin contains two iron-binding sites, one of which can hold iron more tightly at acid pH values (Lestas, 1976), and lactoferrin

can hold its iron down to pH values of 4.0 (Groves, 1960; Johansson, 1960). However, some iron was released to bleomycin at pH 5.3 from pure transferrin, iron-loaded at 5, 10, 20 and 40% saturation (results not shown), when all the iron would be expected to be on the tighter 'acid-binding' site.

The presence of BLM-iron in synovial fluids appears to relate directly to the low concentrations of transferrin and lactoferrin, and possibly to the higher (when compared with other arthritic patients) percentage iron saturation of the transferrin. However, it should be noted that, as a group, arthritic patients have generally a lower percentage iron saturation of transferrin than do normal controls. The possibility that the transferrin is chemically or physically different in the BLM-iron-positive fluids has yet to be established.

This study suggests that the bleomycin assay when carried out at pH 5.3 provides an important diagnostic test to distinguish arthritic patients with low degrees of anti-oxidant protection against iron-stimulated damage and a propensity to release catalytic iron in the microenvironment. The use of an iron chelator, such as lactoferrin, effective at low pH values may be beneficial in the treatment of this group of patients.

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