Effect of iron complexes on adjuvant arthritis in rats

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Abstract

When a total dose infusion of iron dextran is given to anaemic rheumatoid patients an exacerbation of inflammatory synovitis in previously affected joints is observed. The adjuvant arthritis model of inflammation in rats has been used to investigate the mechanism of iron promoted synovitis. Either iron dextran (5 mg injected intravenously) with a dextran C control, or iron sorbitol (7.5 mg injected intramuscularly) with a sorbitol citrate complex control was given at the onset of clinical joint inflammation. Iron dextran significantly increased joint inflammation (assessed by joint scoring) at days 12, 13, 14, and 16 after injection. Similarly, iron sorbitol produced a significant increase in the joint score at days 17, 18, 19, and 21. In addition, extensive osteoporosis was observed in the rats treated with iron sorbitol. These pro-inflammatory effects of iron coincide with the presence of positive results for synovial iron (III) using Perl's test and neutrophil infiltration. The results of this study suggest that the iron induced increase in synovitis in adjuvant arthritis is a result of iron promoted oxidative damage and is not likely to be due to the dextran C or the sorbitol citric acid components. It is suggested that a similar mechanism may occur in rheumatoid patients given iron supplements.

Iron is of fundamental importance in the inflammatory process. Infusion of iron dextran (about 15 mg/kg) into patients with rheumatoid arthritis (RA) results in the exacerbation of joint symptoms^{1,2} and the effect appears to be mediated by iron (rather than the dextran component) affecting the promotion of lipid peroxidation. In an attempt to understand the part iron plays in inflammation, various workers have studied the effects of the manipulation of iron stores in animal models of inflammation. Mild iron deficiency induced by a low iron diet and treatment with iron chelators reduce the severity of adjuvant induced joint inflammation.^{3,4} Such mild iron deficiency did not affect the systemic parameters of adjuvant arthritis or the immune functions. This suggests that iron deficiency or chelation may specifically protect the joint in adjuvant arthritis by reducing the amount of iron available for the promotion of hydroxyl radical (OH·) formation and subsequent tissue damage. Mowat and Garner⁵ gave 1 mg intramuscular iron dextran to male rats three days before adjuvant inoculation and then daily up to day 25 after adjuvant induction

(total dose of 80 mg/kg). Iron dextran exacerbated joint inflammation at days 17 to 26. This study is informative in terms of iron and joint inflammation; however, it is not comparable with the clinical situation in terms of the dose used, the form of treatment, and the time at which the iron was given in relation to disease activity.

This study aimed to investigate the effects of iron complexes on adjuvant arthritis in a study specifically designed to mimic the clinical situation in which iron was given in the presence of, rather than before, clinical manifestations of joint symptoms.

Materials and methods

ANIMALS

Male Wistar rats weighing 300-350 g (Bantin and Kingman, Hull, UK) were used. The rats were fed on standard laboratory diet ad libitum.

INDUCTION OF DISEASE

Adjuvant disease was induced using the method of Kaibara *et al.*⁶ All rats received a single intradermal injection in the base of the tail of 0.1 ml of a 10 mg/ml suspension of freeze dried *Mycobacterium butyricum* (Difco, Surrey, UK) in liquid paraffin.

JOINT SCORE

Joint inflammation was assessed daily by two blinded observers by a method based on the foot scoring system described by Currey and Ziff.⁷ A subjective score of 0-4 was allocated to each hind and fore paw depending on the extent of inflammation. Zero represented no inflammation; 1, slight redness and swelling of the foot; 2, swelling of the foot such that the tendons were no longer visible; 3, swelling extending to the ankle joint; and 4, gross inflammation and deformity of the ankle joint. The joint score for each animal is the total sum of its limb scores.

HISTOLOGICAL ASSESSMENT

Liver samples from iron studies and synovial samples from the iron dextran study were removed and fixed in formal saline for histological analysis. The tissues were dehydrated, embedded in paraffin wax and sections were stained with haematoxylin and eosin. The presence of iron (III) was detected using Perl's Prussian blue stain⁸ and iron was determined using computer aided image analysis (Seescan Systems, Cambridge, UK).

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BIOCHEMICAL ASSESSMENT

Using the method of Williams *et al.*,⁹ iron was measured in serum samples using a COBAS BIO centrifugal microanalyser in the biology department of Roche Products (Welwyn Garden City) in conjunction with Dr E J Lewis.

MICROFOCAL RADIOLOGY

Bone changes were assessed using microfocal radiology. This radiological system consisted of a demountable x ray unit, an x-y positioning frame, and a Marconi image intensifier and monitor. The special 25 µm microfocal x ray set had an effective resolution of >20 l/p/mm. Radiographs were obtained at a magnification of ×10 for the feet using standard x ray cassettes positioned in front of the image intensifier. Exposures were at 60 kV and ImAS (0.5 mA).

EFFECT OF IRON DEXTRAN ON ADJUVANT ARTHRITIS IN RATS

The rats were divided into three groups. Adjuvant disease was initiated as described. The animals were injected with 0.1 ml saline, 20% w/v dextran C (the low molecular weight dextran component of iron dextran), or iron dextran (5 mg iron; Imferon, Fisons, UK). All the injections were given intravenously at the time of the appearance of clinical symptoms in the joints (day 9 in this study). Six rats from each group were killed on day 10, and 12 rats from each group on each of days 12, 14, and 16.

EFFECT OF IRON SORBITOL ON ADJUVANT ARTHRITIS IN RATS

Intramuscularly injected iron sorbitol (Jectofer, Astra Pharmaceutical Products, United Kingdom) is eliminated by urinary excretion at a much higher rate than intravenously injected iron dextran.¹⁰ To achieve comparable concentrations of iron to those obtained in the iron dextran study, we performed a comparative study in normal rats. The results of this study (data not shown) have shown that 7.5 mg of intramuscularly injected iron sorbitol was needed to produce comparable serum iron concentrations to those achieved by 5 mg of intravenously injected iron dextran.

The rats were divided into three groups and adjuvant disease was induced as described. The rats were injected intramuscularly with 0.15 ml of either saline, sorbitol control, or iron sorbitol (7.5 mg elemental iron). All the injections were carried out at the onset of clinical symptoms (day 12 in this experiment). Ten rats of each group were killed on each of days 13, 14, 17, 19, and 21.

STATISTICS

All parametric data were assessed for significance using Student's *t* test. The results are shown for each group as the mean (one standard error (SE)). Non-parametric data (the joint scores in the adjuvant experiment) were assessed using the Mann-Whitney U test; however, for ease of presentation the results are shown as means (1 (SE)).

Results

EFFECT OF IRON DEXTRAN INJECTED INTRAVENOUSLY

Joint score

Figure 1 shows that the mean score of the groups treated with saline, dextran C, and iron dextran increased as the adjuvant disease progressed. The mean joint score of the rats treated with iron dextran was significantly ($p < 0.05$) higher than the controls treated with saline at days 12, 13, and 14, as analysed by the Mann-Whitney U test. When compared with the rats treated with dextran C, iron dextran treatment significantly ($p < 0.025$) increased the mean joint scores at days 12, 13, 14, and 16. Dextran C has a significant inhibitory effect at days 13 and 16 ($p < 0.05$) when compared with the rats treated with saline.

Concentrations of iron in serum

Serum iron concentrations were measured in six healthy rats from the batch used in this experiment (mean (SE) 56.1 (0.8) µmol/l). All adjuvant rats showed lower ($p < 0.01$) serum iron concentrations than non-adjuvant controls (mean serum iron concentrations ranged from 15.0 to 26.3 µmol/l with standard errors ranging from 0.6 to 3.6).

Concentrations of iron (III) in liver

Livers of rats treated with iron dextran contained significantly higher iron concentration than those treated with dextran C on days 1 ($p < 0.05$), 3 ($p < 0.001$), and 5 ($p < 0.001$) after injection intravenously. Similarly, the rats treated with iron dextran had significantly higher concentrations of iron (III) in the liver on days 1 ($p < 0.01$), 3 ($p < 0.02$), 5 ($p < 0.001$), and 7 ($p < 0.01$) when compared with adjuvant controls treated with saline (see fig 2).

Perl's Prussian blue staining showed the presence of iron (III) in nine of 12 (75%) synovial sections in the rats treated with iron dextran. Most of these iron (III) deposits were contained within the synovial lining cells. However, only four of 12 (33.3%) and one of 12 (8.3%) sections respectively from rats treated

Figure 1 Effect of iron dextran and dextran C injected intravenously on the mean joint score of adjuvant rats. Mann-Whitney U test compares iron dextran v dextran C. ** $p \leq 0.025$. Standard errors less than 0.1 are not included. $n \geq 6$.

Figure 2 Concentrations of iron (III) in the liver of adjuvant rats treated with iron dextran, dextran C, or saline injected intravenously. Iron concentrations are expressed as the area times the intensity of blue. $n=3-4$. $p\leq 0.05$; $**p\leq 0.01$ v the dextran C control.

Figure 3 Effect of iron sorbitol on the mean joint score in rats with adjuvant arthritis. $n\geq 10$. $*p\leq 0.05$, $**0.025$ v the sorbitol control.

with saline and dextran C contained iron (III) deposits. Neutrophil infiltration was a feature of the synovial histology in all groups from days 12 to 16.

Except for considerable soft tissue swelling in the group treated with iron dextran, microfocal radiography showed no obvious bone changes such as osteoporosis, new bone formation, nor joint space narrowing in any of the groups studied.

EFFECT OF IRON SORBITOL INJECTED INTRAMUSCULARLY

Joint score

The mean joint score of all three groups increased as the inflammation progressed. After an initial decrease in the joint score at day 13, iron sorbitol produced a statistically significant potentiation of joint inflammation at days 17 ($p\leq 0.05$), 18 ($p\leq 0.05$), 19 ($p\leq 0.025$), and 21 ($p\leq 0.025$) when compared with the sorbitol control group (fig 3). The potentiation was also significant when compared with the saline control at days 18 and 19 ($p\leq 0.05$).

are significantly higher ($p\leq 0.01$) on day 13 (24 hours after injection intramuscularly) when compared with the saline and sorbitol treated control groups.

Figure 4 Concentrations of iron (III) in the liver of adjuvant rats treated with iron sorbitol, sorbitol, or saline injected intramuscularly. $n=5-7$. $**p\leq 0.001$.

Concentration of iron in serum

Serum iron concentrations were determined in six healthy rats from the batch used for this experiment (mean (SE) 36.9 (5.3) $\mu\text{mol/l}$). All three treatment groups showed a decrease in serum iron concentrations compared with healthy controls (table). Serum iron concentrations following treatment with iron sorbitol

Effect of iron sorbitol on concentrations of iron in serum samples from rats with adjuvant arthritis

Mean (SE) iron concentrations in serum ($\mu\text{mol/l}$)			
Day	Saline	Sorbitol	Iron sorbitol
13	22.6 (1.3)*	21.3 (0.8)	32.8 (1.8)†
14	23.1 (1.7)	18.9 (1.4)	21.5 (0.6)
17	22.6 (1.8)*	22.7 (0.9)†	24.3 (1.4)
19	20.3 (1.6)†	21.8 (2.0)†	20.6 (1.4)
21	20.9 (1.6)†	17.7 (1.2)	20.8 (1.2)

$p\leq 0.01$ v the sorbitol and saline controls. Number of determinations was seen except for *eight, †nine.

Figure 5 Radiograph of an ankle joint of a control rat treated with saline. Day 21 after the induction of adjuvant disease. Note osteoporosis (arrows) and formation of new bone (arrow head).

Concentrations of iron (III) in liver

Rats treated with iron sorbitol contained significantly higher ($p < 0.001$) concentrations of iron (III) in the liver than either the sorbitol or saline treated controls (fig 4).

Figures 5 and 6 show microfocal radiographs of the adjuvant rats treated with saline and sorbitol. They show the presence of some osteoporosis compared with the non-adjuvant controls. The group treated with iron sorbitol (fig 7) shows extensive osteoporosis with a complete loss of bone structure.

Discussion

This study has been specifically designed to mimic the clinical situation in which anaemic rheumatoid patients are given iron supplements in the presence of clinical joint symptoms. In these patients, parenteral iron leads to a flare of joint inflammation in previously affected joints

Figure 6 Radiograph of an ankle joint of a control rat treated with sorbitol. Day 21 after the induction of adjuvant disease.

Figure 7 Radiograph of an ankle joint of a rat receiving iron supplements (7.5 mg iron sorbitol). Day 21 after the induction of adjuvant disease. Note osteoporosis (arrow) and formation of new bone (arrow heads).

at about three days after treatment with iron. Our animals showed the same response as that observed in humans; the two iron complexes produced a significant increase in the joint score three to five days after injection (figs 1 and 3). This delay is probably a result of the time taken for the iron to be processed in the reticulo-endothelial cells before it becomes available to plasma transferrin. Such proinflammatory effects of iron coincide with the presence of positive results for Perl's test in synovial tissue and neutrophil infiltration. The presence of neutrophils within the joints at the time of the clinical exacerbation of joint inflammation supports the idea of their part in the pro-inflammatory effects of iron. Superoxide radicals (O_2^-) may be generated within the inflamed joint either by activated neutrophils undergoing a respiratory burst¹¹ or as a result of hypoxia induced metabolic changes, followed by reperfusion.¹²⁻¹⁴ In the presence of the catalytic enzyme superoxide dismutase, dismutation of O_2^- occurs with the production of hydrogen peroxide, which in the presence of catalytic iron produces the highly reactive hydroxyl radical ($OH^\cdot$). In this study iron has been shown to be present in the synovium of the knee joints of rats given iron dextran at the clinical onset of adjuvant disease. Iron deposits have been shown in the inflamed knee and hind paw joints of rats with adjuvant disease.¹⁵ Such iron deposition was apparent in the synovial membrane of the knee joint of our adjuvant rats. However, it occurred more often following iron dextran injection than in the rats treated with saline or dextran C. Such iron may be a result of activated synovial macrophages removing iron from iron saturated transferrin.¹⁶ In addition, the uptake of ferritin¹⁷ or dextran itself¹⁸ from the synovial cavity may contribute to increased synovial iron deposition in the iron loaded rats. Catabolised erythrocytes arising from intermittent intra-articular haemorrhages¹⁹ may also contribute to the iron present in synovial macrophages.²⁰

One of the most constant findings in chronic diseases (such as adjuvant arthritis) is hypoferreaemia.²¹ Plasma iron concentrations in animals with adjuvant disease have been shown to decrease 14 days after adjuvant induction,²² a change also observed in our adjuvant rats (see results and table).

In adjuvant arthritis iron is deposited in the macrophages of tissue which do not normally participate in iron turnover—for example, the liver, spleen, and synovium.²³ Using Perl's Prussian blue staining, the presence of iron (III) in the liver was shown in all the adjuvant groups compared with normal rats, in which iron was not usually detectable by this method (figs 2 and 4). Such an increase in the concentration of iron (III) in the liver in adjuvant arthritis has been shown by other workers,²² and was shown to correlate with oedema of the paw.²⁴ As expected, rats injected with either of the iron complexes showed a further increase in the concentration of iron (III) in the liver, which was significant when compared with control groups.

Bone changes were studied using microfocal radiology. In the iron dextran study bone

abnormalities were not present. This was probably because the rats were killed early (day 16), before the development of bone damage. However, in the iron sorbitol study, bone abnormalities were visible from day 17. Focal osteoporosis was observed in all groups but was very extensive in the rats treated with iron sorbitol (figs 5–7). Within the inflamed joint, an inflammatory cellular infiltrate may lead to periosteal proliferation and bone remodelling via mediators such as interleukin 1 and prostaglandin E_2 .²⁵ In addition, the increased amount of prostaglandins in arthritic joint fluids²⁶ may contribute to the destruction of juxta-articular bone in patients with RA.²⁷ This may be a contributing factor in the osteoporosis observed in the ankle joints of adjuvant rats (figs 5 and 6). Certainly, iron induced prostaglandin E_2 production by synovial fibroblasts²⁸ could account for the increased bone resorption and osteoporosis observed in the rats treated with iron sorbitol (fig 7). Prostaglandins increase osteoblastic cyclic adenosine 5'-phosphate, which in turn causes osteoblasts to stimulate osteoclastic bone resorption.^{29–30} Recently, Garret *et al*³¹ have shown that the generation of reactive oxygen species, either in cultured bone or adjacent to bone surfaces *in vivo* leads to the proliferation and recruitment of osteoclasts and enhanced bone resorption. Ionic iron in the iron sorbitol complex³² may catalyse free radical production, which may contribute to the massive osteoporosis observed in the rats treated with iron sorbitol.

The results of this study suggest that the iron complexes caused an exacerbation of the synovitis in adjuvant arthritis, which is probably not a result of the dextran C or the sorbitol citric acid complex. This view is supported by the following observations. Firstly, arthritis associated with idiopathic haemochromatosis³³ and with transfusional secondary haemochromatosis³⁴ has been linked to the presence of synovial iron deposits. In addition, synovial iron deposits have been shown in RA,^{35–36} and in articular cartilage in patients with degenerative arthropathy of haemochromatosis.³⁷ Hence, the synovial iron deposits observed in the iron loaded adjuvant rats is likely to be related to the exacerbation of joint score.

Most previously reported work suggests that iron is proinflammatory via a free radical mediated mechanism in diseases such as RA^{1–2–38} and haemochromatosis arthropathy.³⁹ However, lymphocyte related mechanisms for the iron promoted synovitis in RA have been suggested.^{40–41}

As lymphocyte infiltration into the synovium is not a major feature of adjuvant arthritis,⁴² then iron mediated exacerbation of joint score is not likely to involve lymphocytes in this disease. Furthermore, the parts played by free radical and lipid peroxidation in adjuvant arthritis^{43–44} together with the increased amount of synovial iron (III) resulting from iron overload suggests that iron induced free radical production and subsequent lipid peroxidation is a possible mechanism for iron promoted exacerbation of joint symptoms in adjuvant arthritis. The exacerbation observed in adjuvant arthritis is

comparable in nature with that in RA in terms of the dose and nature of the iron complex used, the time the iron is given, and the time of onset of joint exacerbation. It is probable that the exacerbation of the synovitis observed in RA after treatment with iron is not a result of the effect of iron on the lymphocytes, but probably occurs via an iron promoted free radical mechanism.

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