

## CONCISE REPORT

# Expression of ferritin, transferrin receptor, and non-specific resistance associated macrophage proteins 1 and 2 (Nramp1 and Nramp2) in the human rheumatoid synovium

J F Telfer, J H Brock

*Ann Rheum Dis* 2002;**61**:741–744

**Objective:** To gain a better understanding of how iron accumulates in human rheumatoid synovium.

**Methods:** The distribution of ferritin, transferrin receptor, and non-specific resistance associated macrophage proteins 1 and 2 (Nramp1 and Nramp2) in the human rheumatoid synovium was investigated by immunocytochemistry and reverse transcriptase-polymerase chain reaction (RT-PCR).

**Results:** Both heavy and light ferritin subunit types were detected in the lining layer and the subintimal zone of rheumatoid synovium, heavy ferritin generally being more abundant than light. Both heavy and light ferritin were detected in isolated synovial macrophages and fibroblasts. Transferrin receptor expression was largely confined to fibroblasts of the synovial lining layer. Nramp2 was detected by PCR in both isolated synovial macrophages and fibroblasts, whereas Nramp1 was detected by PCR and immunocytochemistry in macrophages and neutrophils in the lining and subintimal zone, and in inflammatory infiltrates, but was absent from fibroblasts.

**Conclusion:** A complex chain of events, perhaps initiated by proinflammatory cytokines, may culminate in a toxic build up of iron in the rheumatoid joint.

Finchampstead, UK), ferritin heavy and light chains (provided by Dr Paolo Santambrogio, Milan, Italy), CD68 (Dako) for macrophages, or a polyclonal antibody to murine Nramp1 (and found to react with human Nramp1), provided by Dr Howard Barton, University of Southampton. An antibody to yeast glucose oxidase (Dako) or rabbit IgG was used as control for monoclonal and polyclonal antibodies, respectively. Antibody binding was detected with an IgG peroxidase kit (Vectastain Elite ABC kit, Vector, Peterborough, UK). Iron was detected by Perls' Prussian blue reaction.

### Separation and staining of macrophages and fibroblasts from synovial tissue

Fresh synovial tissue (5–10 g) was washed four times in Hanks's balanced salts solution (Gibco, Paisley, UK), cut into small pieces, and digested in 0.2% (w/v) collagenase type IV (Sigma) in RPMI 1640 medium containing 10% (v/v) fetal bovine serum and penicillin-streptomycin for three hours at 37°C with occasional gentle shaking. After filtration, CD14 positive cells were selected using MCAs CD14 microbeads (Miltenyi, Woking, UK), according to the manufacturer's instructions. The flow-through from the column contained fibroblasts, and purity of cells was determined using antibodies against CD68 (macrophages) and human fibroblasts (MCA 1399, both from Serotec, Oxford, UK). After overnight culture, cells were fixed in acetone and examined by immunocytochemistry as above.

### Separation of neutrophils from synovial fluid

Neutrophils were separated from synovial fluid obtained during routine therapeutic aspiration, using magnetic beads coated with a monoclonal CD15 antibody (Dynabeads, Dynal, New Ferry, UK).

### Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was prepared using RNazol (Biogenesis, Poole, UK) and reverse transcription carried out using the Superscript Preamplification system (Gibco) with oligo(dT)12–18 as primer. Amplification was carried out using Amplitaq GoldDNA polymerase (Perkin Elmer, Warrington, UK) and 0.2 μM specific primers (Genosys Biotechnologies, Pampisford, UK). Appropriate controls containing water or primers to glyceraldehyde-3-phosphate dehydrogenase were included.

Considerable deposits of iron are present within the synovial membrane of patients with established rheumatoid disease<sup>1</sup> and may be important in determining the chronicity of the inflammatory response.<sup>2</sup> However, little is known about iron uptake and storage within the synovial membrane and the role of the inflammatory response in these processes. We therefore studied the localisation of ferritin heavy and light chain, transferrin receptor, and non-specific resistance associated macrophage proteins (Nramp)1 and 2 within synovial tissue. It has been suggested that both Nramp1 and Nramp2 act as iron transporters.<sup>3,4</sup> We also localised haemosiderin in the synovial membrane.

## PATIENTS AND METHODS

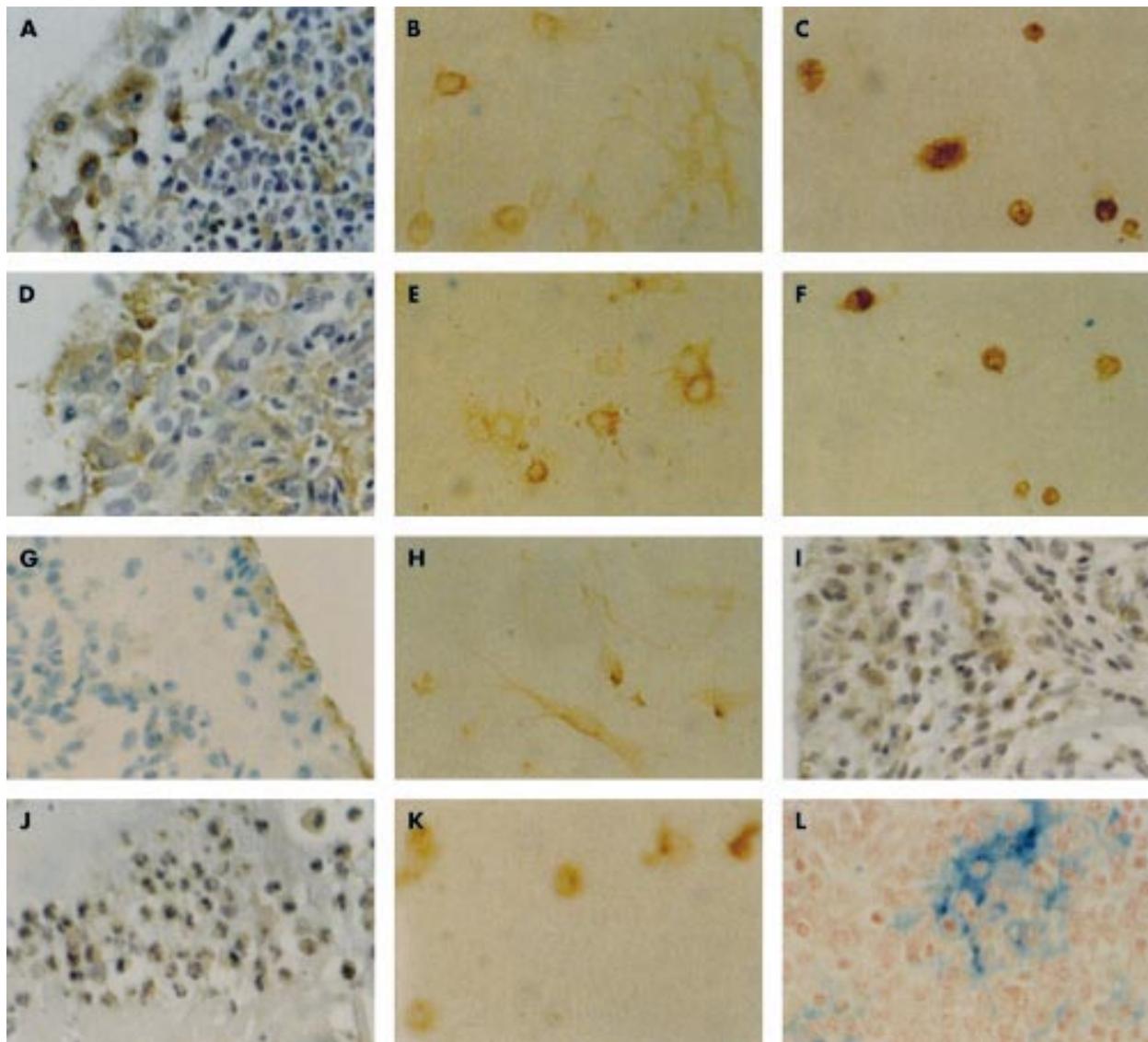
### Patients

Synovial membranes (arthroplasty specimens) were obtained from 20 patients with rheumatoid arthritis satisfying American College of Rheumatology criteria. All subjects gave their informed consent.

### Immunocytochemistry

Formalin fixed sections from biopsy specimens of rheumatoid synovium were stained by using monoclonal antibodies against recombinant human transferrin receptor (Menarini,

**Abbreviations:** IL, interleukin; Nramp, non-specific resistance associated macrophage proteins; RT-PCR, reverse transcriptase-polymerase chain reaction; TfR, transferrin receptor; TNF, tumour necrosis factor



**Figure 1** Immunocytochemistry of human synovial tissue and cells. Synovial tissue (A, D, G, I, J, L) or purified human synovial macrophages (C, F, K) and fibroblasts (B, E, H) were stained with antibodies to heavy ferritin (A–C), light ferritin (D–F), transferrin receptor (G, H), Nrap1 (I–K), or with Perls' reagent (L). Counterstaining was with Harris haematoxylin (A–K) or neutral red (L). Magnification  $\times 500$  (A, D, G, I, J, L) or  $\times 2000$  (B, C, E, F, H, K). For further details see text.

## RESULTS

### Cytochemistry

#### Ferritin

Ferritin heavy chain was localised to macrophages and fibroblasts in the lining layer of synovium and within the subintimal zone (fig 1A) and within areas of lymphocytic infiltrates, where the staining pattern was similar to that with anti-CD68 antibody (not shown), suggesting localisation to macrophages. Immunostaining for light ferritin showed a similar distribution but was generally less intense. Both heavy and light ferritin were detected in isolated fibroblasts (figs 1B and E) and macrophages (figs 1C and F), staining being more intense in macrophages than in fibroblasts. In all cases appropriate controls were negative.

#### Transferrin receptor

Transferrin receptor was restricted to modest staining of cells within the synovial lining layer (fig 1G). Fibroblasts isolated from rheumatoid synovium stained faintly with transferrin receptor antibody (fig 1H), but no staining was seen in isolated synovial macrophages.

#### Nrap1

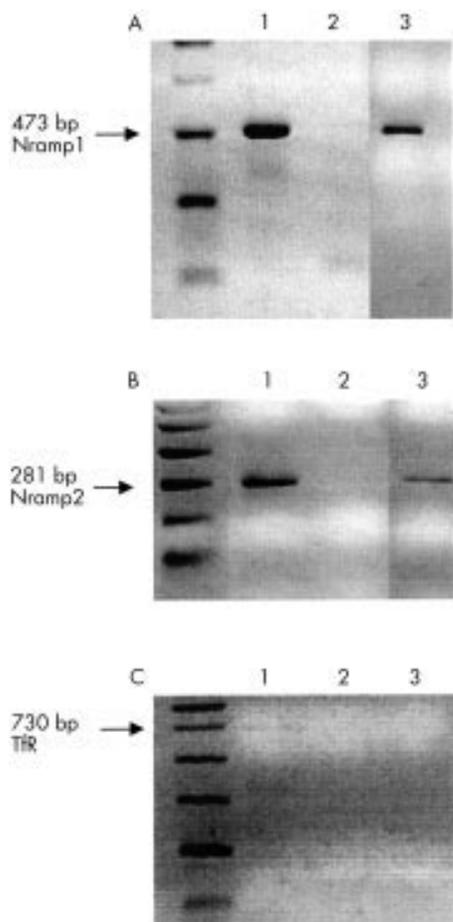
Nrap1 was seen within the synovial lining layer and the subintimal area (fig 1I), and also in neutrophil infiltrates (fig 1J). Isolated macrophages were positive (fig 1K), but fibroblasts were negative.

#### Haemosiderin

Haemosiderin deposits were generally only present within the subintimal area (fig 1L), but were also occasionally associated with fibroblasts, areas of inflammatory infiltrate, or the synovial lining (not shown). There was no correlation between Perls' staining and the intensity of heavy or light ferritin staining.

#### RT-PCR

Nrap1 mRNA was detected in isolated synovial macrophages (fig 2A, lane 3) and synovial fluid neutrophils (lane 1), but synovial fibroblasts were negative (lane 2). Specificity was confirmed by testing activated THP1 monocytic cells and human blood neutrophils, both of which are known to express Nrap1. In contrast, synovial fibroblasts expressed Nrap2



**Figure 2** (A) RT-PCR for Nramp1. Nramp1 was strongly expressed by isolated synovial macrophages (lane 3) but not by isolated fibroblasts (lane 2). Very strong expression was also seen by neutrophils isolated from synovial fluid (lane 1). (B) RT-PCR for Nramp2. Nramp2 was strongly expressed by isolated synovial fibroblasts (lane 1), more weakly by synovial macrophages (lane 3), and not at all by neutrophils from synovial fluid (lane 2). (C) RT-PCR for TfR. TfR was expressed weakly by isolated synovial fibroblasts (lane 1, band is arrowed), and not at all by synovial macrophages (lane 2) or neutrophils from synovial fluid (lane 3)

(fig 2B, lane 1), but synovial fluid neutrophils did not (lane 2). Synovial macrophages, which expressed Nramp1, also expressed Nramp2, albeit rather weakly (lane 3). Transferrin receptor (TfR) mRNA was weakly expressed by synovial fibroblasts (fig 2C, lane 1), while synovial macrophages and synovial fluid neutrophils were negative.

## DISCUSSION

Previous work has suggested that iron contributes to the pathogenesis of rheumatoid arthritis.<sup>1,2</sup> It is therefore important to understand how iron enters and is handled within the synovium. This in turn requires information about the presence and activity in the synovium of proteins which play a part in iron metabolism. Although there are some previous data describing the distribution of iron and ferritin in the synovium,<sup>2</sup> there has been no previous systematic attempt to define expression of iron binding proteins in the synovium.

Ferritin, which is responsible for iron storage and detoxification, was found in macrophages, fibroblasts, and areas of lymphocytic infiltrate. The abundance of heavy ferritin, primarily responsible for iron detoxification, was generally greater than light chain, which is mainly involved in long term storage. Light ferritin is selectively induced by interleukin 1 $\beta$  (IL1 $\beta$ ) and tumour necrosis factor  $\alpha$  (TNF $\alpha$ ),<sup>5,6</sup> which are

present in rheumatoid synovium.<sup>7</sup> We found no correlation between Perls' positive iron and ferritin, suggesting that iron is not the main stimulus for ferritin synthesis.

TfR was detected only in synovial lining layer cells, and RT-PCR showed that TfR mRNA is present only in synovial fibroblasts (at very low abundance) and not at all in synovial macrophages. Furthermore, fibroblasts isolated from the synovium stained with TfR antibody, but macrophages did not. TfR expression is associated with cell proliferation<sup>8</sup> and fibroblasts in rheumatoid synovium are proliferating,<sup>9</sup> so one might expect that fibroblasts would express high levels of TfR. However, TfR expression is also down regulated by iron,<sup>10</sup> which would counteract proliferative up regulation. The absence of detectable TfR expression by synovial macrophages indicates that the transferrin-TfR uptake system is unlikely to be involved in accumulation of iron by these cells. This is consistent with experiments showing that erythrocytes from intermittent intra-articular haemorrhages, rather than transferrin itself, were the source of iron deposits in rheumatoid synovium.<sup>11</sup>

Nramp2 mRNA was expressed in synovial fibroblasts and macrophages, but not neutrophils, suggesting a possible role in uptake of non-transferrin-bound iron, known to be present in the synovium. Nramp1 and Nramp2 (also known as DCT1 and DMT1) are two highly homologous transmembrane proteins involved in divalent cation transport. Nramp2 is widely expressed, and has been shown to mediate iron uptake from the intestinal lumen into mucosal cells,<sup>4</sup> whereas Nramp1 is the human homologue of the murine macrophage resistance gene, *Ity/Lsh/Bcg*, which controls susceptibility to intracellular pathogens.<sup>12</sup> Nramp1 is found exclusively in late endosomes and phagosomes of phagocytes.<sup>13</sup> Consistent with this, we detected Nramp1 mRNA and protein in synovial fluid neutrophils and synovial macrophages, but no expression in synovial fibroblasts. Unlike Nramp2, the exact function of Nramp1 in iron metabolism is unclear, but it may promote iron release from macrophages in vitro (Mulero V, *et al*, unpublished data), suggesting that it might influence iron accumulation within the synovium, especially in macrophages.

From our results it is possible to suggest tentatively how iron accumulates in the synovium. Initial inflammation and microbleeding leads to iron accumulation in synovial macrophages through erythrophagocytosis. Proinflammatory cytokines such as IL1 and TNF $\alpha$  cause and increase in ferritin (especially heavy ferritin) synthesis, but this may be insufficient to sequester all the iron, and these cytokines can, moreover, lead to increased macrophage iron retention.<sup>14</sup> Perls' positive iron will appear, and at this stage synovial ferritin synthesis appears to be down regulated by unknown factors, as suggested previously,<sup>15</sup> preventing sequestration of the Perls' positive iron. Macrophages will release some of this iron into the joint, probably regulated by Nramp1, where it accumulates as non-transferrin-bound iron in the synovial fluid. This, in turn, will be taken up by fibroblasts through Nramp2, allowing these cells too to become iron overloaded. As a result, potentially toxic iron builds up in the joint, and contributes to tissue damage. Further studies may help to determine the validity of this sequence of events.

## ACKNOWLEDGEMENTS

This work was supported by the Arthritis and Rheumatism Council. We thank Dr A Kinninmonth and Dr A Gracie for providing tissue samples, Dr CH Barton for providing anti-Nramp1 antibody and Dr P Santambrogio for antiferritin antibodies.

## Authors' affiliations

J F Telfer, J H Brock, Department of Immunology and Bacteriology, University of Glasgow, UK

Present address of Dr J F Telfer: Biomedical Research Centre, Ninewells

Hospital, Dundee DD1 9SY, UK

Correspondence to: Dr J F Telfer, Biomedical research Centre, Ninewells Hospital, Dundee, DD1 9SY, UK; telferjoan@hotmail.com

Accepted 23 January 2002

## REFERENCES

- 1 **Muirden KD**, Senator GB. Iron in the synovial membrane in rheumatoid arthritis and in other joint diseases. *Ann Rheum Dis* 1968;27:38–48.
- 2 **Blake DR**, Gallagher PJ, Potter AR, Ball MJ, Bacon PA. The effect of iron on the progression of rheumatoid disease: a histologic assessment of patients with early rheumatoid synovitis. *Arthritis Rheum* 1984;27:495–501.
- 3 **Atkinson PGP**, Barton CH. Ectopic expression of Nramp1 in COS-1 cells modulates iron accumulation. *FEBS Lett* 1998;425:239–42.
- 4 **Gunshin H**, Mackenzie B, Berger UV, Gunshin Y, Romero MF, Boron WF, *et al*. Cloning and characterization of a mammalian proton-coupled metal-ion transporter. *Nature* 1997;388:482–8.
- 5 **Miller LL**, Miller SC, Torti SV, Tsuji Y, Torti FM. Iron-independent induction of ferritin H chain by tumor necrosis factor. *Proc Natl Acad Sci USA* 1991;88:4946–50.
- 6 **Wei Y**, Miller SC, Tsuji Y, Torti SV, Torti FM. Interleukin 1 induces ferritin heavy chain in human muscle cells. *Biochem Biophys Res Commun* 1990;169:289–96.
- 7 **Klimuik PA**, Goronzy JJ, Bjornsson J, Beckenbaugh RD, Weyand CM. Tissue cytokine patterns distinguish variants of rheumatoid synovium. *Am J Pathol* 1997;151:1311–19.
- 8 **Sutherland R**, Delia D, Schneider C, Newman R, Kemshead J, Greaves M. Ubiquitous cell-surface glycoprotein on tumor cells is proliferation-associated receptor for transferrin. *Proc Natl Acad Sci USA* 1981;78:4515–19.
- 9 **Mohr W**, Beneke G, Mohing W. Proliferation of synovial lining cells and fibroblasts. *Ann Rheum Dis* 1975;34:219–24.
- 10 **Casey JT**, Hentze M, Koeller DM, Caugman SW, Rouatt TA, Klausner RD, *et al*. Iron-responsive elements: regulatory RNA sequences that control mRNA and translation. *Science* 1988;240:924–8.
- 11 **Zvaifler NJ**. New perspectives on the pathogenesis of rheumatoid arthritis. *Am J Med* 1988;85(suppl. 4A):12–17.
- 12 **Atkinson PGP**, Blackwell JM, Barton CH. Nramp1 locus encodes a 65kDa interferon-gamma-inducible protein in murine macrophages. *Biochem J* 1997;325:779–86.
- 13 **Gruenheid S**, Pinner E, Desjardins M, Gros P. Natural resistance to infection with intracellular pathogens: The Nramp1 protein is recruited to the membrane of the phagosome. *J Exp Med* 1997;185:717–30.
- 14 **Alvarez-Hernández X**, Licéaga J, McKay IC, Brock JH. Induction of hypoferrremia and modulation of macrophage iron metabolism by tumor necrosis factor. *Lab Invest* 1989;61:319–22.
- 15 **Hershko C**, Konijn AM. Pathogenesis of impaired iron release in inflammation. In: Brown EB, Aisen P, Fielding J, Crichton RR, eds. *Proteins of iron metabolism*. New York: Grune and Stratton, 1977:417–25.

# Direct Access to Medline

Medline

Link to Medline from the homepage and get straight into the National Library of Medicine's premier bibliographic database. Medline allows you to search across 9 million records of bibliographic citations and author abstracts from approximately 3,900 current biomedical journals.

[www.annrheumdis.com](http://www.annrheumdis.com)