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# Expression of growth factors, cytokines and matrix metalloproteinases in frozen shoulder

T. D. Bunker, J. Reilly, K. S. Baird, D. L. Hamblen

*From the University of Glasgow, Scotland*

**Frozen shoulder is a chronic fibrosing condition of the capsule of the joint. The predominant cells involved are fibroblasts and myofibroblasts which lay down a dense matrix of type-I and type-III collagen within the capsule. This subsequently contracts leading to the typical features of pain and stiffness. Cytokines and growth factors regulate the growth and function of the fibroblasts of connective tissue and remodelling of the matrix is controlled by the matrix metalloproteinases (MMPs) and their inhibitors.**

**Our aim was to determine whether there was an abnormal expression or secretion of cytokines, growth factors and MMPs in tissue samples from 14 patients with frozen shoulder using the reverse transcription/polymerase chain reaction (RT/PCR) technique and to compare the findings with those in tissue from four normal control shoulders and from five patients with Dupuytren's contracture.**

**Tissue from frozen shoulders demonstrated the presence of mRNA for a large number of cytokines and growth factors although the frequency was only slightly higher than in the control tissue. The frequency for a positive signal for the proinflammatory cytokines Il-1β and TNF-α and TNF-β, was not as great as in the Dupuytren's tissue. The presence of mRNA for fibrogenic growth factors was, however, more similar to that obtained in the control and Dupuytren's tissue. This correlated with the histological findings which in most specimens showed a dense fibrous tissue response with few cells other than mature fibroblasts and with very little evidence of any active inflammatory cell process.**

**Positive expressions of the mRNA for the MMPs were also increased, together with their natural inhibitor TIMP. The notable exception compared with control and Dupuytren's tissue was the absence of MMP-14, which is known to be a membrane-type MMP required for the activation of MMP-2 (gelatinase A).**

**Understanding the control mechanisms which play a part in the pathogenesis of frozen shoulder may lead to the development of new regimes of treatment for this common, protracted and painful chronic fibrosing condition.**

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The term 'frozen shoulder' was first used by Codman[1] who described the common features such as pain of gradual onset, which is felt near the insertion of the deltoid, inability to sleep on the affected side, painful restriction of elevation and external rotation and a normal radiological appearance.

The disorder is characterised by dense fibrosis of the capsule of the shoulder,[2-4] in which the cellular element consists of fibroblasts and myofibroblasts,[5,6] leading to a contracture of the rotator interval and the coracohumeral ligament, which restricts movement.[7-9]

Cytokines and other cellular growth factors are known to regulate the growth and function of fibroblasts in connective tissue. They are cell messengers derived from lymphoid cells, platelets, epithelial cells, endothelial cells, mesangial cells and fibroblasts and act in minute concentrations (nanomolar and femtomolar) by binding to cell receptors, causing a hormone-like action. One of their basic functions is the control of healing in damaged tissues. This process involves the accumulation of fibroblasts at the site of healing and is brought about by chemotaxis (attracting fibroblasts from elsewhere) and proliferation of fibroblasts. Cytokines and growth factors can also modulate the synthesis of collagen by fibroblasts.

The histological feature of frozen shoulder is a matrix of type-I and type-III collagen populated by fibroblasts and myofibroblasts which suggests that the condition may be modulated by an abnormality in the production of cytokines and growth factors. Previous studies have shown a striking resemblence between the histology, immunocytochemistry,

T. D. Bunker, BSc, MCh Orth, FRCS, FRCS Ed, Consultant Orthopaedic Surgeon
Princess Elizabeth Orthopaedic Centre, Barrack Road, Exeter EX2 5DW, UK.

J. Reilly, PhD
K. S. Baird, FRCS Ed, FRCS Orth
D. L. Hamblen, PhD, FRCS, Professor
Department of Orthopaedic Surgery, University of Glasgow, Western Infirmary, Dumbarton Road, Glasgow G11 6NT, UK.

Correspondence should be sent to Mr T. D. Bunker.


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and cytogenetics of tissues from frozen shoulder and those of Dupuytren's disease[5,9] and the expression of cytokines and growth factors has been shown to be abnormal in Dupuytren's disease.[10] We have therefore compared the expression of mRNA for cytokines, growth factors, and metalloproteinases (MMPs) in frozen shoulder with that of material from Dupuytren's disease and normal control tissues. The genes were identified using the technique of the reverse transcription/polymerase chain reaction (RT/PCR) in RNA extracted from the fibrosed capsule of patients with frozen shoulder.

## Patients and Methods

Of 1800 new patients presenting to one of the authors (TDB) with disorders of the shoulder, 93 fitted Codman's criteria[1] for the diagnosis of frozen shoulder. These patients had all had conservative treatment consisting of analgesia and physiotherapy, with or without injections of steroid. Those who failed to improve with conservative treatment underwent shoulder arthroscopy and manipulation under anaesthesia. A resistant group whose shoulders remained stiff at the time of manipulation had surgical release, using the method described by Ozaki et al.[7] In this operation the diseased and contracted coracohumeral ligament and interval are excised, releasing the contracted shoulder.

We studied 14 consecutive patients undergoing surgical release. There were nine women and five men with a mean age of 54.6 years. The duration of the disorder was 21.8 months. These patients were preselected as having severe disease by their failure to respond to conservative treatment. The initial treatment consisted of physiotherapy and manipulation in all, with additional injection of steroid in 50%. The mean range of movement was combined passive flexion of 86°, external rotation of only 5°, and internal rotation to reach to buttock level or below in all cases. Seven had had arthroscopy before surgical release. Of these, all had obliteration of the area of the rotator interval; four showed vascular villous synovitis at the site and the other three had mature scar tissue in the area.

At operation a small deltoid-splitting incision was made and the coracoacromial ligament excised to reveal the area of the rotator interval. In all patients there was abnormal scar tissue at this site which acted as a checkrein to passive movement of the joint. Excision of this scar tissue restored a functional range of passive movement. At follow-up three months after surgical release the mean combined passive flexion was 136°, external rotation 35°, and internal rotation L3 (T6 to L5).

**Collection of tissue.** The tissue excised from the area of the rotator interval was snap-frozen in liquid nitrogen until the RNA was extracted. For comparison RNA was obtained from fresh pathological material excised from the thickened palmar fascia of five patients with typical Dupuytren's contracture. Tissue removed at arthroscopy from the capsule of the shoulder of four patients with mechanical problems was stored under similar conditions to give a control group.

The samples were divided into two. One half was stored for histological examination and in the other the total RNA was extracted using the acid phenol-guanidium thiocyanate chloroform method. The RNA was quantitated by spectrophotometry and a standard amount (5 μg) reverse transcribed using a genetically-engineered M-MLV reverse transcriptase (Superscript, Gibco/BRL). The resulting complementary DNA (cDNA) was dissolved in 100 μl of sterile distilled water and 1 in 20 of the cDNA (5 μl) was used as a template for each PCR. This was carried out under standard reaction conditions in a volume of 50 μl. Appropriate safeguards were used to avoid false-positive results caused by external contamination. Purified Taq polymerase and reaction buffer were supplied by Gibco/BRL and the oligonucleotide primers were used at a final concentration of 0.5 μM each. Thirty-five cycles of amplification were used as standard, one cycle comprising a 'melting' step of 94°C for 50 seconds, an 'annealing' step of 65°C for 50 seconds and an 'extension' step of 72°C for 90 seconds. During the first cycle, the melting step was extended to 2.5 minutes and during the last cycle, the extension step was extended to 5 minutes.

Oligonucleotide primers (20 to 26 mer) were designed on the basis of published sequences with the aid of a CGC sequence-analysis software package and prepared using an automated oligonucleotide synthesiser. Primer recognition sites on the respective mRNAs and predicted PCR product sizes are shown in Table I.

In all cases the primer recognition sites were designed to reside in separate exons or at intron-exon boundaries of the genomic sequence, ensuring specificity for amplification of

**Table I.** Primer recognition sites on the respective mRNAs and predicted product sizes

| Primers | Recognition sites (base pairs) | Product size (bp) |
|---|---|---|
| IL-1α | 299 to 612 | 313 |
| IL-1β | 93 to 461 | 368 |
| IL-6 | 187 to 485 | 298 |
| TNF-α | 13 to 452 | 439 |
| TNF-β | 84 to 412 | 328 |
| MMP-1 | 335 to 742 | 407 |
| MMP-2 | 231 to 599 | 368 |
| MMP-3 | 381 to 896 | 515 |
| MMP-9 | 1438 to 1700 | 262 |
| MMP-14 | 1354 to 1535 | 182 |
| TIMP-1 | 80 to 506 | 426 |
| PDGF-α | 490 to 747 | 257 |
| PDGF-β | 1310 to 1642 | 332 |
| TGF-β | 1252 to 1551 | 299 |
| Acidic FGF | 151 to 455 | 304 |
| Basic FGF | 575 to 778 | 203 |
| Beta-2-M (internal control) | 24 to 345 | 321 |

mRNA (cDNA), rather than genomic DNA. The identity of the PCR products was confirmed by their predicted size on agarose-gel electrophoresis and by restriction analysis. In this technique, diagnostic fragments of a specific size are obtained when the PCR product is digested by a restriction enzyme with a recognition site within the product in question (data not shown, full information on restriction enzymes used, and fragments generated available on request). Reaction products (12 μl) were analysed by electrophoresis through 1.5% agarose gels stained with ethidium bromide and visualised by transillumination under ultraviolet light at a wavelength of 302 nM.

Amplification of the mRNA for beta-2-microglobulin (beta-2-M), a component of the HLA class-1 antigen present on all nucleated cells, was used as an internal control in all cases. This provided confirmation of the integrity of the extraction, reverse transcription and PCR processes.

## Results

**Cytokines and growth factors.** The cytokines and growth factors studied in the excised and control material were interleukin-1 (Il-1)α and Il-1β, interleukin-6 (Il-6), tumour necrosis factors (TNF)-α and TNF-β, platelet-derived growth factor (PDGF)-α and PDGF-β, transforming growth factor (TGF)-β and the acidic and basic fibroblast growth factors (FGF).

The results were recorded as the frequency and percentage of tissues showing a positive PCR result for the genetic expression of the individual cytokine or growth factor.

The tissue from the patients with frozen shoulder showed strong expression of the mRNA for some, but not all, the cytokines tested (Table II and Fig. 1). All tissues expressed IL-1α and Il-6, as did the tissue from the Dupuytren's contractures and most of the control group. By contrast, 80% of shoulder tissue was positive for TNF-β compared with 100% of Dupuytren's tissue and 50% of the control group. The signal for Il-1β was present in 100% of Dupuytren's tissue, but only in 42.9% of frozen shoulder tissue and 25% of the control group. Even lower positive results were obtained for TNF-α, which was only expressed in 7.1% of frozen shoulder tissue compared with 40% in Dupuytren's tissue and none in the control tissue.

Table III and Figure 2 show that the results for growth factors were equally variable with only acidic FGF expressing in 100% of both shoulder and Dupuytren's tissue, as well as 75% of the control group. Of interest was a positive result of 100% for PDGF-α, PDGF-β and TGF-β in both Dupuytren's and control tissues, compared with equivalent levels of 86%, 50% and 93%, respectively, in frozen shoulder tissue. By contrast, the level of basic-FGF was higher at 86% compared with 75% in the control group and 60% in Dupuytren's tissue.

The results for the frequency of the mRNAs for the MMPs and their inhibitor TIMP-1 is shown in Table IV and

**Table II.** Expression of cytokines in tissues from frozen shoulder, Dupuytren's contracture and the control group

| | **Cytokines** | | | | | |
|---|---|---|---|---|---|---|
| | **IL-1α** | **IL-1β** | **IL-6** | **TNF-α** | **TNF-β** | **Beta-2-M** |
| Frozen shoulder | | | | | | |
| Frequency | 14/14 | 6/14 | 14/14 | 1/14 | 12/14 | 14/14 |
| Percentage positive | *100.0* | *42.9* | *100.0* | *7.1* | *85.7* | *100.0* |
| Control shoulder | | | | | | |
| Frequency | 3/4 | 1/4 | 4/4 | 0/4 | 2/4 | 4/4 |
| Percentage positive | *75.0* | *25.0* | *100.0* | *0.0* | *50.0* | *100.0* |
| Dupuytren's tissue | | | | | | |
| Frequency | 5/5 | 5/5 | 5/5 | 2/5 | 5/5 | 5/5 |
| Percentage positive | *100.0* | *100.0* | *100.0* | *40.0* | *100.0* | *100.0* |

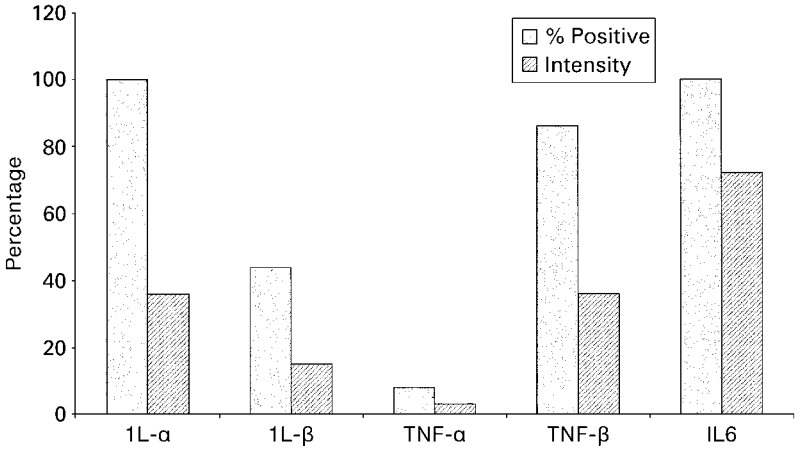


Fig. 1

Expression of cytokines in tissue samples of frozen shoulder.

**Table III.** Expression of growth factors in tissues from frozen shoulder, Dupuytren's contracture and the control group

| | **Growth factors** | | | | | |
|---|---|---|---|---|---|---|
| | **PDGF-α** | **PDGF-β** | **TGF-β** | **Acidic FGF** | **Basic FGF** | **Beta-2-M** |
| Frozen shoulder | | | | | | |
| Frequency | 12/14 | 7/14 | 13/14 | 14/14 | 12/14 | 14/14 |
| Percentage positive | *85.7* | *50.0* | *92.9* | *100.0* | *85.7* | *100.0* |
| Control shoulder | | | | | | |
| Frequency | 4/4 | 4/4 | 4/4 | 3/4 | 3/4 | 4/4 |
| Percentage positive | *100.0* | *100.0* | *100.0* | *75.0* | *75.0* | *100.0* |
| Dupuytren's tissue | | | | | | |
| Frequency | 5/5 | 5/5 | 5/5 | 5/5 | 3/5 | 5/5 |
| Percentage positive | *100.0* | *100.0* | *100.0* | *100.0* | *60.0* | *100.0* |

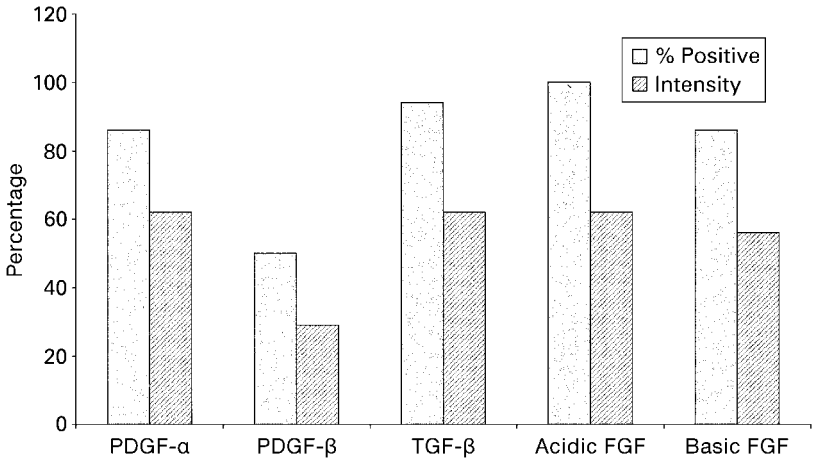


Fig. 2

Expression of growth factors in tissue samples of frozen shoulder.

**Table IV.** Expression of MMPs and TIMP-1 in tissue from frozen shoulder, Dupuytren's contracture and the control group

| | **Metalloproteinases** | | | | | | |
|---|---|---|---|---|---|---|---|
| | **MMP-1** | **MMP-2** | **MMP-3** | **MMP-9** | **MMP-14** | **TIMP-1** | **Beta-2-M** |
| Frozen shoulder | | | | | | | |
| Frequency | 9/14 | 13/14 | 5/14 | 8/14 | 0/14 | 14/14 | 14/14 |
| Percentage positive | *64.3* | *92.9* | *35.7* | *57.1* | *0.0* | *100.0* | *100.0* |
| Control shoulder | | | | | | | |
| Frequency | 3/4 | 4/4 | 1/4 | 1/4 | 2/4 | 4/4 | 4/4 |
| Percentage positive | *75.0* | *100.0* | *25.0* | *25.0* | *50.0* | *100.0* | *100.0* |
| Dupuytren's tissue | | | | | | | |
| Frequency | 5/5 | 5/5 | 0/5 | 3/5 | 4/5 | 5/5 | 5/5 |
| Percentage positive | *100.0* | *100.0* | *0.0* | *60.0* | *80.0* | *100.0* | *100.0* |

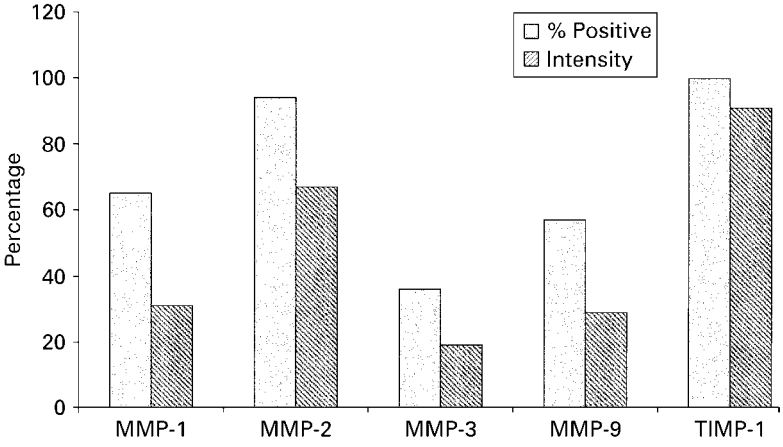


Fig. 3

Expression of metalloproteinases in tissue samples of frozen shoulder.

Figure 3. With the exception of MMP-3 (stromelysin), the MMP positive expression was lower in the frozen shoulder tissue than in that from Dupuytren's disease. None of the shoulder specimens showed the mRNA for MMP-14, compared with 80% of Dupuytren's tissue and 50% of the control group. The mRNA for MMP-3 was not found in any of the five specimens of Dupuytren's disease. All three types of tissue showed 100% expression for the natural inhibitor TIMP-1.

## Discussion

Frozen shoulder is a very protracted condition which only resolves after years rather than months.[11,12] Previous reports of the pathological process have suggested that both an inflammatory condition[2] and a fibrotic element[5] may be responsible. The prolonged clinical history with an initial acute painful phase followed by a prolonged period of stiffness suggests a sequential adhesive capsulitis with an inflammatory synovial process followed by subsequent fibrosis of the capsule.

The advent of shoulder arthroscopy has aided research into shoulder pathology. Arthroscopy in patients with frozen shoulder showed that the capsule was contracted, there were no adhesions, and that the maximal area of abnormality was at the rotator interval. This was usually obliterated by granulating scar tissue, which was covered by an extremely vascular villous synovitis showing discoloration and small petechial haemorrhages.[13,14] Arthroscopic biopsies from the capsule showed histological changes of diffuse capsular fibroplasia, thickening and contracture.[6] Bunker and Anthony[5] carried out histological and immunocytochemical studies on the contracted area of the capsule. Their findings showed that the pathological process is active fibroblastic proliferation, accompanied by some transformation to a smooth muscle phenotype (myofibroblasts). The fibroblasts lay down collagen which appears as thick nodules or bands, macroscopically resembling a fleshy mass. These findings are similar, but not identical, to those found in Dupuytren's disease of the hand.

Fibroblasts are controlled by certain cytokines, the peptide molecules of which act as cell messengers. These control many aspects of cell migration and growth, acting in minute concentrations by binding to receptors on the target cell, to regulate chemotaxis and proliferation of fibroblasts and the synthesis of collagen.[15] Cytokines and growth factors are also known to have a key role in the transcription of the enzymes of MMPs which control the turnover of connective tissue.

Using monoclonal antibody techniques Rodeo et al[16] demonstrated elevated levels of cytokines in frozen shoulder. They found increased levels of TGF-β and PDGF and suggested that they act as a persistent stimulus causing capsular fibrosis and the development of the frozen shoulder. We have used PCR rather than immunocytochemistry techniques and have shown that these cell messengers are expressed locally in most of the tissues tested and are not secreted at some other site and transported to the shoulder. The mRNA for these growth factors, however, was also present in the tissue from control shoulders and Dupuytren's disease.

The protracted natural history of frozen shoulder suggests that these factors act as a persistent stimulus to scar formation and block attempts at remodelling. MMPs are known to have an important role in this remodelling process.[17] They constitute a family of zinc-dependent endopeptidases which may be normally expressed at low levels in adult tissues, but are upregulated during pathological remodelling processes. The expression of MMPs is tightly controlled by cytokines and other growth factors which either stimulate or repress their genetic transcription. Their actions may also be inhibited by the formation of complexes with their naturally occurring inhibitors, TIMP-1 and TIMP-2. In this study MMP-2 (gelatinase A) was expressed more frequently than MMP-1 (collagenase) or MMP-3 (stromelysin-1). The membrane-bound MMP-14 is known to have a key role in the activation of MMP-2 (gelatinase A). The surprising absence of the mRNA for MMP-14 in all 14 specimens of frozen shoulder may suggest a possible mechanism for the slow resolution of the fibrosis. The lack of the gene for this MMP could result in a failure to secrete and activate MMP-2 (gelatinase A), which is known to be essential for the degradation of collagen in connective tissues.[18] The mRNA for MMP-2 was also found to be present in all the five specimens of Dupuytren's tissue and the four normal control specimens. The mRNA for collagenase was also expressed in all the samples of Dupuytren's tissue suggesting a more active inflammatory cellular process although the absence of the genetic message for MMP-3 (stromelysin) in all five specimens suggests an imbalance in the remodelling of connective tissue. The universal expression of the TIMP-1 gene in all tissues suggests an alternative mechanism for the failure to remodel fibrosis in frozen shoulder because of the persistent presence of this natural inhibitor. The recent report by Hutchinson et al[19] of the use of a synthesised broad-spectrum TIMP (Marimastat) in patients with inoperable gastric carcinoma would support this hypothesis. He studied 12 patients to determine the ability of TIMP to slow down the progression of the disease. Six developed bilateral frozen shoulder within four months of starting treatment, and three also had a Dupuytren-type palmar contracture. This remarkable in-vivo experiment would appear to confirm that the high levels of TIMP-1 expression, which were found in the tissues in our study, may also have an important part in the formation and continuation of fibrous contractures in frozen shoulder.

It seems likely that frozen shoulder is initiated by one of a number of triggering factors which occur in patients with a predisposition to contracture. This may be local minor trauma, minor injury in the same limb, as occurs in Colles' fracture, or from other surgery in the area such as mastecto-

my. This predisposition to contracture may be genetic and could explain the strong association with Dupuytren's contracture. An inflammatory healing response appears to be produced within the capsule of the shoulder. This is moderated by cytokines and growth factors, leading to the accumulation and propagation of fibroblasts which typically lay down an excess of type-III collagen. The cytokine response may also lead to angiogenesis within the capsule giving the typical arthroscopic appearances of frozen shoulder. Some of the fibroblasts differentiate into myofibroblasts which lead to contracture of the newly laid down type-III collagen. At the same time there may be a failure of collagen remodelling, which in part may result from a genetic failure to activate gelatinase A, or from elevation of the levels of the natural inhibitor TIMP in the capsule. This imbalance between aggressive healing, scarring, contracture and a failure of remodelling may lead to the protracted stiffening of the capsule.

Further immunolocalisation studies are required to identify the sites and type of cytokine and MMP secretion in order to provide a better understanding of the cellular and enzymatic control mechanisms which play a part in the pathogenesis of frozen shoulder. These could lead to the development of new regimes of treatment for patients suffering from this common, protracted and painful chronic condition.

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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