We collected 16 samples of the membrane which surrounds loose hip prostheses from patients undergoing revision operations for aseptic loosening. To serve as the control group, samples of the synovial tissue and the fibrous capsular tissue were collected from 11 patients undergoing primary hip arthroplasties. Analyses of the expression levels of inducible nitric oxide synthase (iNOS), tumour necrosis factor-alpha (TNF-α), and cytosolic phospholipase A₂ (cPLA₂) mRNAs were performed by a reverse transcription polymerase chain reaction, and the content of nitrite was measured by the Griess reaction using sodium nitrite as the standard.

The expression levels of iNOS, TNF-α, and cPLA₂ mRNAs in the membranes were significantly higher than those in the control samples (p < 0.05). The expression levels of iNOS mRNA and the nitrite content in the membranes significantly correlated with those of TNF-α and cPLA₂ mRNAs, respectively. In addition, the expression levels of iNOS, TNF-α, and cPLA₂ mRNAs were significantly higher in membranes from cementless than in those from cemented implants (p < 0.05).

Our results suggest that the expression levels of iNOS, TNF-α, and cPLA₂ mRNAs in the membranes are regulated by closely-related mechanisms and that these have a significant role in aseptic loosening.

Received 3 April 2001; Accepted after revision 7 August 2001

Aseptic loosening is the most common mode of failure of total hip arthroplasty (THA) and is thus a major clinical problem. The causes are unclear, but factors which interact to produce aseptic loosening can be classified into several independent processes, including those involving mechanical factors, the material properties of the implants and biological and host factors. In recent years, attention has focused on the potential pathophysiological importance of a synovial-like membrane which develops at the interface between the implant and bone or between the cement and bone. There are a number of cytokines with important effects on the immune and haematological systems which also act on bone cells. Tumour necrosis factor-alpha (TNF-α) is a cytokine known for its cytostatic, cytolytic, and antiviral actions, but in contrast to its growth-inhibiting effects on tumour cells, it stimulates bone resorption by enhancing the recruitment of osteoclasts, and several studies have shown that it plays an important role in the pathogenesis of periprosthetic osteolysis. Reports suggest that interleukin (IL)-1 and prostaglandins released by the cells of the membranes of loosened total hip arthroplasties are involved in bone resorption, and recent studies have shown that inducible nitric oxide synthase (iNOS) is present in the cells of the membranes and that nitric oxide (NO) may also be involved. It has been reported that cytokines activate both iNOS and cytosolic phospholipase A₂ (cPLA₂), suggesting their interaction during the process of bone resorption.

We have attempted to examine the relationship between the levels of iNOS, TNF-α, and cPLA₂ mRNAs in the membrane around THAs and their role in aseptic loosening.

Patients and Methods

Tissue sampled from patients. Between December 1998 and July 2000, samples of the membrane were obtained from overt osteolytic areas during revision surgery for aseptic loosening in 16 patients. We excluded patients with infections or other systemic diseases from the study. The mean age at the time of revision was 56.7 years (24 to 76) and the mean interval between primary THA and revision was 108.9 months (18 to 209). Eight had initially undergone THA because of avascular necrosis of the femoral head, four because of a fracture of the femoral neck or associated sequelae and four because of osteoarthritis of the hip. Ten implants were cementless and six cemented. As a
control group, 11 samples of synovial membrane and joint capsule were obtained from patients during primary hip arthroplasty. The mean age of the control group was 57.6 years (33 to 81). Seven had avascular necrosis of the femoral head and required primary THA, and four had a fracture of the femoral neck and underwent bipolar hemiarthroplasty.

**Measurement of the extent of osteolysis.** The extent of the osteolysis was analysed on the postoperative radiographs by measurement of the sum of each surface area of osteolysis by point-counting with a grid on the true anteroposterior radiograph of the hip. All the measured values were corrected for the radiological magnification by using a magnification marker.

**Isolation of the total RNA and the reverse transcription polymerase chain reaction (RT-PCR).** The total RNA was isolated from the tissue samples by RNAzol B (Tel Test Inc, Friendswood, Texas), reverse transcribed into the first strand cDNA by using oligo dT primer and amplified by 35 cycles (94°C, 1 minute; 50°C, 1 minute; 72°C, 1 minute) of PCR with 20 picomoles of specific primers (Table I). On completion of the PCR reaction, the products were examined on 2% agarose gel. β-actin primers were used as the internal standard. The amounts of the PCR products were quantified by an image analyser (Biorad, Hercules, California) and normalised to β-actin signals.

**Measurement of the total nitrite production.** Production of NO was assessed by measuring nitrite (a stable oxidation product of NO) in a conditioned medium, based on the Griess reaction. Conditioned medium was obtained by incubating tissue samples in Dulbecco's modified eagle medium for 24 hours. Sodium nitrite was used as the standard. Levels of nitrite were measured by mixing 100 μl aliquots of samples with 50 μl of 1% sulphanilamide in water plus 50 μl of 0.1% N-1-naphthylethylenediamine dihydrochloride in 5% phosphoric acid, then incubating the entire solution for ten minutes at room temperature. The absorbance at 540 nm was then measured by using a Titertek Multiskan MC plate reader (ICN/Flow Biochemicals, Huntsville, Alabama). The results were expressed as picomoles of nitrite per mg of protein.

**Statistical analysis.** The mean and the SEM were used to express the dispersion of the data. Using the Systat software package (SPSS Science, Chicago, Illinois), the group means were compared by the Mann-Whitney U test. A p value of 0.05 or less was considered to be significant. Pearson’s correlation coefficient was used to measure correlations.

### Results

Table II gives the clinical data, the extent of osteolysis and the expression levels of iNOS, TNF-α, cPLA2 mRNAs and nitrite in the membranes of patients with aseptic loosening.

**Expression of iNOS, TNF-α and cPLA2 mRNAs in the membranes.** The expression levels of iNOS, TNF-α, and cPLA2 mRNAs were compared with those of the control tissues and those of the membranes obtained from patients with aseptic loosening by RT-PCR (Fig. 1). They were higher in the membranes than in the control tissues. Quantification of the amplified PCR products by image analysis showed an increase of 200% to 300% in the membranes (p < 0.05) (Fig. 2).

A strong correlation was observed between the expres-
### Table 1

<table>
<thead>
<tr>
<th>Case</th>
<th>Control</th>
<th>Interfacial Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS</td>
<td><img src="image1" alt="Image" /></td>
<td><img src="image2" alt="Image" /></td>
</tr>
<tr>
<td>TNF-α</td>
<td><img src="image3" alt="Image" /></td>
<td><img src="image4" alt="Image" /></td>
</tr>
<tr>
<td>cPLA2</td>
<td><img src="image5" alt="Image" /></td>
<td><img src="image6" alt="Image" /></td>
</tr>
<tr>
<td>β-actin</td>
<td><img src="image7" alt="Image" /></td>
<td><img src="image8" alt="Image" /></td>
</tr>
</tbody>
</table>

### Figures

**Fig. 1**
Photograph of the RT-PCR products of total RNAs isolated from the control tissues and the membranes of patients with aseptic loosening.

**Fig. 2**
Mean (± SEM) expression levels of iNOS, TNF-α, and cPLA2 mRNAs in the control tissues and the membranes obtained from patients with aseptic loosening (*p < 0.05*).

**Fig. 3**
Correlation between iNOS mRNA expression and nitrite levels in the membranes.

**Fig. 4**
Correlation between expression levels of TNF-α mRNA and iNOS mRNA in the membranes.

**Fig. 5**
Correlation between expression levels of cPLA2 mRNA and iNOS mRNA in the membranes.
sion level of iNOS mRNA and the level of nitrite in the membranes (r = 0.94; p < 0.01; Fig. 3).

**Relationship of the expression levels of iNOS, TNF-α, and cPLA₂ mRNAs.** The TNF-α mRNA expression in the membranes was found to be strongly correlated with that of the iNOS (r = 0.78; p < 0.01; Fig. 4). The latter also correlated with the expression level of cPLA₂ mRNA (r = 0.67; p < 0.01; Fig. 5) and this in turn significantly correlated with TNF-α mRNA (r = 0.85; p < 0.01; Fig. 6).

**Relationship between the extent of osteolysis and the expression levels of iNOS, TNF-α and cPLA₂ mRNAs.** There was no significant correlation between the extent of osteolysis and the interval between primary THA and revision (r = 0.17) or with the expression levels of iNOS, TNF-α and cPLA₂ mRNAs (r = 0.26, r = 0.2 and r = 0.22, respectively).

Although there was no significant difference in the extent of osteolysis between the cemented and cementless implants, the expression levels of iNOS, TNF-α, and cPLA₂ mRNAs were significantly higher in the cementless group (p < 0.05) (Table III).

**Discussion**

The pathophysiological mechanism of aseptic loosening has yet to be defined, although there is increasing evidence that cyclic mechanical loosening, production of wear particles, and the ensuing adverse tissue response are all significant contributors to local osteolysis at the prosthesis-bone interface. Recent studies have shown that the levels of IL-1, TNF-α, prostaglandin E₂, and NO in the membranes are strongly implicated in the induction and maintenance of bone resorption.

**Table III.** Mean (± SEM) expression levels of iNOS, TNF-α and cPLA₂ mRNAs and the extent of osteolysis in cementless and cemented implants

<table>
<thead>
<tr>
<th></th>
<th>iNOS/β-actin ratio</th>
<th>TNF-α/β-actin ratio</th>
<th>cPLA₂/β-actin ratio</th>
<th>Osteolysis (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cementless</td>
<td>0.78 ± 0.09*</td>
<td>0.73 ± 0.11*</td>
<td>0.88 ± 0.12*</td>
<td>958.9 ± 291.4</td>
</tr>
<tr>
<td>(n = 10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cemented</td>
<td>0.44 ± 0.08</td>
<td>0.43 ± 0.06</td>
<td>0.55 ± 0.1</td>
<td>1262.5 ± 831.7</td>
</tr>
<tr>
<td>(n = 6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* p < 0.05

**Correlation between expression levels of TNF-α mRNA and cPLA₂ mRNA in the membranes.**

**Fig. 6**

Our study has shown that the expression levels of iNOS, TNF-α and cPLA₂ mRNAs were altered in the membrane of patients with aseptic loosening, were higher than those of the control tissues, and that they correlated with each other. Several cytokines can cause cellular recruitment in inflammation and these are grouped in the chemokine family. Some of these are produced in the tissue of the membrane and recent studies have clearly demonstrated interaction between these types of cytokine. TNF-α increases the iNOS expression in various tissues and activates phospholipase A₂, which has been involved in the expression of iNOS mRNA in various types of tissue. Peroxynitrite was found to promote activation of phospholipase A₂ in PC12 cells.

These studies, taken together with our data, suggest that the expression of cytokines and cytokine-producing enzymes in the membranes as well as other tissues may be closely linked to each other. Thus, the cytokine network in the tissues of the membrane may have an important role in the loosening of total hip implants, are involved in the activation of the macrophages and are released from activated macrophages. Our results support the notion that aseptic loosening is caused in part by a host response to foreign material.

Zicat, Engh and Gokcen showed that the osteolysis around cementless cups was localised and expansile, that it was not associated with loosening of the component, and that it produced more loss of bone than did the linear pattern of osteolysis around cemented cups. Goodman et al., using immunohistochemistry and in situ hybridisation techniques on periprosthetic membranes, showed increased quantities of macrophages, T-lymphocyte subgroups, and IL-1 and IL-6 expression levels in osteolytic lesions around cemented implants. They also found, however, that the osteolysis around cementless implants was associated with elevated levels of T-lymphocyte subgroups and TNF-α, suggesting that there were different biological mechanisms of loosening for cemented and cementless implants. Our findings showed that the expression levels of iNOS, TNF-α, and cPLA₂ mRNAs were significantly higher in the cementless than in the cemented implants, suggesting that cementless fixation may exaggerate a host reaction.

The initial stimulus of iNOS expression in patients with aseptic loosening is still not clear. Recent studies suggest that the loading of the prosthesis and the generation of wear particles activate the inflammatory phagocytic cells and that phagocytosis of the wear debris is a key element in the induction of cytokines, including NO in human macrophages. The interactions between these cytokines play a synergistic role in the processes of bone resorption.

THE JOURNAL OF BONE AND JOINT SURGERY
One area of intense research regarding inflammation is the regulation of cyclo-oxygenase-2 (COX-2) and the prostanoid production by cytokines by way of the synthesis of NO. This is of high clinical relevance because COX-2 is the primary target for non-steroidal anti-inflammatory agents and may have implications in the management of aseptic loosening by anti-inflammatory agents which are capable of suppressing the synthesis or the activity of iNOS and COX. The COX family of enzymes catalyses the breakdown of arachidonic acid into prostanooids such as the leukotrienes and the prostaglandins. There are two isoforms of COX, the constitutive isoform (COX-1) which maintains the physiological vascular and gastric functions and the inducible isoform (COX-2), of which mRNA and protein synthesis of which are stimulated largely by the same cytokines as for iNOS, IL-1, TNF-α, and interferon-γ. In our study, the expression level of cPLA2 was also increased in the membranes. Therefore an increase in the expression level of cPLA2 may also have a role in the activation of COX-2 and subsequent synthesis of prostanooid in addition to NO-induced COX-2 activation. Although increased mRNA for cPLA2 can be predicted from the increased TNF-α, we have shown changes in cPLA2 mRNA expression on the basis of experimental data in tissue samples obtained from patients with aseptic loosening.

Our results suggest that the expression of iNOS, TNF-α, and cPLA2 mRNAs in the membranes is regulated by closely-related mechanisms, and that these mechanisms have a significant role in the aseptic loosening of joint implants. No benefits in any form have been received or will be received from a company related directly or indirectly to the subject of this article.

References


