Major Role for Interleukin 1 but Not for Tumor Necrosis Factor in Early Cartilage Damage in Immune Complex Arthritis in Mice

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ABSTRACT. Objective. To determine the regulating role of interleukin-1 α and β (IL-1α,β) and tumor necrosis factor α (TNF-α) on inhibition of proteoglycan synthesis and proteoglycan degradation in early immune complex arthritis (ICA) in the mouse.

Methods. In the early phases of arthritis, IL-1 and TNF were measured using cytokine specific bioassays, the NOB.1 EL-4 and L929 assay, respectively. The impact of IL-1 in proteoglycan synthesis was studied by neutralizing the formed IL-1 during early arthritis either by giving anti-IL-1 specific antibodies intravenously or IL-1 receptor antagonist (IL-1ra) intraperitoneally by osmotic pumps. TNF-α was neutralized by giving monoclonal antibodies directed against murine TNF-α. Synthesis of proteoglycans was measured ex vivo by uptake of 35S-sulfate by patellae derived from inflamed and control, noninflamed knee joints. In vivo formation of 35S-sulfate labeled proteoglycans was studied by autoradiography. Degradation of proteoglycans was measured by labeling patellae in vivo with 35S-sulfate before arthritis induction.

Results. High levels of IL-1 are formed during the first phase of immune complex arthritis (ICA). Neutralization of either IL-1α or β with specific polyclonal antibodies resulted only in partial blocking, whereas a combination fully blocked inhibition of proteoglycan synthesis. Full blocking was also found after systemic treatment with high amounts of IL-1 receptor antagonist (1.2 mg/day during 3 days). Influx of cells was also significantly reduced both in the anti-IL-1 as well as in the IL-1ra treated groups. Whether infiltrating cells are involved in inhibition of proteoglycan synthesis was further investigated in neutropenic mice. Significantly higher levels of IL-1 were found in arthritic joints of neutropenic compared with control mice. Suppression of proteoglycan synthesis was similar in arthritic knee joints of normal and neutropenic mice. However, only minor proteoglycan degradation was found in the latter. TNF-α was undetectable in the bioassay in early ICA and neutralization of TNF-α did not change either swelling, cell influx, proteoglycan synthesis or proteoglycan degradation.

Conclusion. Local production of IL-1 in ICA in knee joints seems directly responsible for inhibition of proteoglycan synthesis. A direct role of IL-1 in proteoglycan loss is unlikely, but indirectly IL-1 may be involved in proteoglycan breakdown by attracting inflammatory leukocytes and activating synovial cells. TNF-α seemed to have no effect on either cell influx, proteoglycan synthesis or proteoglycan degradation in this model. (J Rheumatol 1995;22:2250–8)

Key Indexing Terms:
EXPERIMENTAL ARTHRITIS IL-1 RECEPTOR ANTAGONIST CARTILAGE DAMAGE

One of the main features of joint inflammation is the alteration of homeostasis of chondrocyte function, which may lead to cartilage destruction. In normal cartilage a balance exists between anabolism and catabolism of proteoglycans, one of the main constituents of cartilage determining its compressive resilience. In experimental arthritides the disturbance of this balance is shown by severe inhibition of proteoglycan synthesis and enhanced degradation of proteoglycan1-3. Tumor necrosis factor α (TNF-α) and interleukin 1 (IL-1) are believed to play a crucial role in these effects. Enhanced cytokine levels are found during the early phases of experimental arthritides4-8. Moreover, it is known from in vitro experiments that both IL-1 and TNF-α added to living explants of cartilage induce breakdown and marked inhibition of proteoglycan synthesis, with IL-1 being more potent9-11. Moreover, relatively high doses of IL-1 are needed to cause breakdown, whereas low doses inhibit pro...
teoglycan synthesis<sup>5-11</sup>. In vivo IL-1 was shown to cause joint inflammation and marked proteoglycan depletion after direct injection in the knee joints of rabbits<sup>1</sup>, rats<sup>12</sup>, and mice<sup>1</sup>. Experiments blocking the influx of neutrophils revealed that cell influx had no direct effect on cartilage<sup>13</sup>. In experimental arthritis the role of IL-1 and TNF in cartilage destruction is less clear. Although IL-1 could be detected in the synovial tissue, the concentrations are probably too low to cause proteoglycan breakdown directly, whereas it may be sufficient to initiate inhibition of proteoglycan synthesis. In antigen induced arthritis it was found that blocking of IL-1 with antibodies<sup>2</sup> or IL-1 receptor antagonist (IL-1ra)<sup>14</sup> prevented inhibition of proteoglycan synthesis, independent of joint inflammation. However, breakdown of proteoglycan was unaffected, suggesting the strong influence of other mediators. In contrast, IL-1 blocking in collagen arthritis was effective on both synthesis and breakdown but the latter result is probably related to the marked suppression of joint inflammation in this model<sup>15</sup>. We attempted to further elucidate the role of IL-1 and TNF in the breakdown and synthesis of proteoglycan, and the influence of infiltrating leukocytes. We used the recently developed model of cationic immune complex arthritis (ICA) in mice<sup>6</sup>, a model characterized by marked IL-1 production, pronounced IL-1 dependent cell influx, and early cartilage damage. Moreover, proteoglycan degradation is clearly dependent on infiltrating leukocytes, although no role could be attributed to PMN elastase<sup>16</sup>. We studied the cartilage destruction and cytokine profiles in both normal and neutropenic mice and analyzed the effect of neutralization of IL-1ra, β and TNF-α using subtype specific antibodies and IL-1ra.

**MATERIALS AND METHODS**

*Animals.* Male C57 B1/6 mice were obtained from our breeding facilities (Ovaresselt, The Netherlands). They were fed a standard diet and tapwater ad libitum. Their age was between 8 and 12 weeks at the time of study.

*Chemicals.* Poly-L-lysine (PLL), lysozyme, and 1-ethyl-3(3-dimethylaminopropyl) carbodiimide (EDC) were obtained from Sigma Chemical Company, St. Louis, MO, USA. N,N-dimethyl-1,3 propanediamine was obtained from BDH Chemicals Ltd., Poole, England.

*Lysozyme coupling to PLL.* Lysozyme was coupled to PLL according to the method of Danon<sup>17</sup> using EDC as an activator and PLL as a nucleophile as described<sup>18</sup>. Free carboxyl groups of the protein are then coupled to aminated groups of PLL. The molecular weight was raised whereas the isoelectric point remained high as was determined in a 5% polyacrylamide gel electrophoresis.

*Induction of ICA.* PLL-lysozyme (3 µg) was injected into the right knee joint of mice previously given specific antisera (0.2 ml) directed against lysozyme intravenously (iv). The antisera, raised in rabbits, were decomplemented by heating at 56°C for 30 min. The left knee joint was injected with saline and served as a control.

*Polymorphonuclear (PMN) depletion.* Mice were depleted of PMN by either total body irradiation or cyclophosphamide treatment. A single dose of 7.5 Gy was applied at a dose rate of 2 Gy/min, with a 13 MV electron beam, from a linear accelerator (CGR Saturne, Buc, France). Treatment was started 4 days before injection of the antisera. Mice were anesthetized by intraperitoneal (ip) injection of sodium pentobarbital, 0.03 mg/g body weight (Narcover®, Aphaarma B.V., Arnhem, The Netherlands) and placed between 2 perspex plates, the upper one 1 cm thick. Cyclophosphamide (Endoxan®; Asta Medica AG, Frankfurt am Main, Germany) was given ip at Day 4 (150 mg/kg) and Day 1 (100 mg/kg body weight) before induction of arthritis. To prevent infection in both irradiated and cyclophosphamide treated groups, mice were kept under aseptic conditions in a steriletite room and received acidified drinking water and sterilized food. The irradiated mice were given daily supplements of 1 ml of glucose solution ip until injection of the antibodies. At this time, most mice had lower skin temperature and bodyweight loss up to 10%.

*Antiserum treatment of mice.* Anti-IL-1 antibodies. Mice were given 400 µl of a mixture: 200 µl of anti-IL-1α and β antisera (1 to 1 ratio) and 200 µl of anti-lysozyme, 16 h before arthritis induction as described<sup>16</sup>. Culturing articular patea of anti-IL-1 treated mice revealed that IL-1 could not be detected in the NOB assay (data not shown).

**IL-1ra.** IL-1ra (100 mg/ml) kindly provided by Synergen, Boulder, CO, USA (Dr. Thompson), was brought into micro-osmotic minipumps (Alzet model 1001D; Alza Corporation, Palo Alto, CA, USA). These pumps release 0.5 µl/h during 7 days in a standard manner. The pumps were implanted 2 days before starting the experiment to minimize the effect of stress. As controls empty pumps were implanted.

*Anti-TNF antibodies.* Neutralizing monoclonal antibodies (Mab) directed against TNF-α were injected iv. The 50,000 units of Mab V1Q9 given were sufficient to fully block biologic activity of 4500 ng of TNF in the L929 bioassay.

*Cytokine production by synovial tissue.* Synovial tissue was isolated in a standard manner by dissection of patellar tendon and patellar plate. The tissue specimen contained the patella, tendons and synovium<sup>5</sup>. Six synovial specimens from knee joints were washed in 2 ml culture medium (RPMI 1640 medium; Dutch modification) for 1 h at room temperature. Washouts (undiluted or diluted 10,20,40 and 80-fold) were tested for IL-1 and TNF activity by sensitive bioassays. Also higher dilutions were tested (160-320-fold) but in most washouts, no IL-1/TNF signal was detected.

**Bioassay for IL-1.** IL-1 activity was measured in the one stage bioassay for IL-1 as described by Gearing, et al<sup>19</sup>. The assay is performed as a coculture of the IL-1 specific subclone of the murine thymoma cell EL-4, designated NOB-1 cell, producing IL-2 and IL-4, with the lymphokine producing murine thymoma cell EL-4. After 20 h, 0.5 µCi <sup>3</sup>H-thymidine (Dupont, NEN products, Boston, MA, USA; specific activity 20 Ci/mmol) was added to each well, and the contents harvested 3 h later, and the incorporated activity determined. The EL-4 6.1 line from which NOB-1 was derived does not incorporate thymidine since it is deficient in thymidine kinase and therefore only CTLL proliferates when incubated with <sup>3</sup>H-thymidine (range 0.6 to 0.0002 ng/ml). Arthritic patella washouts (dilution 0-3) were tested. After incubation of 20 h at 37°C and 5% CO<sub>2</sub>, TNF-α mediated cytopathic effects on L929 cells were evaluated. The above dilution is (160-320-fold) but in most washouts, no IL-1/TNF signal was detected. The assay is performed as a coculture of the IL-1 specific subclone of the murine thymoma cell EL-4, designated NOB-1 cell, producing IL-2 and IL-4, with the lymphokine responding CTLL line. In brief EL-4 cells were washed twice and resuspended at 5 x 10<sup>6</sup> cells per ml RPMI containing 1% fetal calf serum (FCS). The cells were distributed into 96 well microtiter plates at 2 x 10<sup>5</sup> cells/well in 100 µl volumes. CTLT cells (4 x 10<sup>6</sup>) were added in 50 µl RPMI followed by appropriate dilutions of test sample to a final volume of 200 µl. After 20 h, 0.5 µCi <sup>3</sup>H-thymidine (Dupont, NEN products, Boston, MA, USA; specific activity 20 Ci/mmol) was added to each well, and the contents harvested 3 h later, and the incorporated activity determined. The EL-4 6.1 line from which NOB-1 was derived does not incorporate thymidine since it is deficient in thymidine kinase and therefore only CTLL proliferation is measured by <sup>3</sup>H-thymidine incorporation. Maximal <sup>3</sup>H-thymidine incorporation in the bioassay in the presence of IL-1 was between 10-10000 cpm. CTLT alone served as control and incorporated only 100-1000 cpm, indicating that washouts contained only low IL-2 or IL-4 concentrations.

**Bioassay for TNF-α.** TNF-α activity was measured as described<sup>21</sup>. Briefly, 1 x 10<sup>5</sup> L929 cells were brought into a flat bottom 96 well microtiter plate. A standard curve was made by adding serial 2-fold dilutions of recombiant mouse TNF-α (range 0.6 to 0.0002 ng/ml). Arthritic patella washouts (dilution 0-3) were tested. After incubation of 20 h at 37°C and 5% CO<sub>2</sub>, TNF-α mediated cytopathetic effects on L929 cells were evaluated. The above dilution was gently discarded and the remaining cells fixed by adding methanol (96%) during 1 min. After drying of the cells, crystal violet (Sigma Chemical Co., St. Louis, MO) was added, and after 5 min, the excess of crystal violet washed away. The plate was then dried, 100 µl of acidic acid.
added, and, after shaking of the plate, the extinction determined on an ELISA reader at 540 nm.

Measurement of chondrocyte proteoglycan synthesis. Quantitative. Mice were sacrificed by ether anesthesia. The patellae from arthritic joints and the contralateral nonarthritic knee joints either treated with anticytokine antibodies, or controls were dissected leaving the cartilage intact. After incubation for 3 h at 37°C in RPMI with 20 μCi Na₂S₇O₄/ml, tissue specimens were washed, fixed in 4% buffered formalin for 20 h and decalcified in 5% formic acid for 4 h. The patella could thus be punched out and the surrounding connective tissue removed. Punched patellae, consisting of cartilage and bone were digested in solulyte (Lumac LSC bv, Olen, Belgium) for 3 h at 60°C and subsequently dissolved in Lipofluor (Lumac LSC bv, Groningen, The Netherlands) for liquid scintillation counting. The washing, fixation, and decalcification procedures removed all free 35S-sulfate from the tissue. The amount of 35S-sulfate retained is a measure of 35S proteoglycans. More than 90% of the incorporated radioactivity was confined to the patellar cartilage layer. Comparison of radioactivity in the patella and that in quantitatively isolated glycosaminoglycans (GAG) [more than 95% of the incorporated label was liberated by overnight papain digestion (1 mg/ml)] revealed that for 35S-sulfate incorporation studies the whole patella can be used as a reliable measure for sulfated GAG synthesis.

Qualitative. Autoradiography. Whole knee joint sections of joint specimens (6 μm) were prepared 2 days after arthritis induction in immune mice. Three hours prior to sacrifice, 75 μCi 35S-sulfate was injected ip. Knee joint sections were coated on gelatin coated slides. These were dipped in K emulsion (Ilford Basildon, Essex, England) and exposed for 1–4 weeks. After this period the slides were developed and stained with hematoxylin and eosin.

Measurement of proteoglycan degradation. Patellae were prelabelled by ip injection of radioactive (50 μCi/mouse) 4 h before induction. More than 90% of the incorporated radioactivity was confined to the patella cartilage layer if compared to whole patellae. At various days after arthritis induction, patellae were isolated, fixed in 4% buffered formalin for 20 h and decalcified in 5% formic acid for 4 h. The patella could thus be punched out and the surrounding connective tissue removed. Punched patellae, consisting of cartilage and bone were digested in solulyte (Lumac LSC bv, Olen, Belgium) for 3 h at 60°C and subsequently dissolved in Lipofluor (Lumac LSC bv, Groningen, The Netherlands) for liquid scintillation counting. The washing, fixation, and decalcification procedures removed all free 35S-sulfate from the tissue. The amount of 35S-sulfate retained is a measure of 35S proteoglycans. More than 90% of the incorporated radioactivity was confined to the patellar cartilage layer. Comparison of radioactivity in the patella and that in quantitatively isolated glycosaminoglycans (GAG) [more than 95% of the incorporated label was liberated by overnight papain digestion (1 mg/ml)] revealed that for 35S-sulfate incorporation studies the whole patella can be used as a reliable measure for sulfated GAG synthesis.

RESULTS

Production of IL-1 and TNF in ICA. Biologically active IL-1 and TNF production during early arthritis was measured with bioassays using washouts of standardized patella specimens. Detectable IL-1 levels were found as early as 3 h after arthritis induction (data not shown). Markedly enhanced levels were found at 6 h, whereas at 24 h no IL-1 was detected (Figure 1). Production at 6 h varied between 10–30 pg/specimen. Biologically active TNF-α was measured using the L929 bioassay, which has a sensitivity of 1–2 pg/ml. TNF-α could not be detected in inflamed joints, 1,3,6, or 24 h after onset of arthritis (data not shown), indicating that the TNF-α production is below 0.3 pg/patella. No IL-1 nor TNF-α was detected in washouts of contralateral noninflamed joints.

Suppression of proteoglycan synthesis in ICA. ICA was characterized by early joint swelling and suppression of proteoglycan synthesis. Joint swelling, which was correlated to cell influx, reached maximal values already at Day 2 (Figure 2). Swelling varied from 1.5–18 at this time. At Day 7, no significant swelling remained. Marked suppression of proteoglycan synthesis was already seen at Day 1 (Figure 2).

Maximal suppression (55%) was seen at 48 h and declined thereafter. At Day 4, there was no more evidence of suppression, whereas at Day 7 and Day 10, surplus production of proteoglycan of respectively 68 and 44% was found.
In vivo effect of blocking IL-1 on proteoglycan synthesis. IL-1 production was neutralized in 2 ways. Firstly by iv injection of polyclonal antibodies directed against the α and β form iv shortly before arthritis induction; secondly, by giving IL-1 receptor antagonist (IL-1ra) systemically by implanting osmotic pumps ip.

Neutralizing the α form of IL-1 resulted in a significant decrease of swelling (46%). The potency of anti-IL-1β was comparable and neutralization resulted in a 44% decrease in swelling. A combination of anti-IL-1 α and β was most effective and reduced swelling by 90% (Table 1). Total knee joint sections showed that influx of cells was significantly lowered. The α and β form were equipotent in this respect. Both antibodies given together resulted in significantly reduced cell influx.

To confirm findings with neutralizing antibodies, IL-1 bioactivity was blocked with IL-1ra. Osmotic pumps were implanted in the peritoneal cavity and set to release constant amounts of IL-1ra for 7 days (1.2 mg IL-1ra/day). In the control group empty pumps were implanted. Two days after arthritis induction, swelling in the IL-1a treated group was significantly decreased (84% inhibition). Influx of cells was also significantly decreased (Figure 3). Only in the synovial layer small numbers of inflammatory cells were present. Suppression of proteoglycan synthesis was fully blocked by the IL-1ra as measured in the patella assay (Table 2). In both anti-IL-1 and IL-1ra treated groups synthesis of proteoglycan in the contralateral nonarthritic knee joint was higher although values did not reach statistical significance — compared to the nonarthritic joint of control mice. IL-1ra given to normal mice had no effect on proteoglycan synthesis (data not shown).

In additional studies, mice treated with anti-IL-1 antibodies, IL-1ra, or control phosphate buffered saline (PBS) were given 35S-sulfate ip at Day 2 after ICA induction. Three hours thereafter total knee joints were processed for autoradiography and proteoglycan synthesis studied. Figure 3C shows that patellar and femoral cartilage surfaces in control, arthritic knee joints show fewer black spots around the chondrocytes, indicating low proteoglycan synthesis (Figure 3C). Both anti-IL-1 antibodies and IL-1ra treated groups (Figure 3D) showed normal synthesis, comparable to nonarthritic joints injected with PBS.

In vivo effect of blocking TNF on proteoglycan synthesis. Although no free biologically active TNF-α could be detected in early immune complex arthritis, this cytokine might function locally. To investigate the in vivo role of local TNF-α production in suppression of proteoglycan synthesis, an excess of neutralizing anti-TNF-α Mab (50,000 U) was given iv before arthritis induction. Swelling measured by 99mTc uptake was not changed (Table 3). Total knee joint sections stained by hematoxylin/eosin also showed that influx of cells in the synovium and joint cavity was not changed. Measuring proteoglycan synthesis revealed that neutralization of TNF-α could not block suppression (Table 3). Similar suppression was found in control (rat IgG treated) and anti-TNF treated groups.

Table 1. Inflammation and inhibition of proteoglycan synthesis in ICA. Effect of neutralization of α and β IL-1 with anti-IL-1 antibodies

<table>
<thead>
<tr>
<th>99mTc uptake</th>
<th>L/R</th>
<th>Anti-IL-1 α</th>
<th>Anti-IL-18</th>
<th>Anti-IL-1α+β</th>
</tr>
</thead>
<tbody>
<tr>
<td>L:R</td>
<td>1.72 ± 0.02</td>
<td>1.39 ± 0.16</td>
<td>1.40 ± 0.02</td>
<td>1.07 ± 0.03</td>
</tr>
<tr>
<td>Proteoglycan</td>
<td>L</td>
<td>915 ± 259</td>
<td>1431 ± 238</td>
<td>1432 ± 264</td>
</tr>
<tr>
<td>synthesis (cpm)</td>
<td>R</td>
<td>1735 ± 236</td>
<td>2012 ± 64</td>
<td>1979 ± 358</td>
</tr>
<tr>
<td>Inhibition (%)</td>
<td>47</td>
<td>29*</td>
<td>28*</td>
<td>–29*</td>
</tr>
</tbody>
</table>

Swelling was measured using the 99mTc uptake method. L:R — ratios indicating amount of swelling; > 1.1 indicates significant swelling. Proteoglycan synthesis was measured by uptake of 35S by patellae from the arthritic (L) knee joint compared to the contralateral nonarthritic (R) knee joint. Values represent the mean ± SD of 3 experiments. In each experiment at least 6 mice were tested. Data were statistically evaluated by Mann-Whitney U test. * p < 0.05.

Table 2. Inflammation and inhibition of proteoglycan synthesis in ICA. Effect of neutralization of IL-1 by IL-1 ra

<table>
<thead>
<tr>
<th>99mTc uptake</th>
<th>L/R</th>
<th>IL-1 ra</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteoglycan</td>
<td>L</td>
<td>667 ± 43</td>
</tr>
<tr>
<td>synthesis (cpm)</td>
<td>R</td>
<td>1241 ± 43</td>
</tr>
<tr>
<td>Inhibition (%)</td>
<td>50</td>
<td>–10*</td>
</tr>
</tbody>
</table>

IL-1 was neutralized by giving IL-1 ra before arthritis induction. IL-1 ra containing osmotic pumps were implanted in the peritoneal cavity. A constant release of 1.2 mg IL-1 ra/day was set. Joint swelling was measured by 99mTc uptake. Values represent the mean ± SD of 3 experiments. In each experiment at least 6 mice were tested/group. Data were statistically evaluated by Mann-Whitney U test. * p < 0.05.

Table 3. Inflammation and inhibition of proteoglycan synthesis in ICA. Effect of anti-TNF

<table>
<thead>
<tr>
<th>99mTc uptake</th>
<th>L/R</th>
<th>Anti-TNF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteoglycan</td>
<td>L</td>
<td>667 ± 43</td>
</tr>
<tr>
<td>synthesis (cpm)</td>
<td>R</td>
<td>1241 ± 43</td>
</tr>
<tr>
<td>Inhibition (%)</td>
<td>46</td>
<td>45</td>
</tr>
</tbody>
</table>

TNF was neutralized by giving anti-TNF antibodies (100 μl) before arthritis induction. Values represent the mean ± SD of 2 experiments. In each experiment at least 6 mice/group were tested. Data were statistically evaluated by Mann-Whitney U test.
Continental immigration (100 x P. 30 % and 30% to 15 years) is, for a variety of reasons, a topic of interest. It has been observed that the rate of immigration varies from region to region, and from year to year. The reasons for this variation are not fully understood. However, it is clear that immigration is a complex process that is influenced by a variety of factors, including economic, political, and social conditions.

The challenges of immigration are particularly acute in some countries, where the influx of immigrants can put a strain on local resources and social services. In such cases, it is important to consider the needs of both the immigrants and the host community, and to develop policies that are fair and effective. This is a complex task, but one that is essential if we are to ensure that immigration is a positive force for change.
Cell influx and inhibition of proteoglycan synthesis. We investigated whether influx of cells in arthritic knee joints impairs proteoglycan synthesis. To investigate this, mice were made neutropenic either by total body irradiation or cyclophosphamide treatment. Both methods induced more than 98% depletion of peripheral PMN. IL-1 levels in arthritic neutropenic joints, measured at 6 h, were significantly higher compared to control arthritic knee joints. Maximal levels were found at 6 h and remained high at 24 h (Figure 4). Production of IL-1 at 6 h varied between 70-120 pg and at 24 h between 10-30 pg/specimen. At 48 h only minimal amounts of IL-1 were detected in both groups. Again no biologically active TNF-α was detected in neutropenic arthritic joints. No IL-1 nor TNF-α was detected in washouts of contralateral nonarthritic neutropenic joints.

Total knee joint sections showed that only minimal amounts of PMN were observed in arthritic knee joints. Influx of monocytes was also minimal. Total body irradiation had no significant (18% inhibition) side effects on proteoglycan synthesis as measured in the contralateral nonarthritic knee joint (Table 4). Cyclophosphamide treatment raised proteoglycan synthesis by 33%. Arthritis induction in control and neutropenic arthritic knee joints showed comparable suppression of proteoglycan synthesis (Table 4). This suggests that local production of mediators by resident cells is sufficient for proteoglycan suppression.

Table 4. Inhibition of proteoglycan synthesis in neutropenic arthritis mice

<table>
<thead>
<tr>
<th>Animals</th>
<th>Left</th>
<th>Right</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1236 ± 290</td>
<td>540 ± 161</td>
<td>56</td>
</tr>
<tr>
<td>neutropenic</td>
<td>847 ± 175</td>
<td>452 ± 120</td>
<td>47</td>
</tr>
<tr>
<td>Control</td>
<td>814 ± 262</td>
<td>688 ± 143</td>
<td>31</td>
</tr>
<tr>
<td>neutropenic</td>
<td>1188 ± 182</td>
<td>917 ± 224</td>
<td>25</td>
</tr>
</tbody>
</table>

Mice were made neutropenic by either total body irradiation (750 rad) or cyclophosphamide treatment. Patellae were dissected 2 days after induction of ICA. Proteoglycan synthesis was measured by uptake of 35S by patellae derived from the arthritic (L) knee joint and compared to contralateral nonarthritic (R) knee joint. Data represent the mean ± SD of 2 experiments. In each experiment at least 6 mice were tested. Data were statistically evaluated by Mann-Whitney U test.

Direct role of IL-1 in proteoglycan degradation. We studied the degradation of cartilage proteoglycan by prelabeling of the cartilage proteoglycans with 35S-sulfate and analyzing the loss of 35S-proteoglycan after arthritis induction. In irradiated and cyclophosphamide treated mice less 35S uptake by the patellae was observed before induction of ICA, compared to the control group. Proteoglycan loss among groups was compared. Loss of proteoglycan from patella in arthritic joints was compared to that from patella in contralateral nonarthritic joints. At Day 2, a 60% loss of proteoglycan was found in normal mice with ICA. Despite high IL-1 levels...
in neutropenic mice (Figure 1), we observed only minor pro-
teoglycan degradation in neutropenic mice with ICA (Table 5). This suggests that the direct effect of IL-1 on proteogly-
can breakdown is marginal and the main effect of IL-1 is
through indirect regulation of cell influx and amplification
of activation of synovial cells.

DISCUSSION

Our results suggest that IL-1 but not TNF-α is the main
cytokine involved in cartilage damage during experimental
ICA. IL-1 directly mediates suppression of proteoglycan
synthesis. Its direct effect on loss of proteoglycan seems only
limited but as the most important regulator of cell influx IL-1
mediates cartilage degradation indirectly.

Neutralization of IL-1 in ICA leads to full blockade of in-
hibition of proteoglycan synthesis but also to significantly
lowered influx of inflammatory cells. The same observations
were made in the collagen type II induced arthritis\textsuperscript{13,25,26},
This did not exclude the possibility that decreased cell in-
flux might well be responsible for observed blockade of in-
hibition of proteoglycan synthesis. In our study we found,
however, that suppression of proteoglycan synthesis was
similar in both neutropenic and control arthritic knee joints,
indicating that inflammatory cells are not involved in sup-
pression of proteoglycan synthesis. This confirms recent
observations in other arthritis models such as methylated
bovine serum albumin induced arthritis\textsuperscript{2} and zymosan in-
duced arthritis\textsuperscript{14}. All models studied to date indicate IL-1 is
directly responsible for inhibition of proteoglycan synthesis.

The potency of IL-1 as an inhibitor of proteoglycan syn-
thesis was already suggested by single mediator studies. IL-1
is a potent inhibitor of proteoglycan synthesis. Injection of
recombinant IL-1 in murine knee joints induced a severe in-
hibition of proteoglycan synthesis\textsuperscript{9}. IL-1 is released in sig-
nificant amounts shortly after ICA induction. Neutralization
of both forms of IL-1 by anti-IL-1 antibodies or blockade of
its biological effect by IL-1ra fully prevented inhibition of
proteoglycan synthesis. This is in agreement with earlier
studies in which full blockade of proteoglycan inhibition was
found after IL-1 neutralization with a combination of α and
β antibodies or IL-1ra in antigen induced arthritis\textsuperscript{14} and col-
lagen type II arthritis (manuscript in preparation). In the ICA
both IL-1α and β are produced although the α form is more
prominent\textsuperscript{8}. Using anti-IL-1α and anti-IL-1β antibodies
separately showed that each form mediates part of the sup-
pression of proteoglycan synthesis. Both IL-1 forms have
been described to bind IL-1 type I receptor present on chon-
drocytes. The IL-1 type I receptor probably mediates the in-
hibiting effect\textsuperscript{27}.

In contrast to anti-IL-1 antibodies, high amounts of IL-1ra
are needed for optimal neutralization of the IL-1 effects. To
block IL-1 effects on murine cartilage a 1000-fold excess of
IL-1ra was necessary. Like IL-1, 17 kDa IL-1ra has a high
clearance rate of 30 min. We found that the effects of an
intraarticular injection of 1 ng could be blocked by the
IL-1ra levels released by the osmotic pumps\textsuperscript{24}. Since the
amount of IL-1 formed during ICA is much less than 1 ng,
these IL-1ra blood levels were sufficient for neutralization.
A similar level is not easily achieved with repeated injec-
tions. Because of the high removal rate of IL-1ra, multiple
injections are necessary to reach sustained, high concentra-
tions. Multiple injections may, however, alter the course of
arthritis by stress induction. Of interest, proteoglycan syn-
thesis of contralateral nonarthritic joints was higher in IL-1ra
treated mice compared to control mice. This suggests that
IL-1ra may elevate normal proteoglycan synthesis. However,
IL-1ra treatment of normal animals, in which no arthritis was
induced, had no effect on proteoglycan synthesis. This indi-
cates that systemic effects are induced by a surplus of IL-1,
generated in the arthritic knee joint and that this is effect-
ively blocked by IL-1ra.

In contrast to IL-1, NFκB could not be detected in early
arthritis. No detectable biologically active TNF-α levels
could be found in control or neutropenic arthritic knee joints.
This indicates that TNF-α production is below 0.3 pg/patel-
la. High amounts of neutralizing anti-TNF-α antibodies given
before arthritis had no effect on tither cell influx or suppres-
sion of proteoglycan synthesis. Other species of anti-TNF-
α Mab showed similar results (data not shown). In vitro
studies have shown that TNF-α may cause significant pro-
teoglycan suppression. TNF-α was however less potent than
IL-1, and large amounts of TNF-α (100 ng/ml) were neces-
sary to induce suppression\textsuperscript{10}. In our model, TNF-α pro-
duction is too low to induce suppression. In other arthritis
models like collagen type II both TNF-α and IL-1 seem to be im-
portant in cell influx\textsuperscript{13,28,29}. IL-1 appeared, however, to be
the dominant cytokine\textsuperscript{25}, and blockade of IL-1 increased in-
flux of cells and normalized suppression of proteoglycan syn-
thesis, whereas blockade of TNF-α had only moderate ef-
fects. In bacterial induced joint inflammation, TNF-α seems
to be the more dominant cytokine involved in cell influx\textsuperscript{30,31}. Furthermore cytokine production by immune complexes and its effect on tissue damage might be tissue dependent.

Table 5. Proteoglycan degradation in neutropenic arthritic

<table>
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<tr>
<th>Treatment</th>
<th>Control (%)</th>
<th>Neutropenic (%)</th>
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<tbody>
<tr>
<td>Total body irradiation (750 rad)</td>
<td>56 ± 7</td>
<td>10 ± 6*</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>60 ± 7</td>
<td>11 ± 14*</td>
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Effect of PMN depletion on proteoglycan degradation. Mice were made
neutropenic either by total body irradiation (750 rad) or cyclophosphamide
(Day −4, 100 mg/kg; Day −1, 80 mg/kg) treatment. Patellae, prelabeled
with 35S (50 μCi/mouse) in vivo before arthritis induction, were dissected
48 h after ICA induction. The 35S content of the arthritic patella is
expressed as % of 35S content of the contralateral noninflamed patellae.
Values represent the mean ± SD of 2 experiments, each consisting of at
least 7 animals. Data were statistically evaluated by Mann-Whitney U test.
* p < 0.05.

mune complex (IC) mediated inflammation in the skin seemed predominantly regulated by IL-1 whereas in the lung both TNF and IL-1 were important11.

Apart from local IL-1 production by resident cells, infiltrating inflammatory cells may also significantly contribute to the cytokine load since these cells contain small amounts of IL-1 and TNF. Local activation of these infiltrated cells, e.g., with cationic IC32 may release these cytokines. In our study we found however that in the absence of PMN even higher IL-1 levels were expressed for longer periods. This indicates that the PMN, which is the predominant cell during early arthritis, affects biologically active IL-1 levels by clearing the triggering IC responsible for IL-1 production16. Furthermore, apart from releasing proinflammatory cytokines, the PMN also produce cytokine inhibitors such as IL-1ra33 or enhance expression and shedding of IL-1 receptor type II. IL-1 receptor type II may act as a decoy to catch IL-1, thus preventing biological activity34. IL-18 has a higher affinity for type II than the α form27 and binds in larger amounts in the presence of PMN. This may also explain why 80% of the IL-1 signal we detected in the bioassay contained the α form.

So although PMN downregulate IL-1 the remaining IL-1 is still sufficient for maximum suppression of proteoglycan synthesis. Apart from reducing synthesis, IL-1 can resorb cartilage in 2 ways: first, within the cartilage, by activating chondrocytes that resorb cartilage from the inside and second from outside by directing the influx of infiltrating cells. The latter may release enzymes into the synovial fluid, which may degrade cartilage starting at the cartilage surface. Our study indicates that in arthritic joints in the absence of inflammatory cells, IL-1 levels were even higher. Nevertheless IL-1 levels seem insufficient to induce the loss of proteoglycan observed in control arthritic knee joints. Earlier studies revealed that higher doses of IL-1 are needed for cartilage resorption than for suppression of proteoglycan synthesis31. This indicates that IL-1 levels secreted during arthritis may be too low to cause cartilage resorption directly, and influx of cells is necessary to induce degradation. The influx of cells during ICA is for the greater part dependent on IL-18. Blockade of IL-1 by anti-IL-1 antibodies or IL-1ra stops the influx of PMN. Although there is little effect on the influx of monocytes, cartilage degradation remained at 50%8 or even 75% in IL-1ra treated arthritic animals (manuscript in preparation). This suggests that the interaction between infiltrating monocytes and synovial cells may be the most important mechanism in cartilage degradation during ICA. On one hand, synovial macrophages and fibroblasts activated by IC release cartilage degrading enzymes like metalloproteinases39. On the other hand infiltrating monocytes have the capacity to release proteoglycan degrading enzymes. Cationic IC, which strongly attach to synovial tissues due to their charge8, may activate infiltrating monocytes to release these enzymes as well as other factors that activate synovial fibroblasts. Leakage of synovial vessels elevates the presence of fibrinolytic enzymes like plasmin, which may transform latent cartilage degrading enzymes in its active form. Whether monocytes and/or synovial cells in this model are responsible for external cartilage degradation is currently under investigation.

Our results indicate that in ICA, IL-1 is the major cytokine involved in suppression of proteoglycan synthesis. Inflammatory cells seem not to be involved in suppression of proteoglycan synthesis. These cells are however involved in proteoglycan loss16. Since IL-1 is the main cytokine involved in cell influx, this cytokine also indirectly regulates proteoglycan loss. Neutralizing the surplus of IL-1 generated in arthritic joints may be an important approach to stimulate reconstruction of damaged cartilage.

ACKNOWLEDGMENT
The authors thank Dr. Otterness, Pfizer Central Research, Groton, CT, for the gift of murine recombinant IL-1α and IL-1β and Dr. Thompson (Synergen, Boulder, CO, USA) for providing IL-1ra.

REFERENCES


