Abstract

**Background.** The effect of multiple infusions of infliximab (INF), a chimeric anti-tumor necrosis factor alpha antibody, on the concentration of hexosaminidase (HEX) activity in a synovial cell culture derived from human synovial inflamed fluid obtained from patients suffering from rheumatoid arthritis (RA) has been evaluated.

**Material and Methods.** Inflamed synovial fluid was taken from RA patients (a study group) and patients who had undergone knee trauma within 7 days (a control group). The following solutions of infliximab were used: 40, 60 and 140 µg/mL. Determination of the concentration of HEX activity in cell cultures was performed after 24, 48, 72 and 96 h of infliximab administration. To identify synoviocytes in cell culture immunohistochemical staining with vimentin and pancytokeratin was performed.

**Results.** A predominance of fibroblast-like synovial cells has been observed in the study group. In the control group the concentration of HEX activity without adding infliximab to the cell culture was 283.00 nkat/mL. After 96 h of incubation with infliximab, the concentrations of HEX activity in cultured synoviocytes according to infliximab doses of 40, 60 and 140 µg/mL were respectively: 280.00, 271.50 and 293.50 nkat/mL. In the study group, the concentration of HEX activity without adding infliximab to the cell culture was 542.27 nkat/mL. The final concentrations of HEX activity of cultured fibroblast-like synovial cells measured after 96 h of incubation with infliximab were: 471.72, 498.27 and 556.72 nkat/mL, according to infliximab doses of 40, 60 and 140 µg/mL. In all groups (besides the infliximab concentration of 140 µg/mL after 96 h of incubation), the level of concentration of HEX activity was significantly higher in the study group compared to the control group, irrespective of infliximab concentration and time of infliximab incubation.

**Conclusions.** Infliximab changes the concentration of HEX activity depending on the drug dose and time of administration (Adv Clin Exp Med 2015, 24, 5, 807–813).

**Key words:** infliximab, inflamed synovial fluid, fibroblasts *in vitro*, hexosaminidase activity.
an enlarged amount of synovial fluid, increased level of its pressure, impairment of vascular flow within the synovial membrane and increased permeability of blood vessels. The higher activity of lysosomal enzymes, such as N-acetyl-β-glucosaminidase (HEX), has been noted in rheumatoid arthritis, compared to healthy synovial fluid. It is supposed that HEX may take part in joint destruction in RA [4].

The first biological agent shown to be effective in RA was infliximab. Infliximab is a chimeric monoclonal antibody against TNF-α and has demonstrated a reduction in the number of inflammatory cells, including intimal and sublining macrophages, T cells and plasma cells in rheumatoid synovial tissue as soon as 48 h after initiation of treatment [5, 6].

Lysosomal exoglycosidases belong to the group of lysosomal enzymes that catalyzes oligosaccharide chains of glycoproteins, glycolipids and proteoglycans in acid medium [7]. Among them is N-acetyl-β-D-hexosaminidase (HEX). During the course of catalysis, an oxonium ion-like transition state is thought to be generated, which is stabilized by a deprotonated carboxyl group from the enzyme [8]. In the pathogenesis of RA, numerous pathogenetic factors occur. The aggressiveness of the disease is stimulated by a variety of factors, including inflammatory cells, e.g. lymphocytes, macrophages and fibroblasts, which release cytokines such as Il-1α, Il-1β, TNF-α and TNF-β [9]. Owing to the essential role of lysosomal exoglycosidases in inflammatory diseases, it may be assumed that the significance of selected lysosomal exoglycosidases activity is also crucial in the specific behavior of RA. They are mediators of hypersensitivity and inflammatory cells in the pathogenesis of different inflammatory disorders [10]. Release of these enzymes into plasma may be a marker of important changes in the lysosome, whether due to enzyme induction or damage, and could be a primary mechanism of disease processes [11]. In the literature there are studies suggesting the possible role of HEX as a joint destruction marker in rheumatoid arthritis patients. Not all of the studies support this, however. In a study by Casal et al., for example, in a group of 51 patients with an evolution period of rheumatoid arthritis, it did not appear to be a reliable marker of erosion and cartilage degradation in RA patients [12]. However, another study by Pasztoi et al. proved a significant activity of hexosaminidase D found in association with extracellular vesicles released by synovial fibroblasts. It describes the expression and disease relevance of the HEX D gene in humans and demonstrates the expression of this novel enzyme within the joints, and suggests that its activity may significantly contribute to overall local exoglycosidase activity [13]. It is well known that glycosidases play a role in cartilage degradation similarly to matrix metalloproteinases. Carbohydrate cleavage products, generated by glycosidases including HEX, are released from degrading cartilage during arthritis. Some of the cleavage products (such as hyaluronate oligosaccharides) have been shown to bind to Toll-like receptors and provide endogenous danger signals, while others (like N-acetyl glucosamine) are reported to have chondroprotective functions. In the current study, for the first time, we systematically investigated the expression of glycosidases within the joints [14]. The authors conclude that glycosidases expressed by synovial membranes and synovial fibroblasts are under negative regulation by locally expressed cytokines in rheumatoid arthritis and therefore these enzymes may contribute significantly to cartilage degradation in RA if acting in collaboration with the differentially upregulated proteases to deplete cartilage in glycosaminoglycans [14].

The use of anti-inflammatory factors in synovial cell culture and evaluation of HEX activity following the administration of them may contribute to the discovery of new treatment methods for motion organ disorders. Therefore, the present study was performed to investigate the effects of infliximab on hexosaminidase (HEX) activity in the synovial cell culture derived from human synovial inflamed fluid obtained from patients suffering from rheumatoid arthritis (RA). The authors made an attempt to show additional proof of infliximab efficacy in rheumatoid arthritis which has not been shown previously.

**Material and Methods**

The inflamed synovial fluid (10 mL) was taken from 11 patients (6 men and 5 women, 25–60 years old) suffering from rheumatoid arthritis. They met all the criteria for rheumatoid arthritis according to the American College of Rheumatology [15].

In the control group, 10 mL of synovial fluid was taken from 10 patients (6 boys and 4 girls, 11–16 years old) who had undergone knee trauma within 7 days without any inflammation signs.

The study was proved by the local Ethics Committee, and all patients or parents of children signed an informed consent form.

Synovial fluid samples derived from patients with RA and knee injury were digested in petri dishes with 0.1% collagenase (Sigma, St. Louis, MO, USA) for 30 min at 37°C, and then washed twice in 10% RPMI-1640 medium with L-glutamine (Gibco, UK) and HEPES (PAA, Austria), 1% solution of penicillin-streptomycin (Sigma, St. Louis, MO, USA) and 10% FBS (Fetal Bovine Serum) (PAA,
Synovial fluid samples were incubated in 5% CO₂ atmosphere at 37°C and 95% humidity to give a homogenous layer of cultured cells (Fig. 1). Adherent fibroblast-like synoviocytes were trypsinized with Trypsin/EDTA (Gibco Laboratories, Grand Island, NY), diluted and then cultured in 24-well microplates (Fig. 2). To identify synoviocytes in cell cultures, immunohistochemical staining with vimentin and pancytokeratin was performed. After the identification of synoviocytes in the cell culture obtained from both the study and the control group, the concentration of HEX activity was obtained before INF administration. Then the cell cultures were divided into three equal parts in order to perform incubation with 40 ug/mL to the first part, 60 ug/mL to the second and 140 ug/mL infliximab to the third, respectively. The concentration of HEX activity was obtained by the method described by Zwierz et al. [16] after cell homogenization in Ultra-Turrax T25 at 0°C after 24 h, 48 h, 72 h and 96 h of incubation with infliximab. In addition, the concentration of HEX activity without adding infliximab to the cell cultures was measured as well. Determination of the concentration of HEX activity was performed in 96-well microplates. The following substances were added to each well: 10 µL of cell lysate, 30 µL of substrate: 4-nitrophenyl-2-acetamide-2-deoxy-β-D-glucopyranoside and 40 µL of 0.1 M citrate-phosphate buffer pH 4.7. After 60 min of incubation at 37°C with constant shaking, the reaction was stopped by adding 200 µL of 0.2 M borate buffer pH 9.8. Spectrophotometric measurements were carried out at 405 nm using a microplate reader Elx800™.

The results were analyzed using STATISTICA 7.0 StatSoft (StatSoft, Kraków, Poland) using two nonparametric tests: Kruskal-Wallis (ANOVA) and U Mann-Whitney. Statistical significance was set at p < 0.05.

**Results**

Monolayers of the fibroblast-like synoviocyte culture obtained from the synovial fluid taken from RA patients were observed while performing the cell culture. To identify synoviocytes in the cell cultures, immunohistochemical staining with vimentin and pancytokeratin was performed. A predominance of fibroblast-like synovial cells was observed in the study group and is shown in Figure 1. After trypsinization, the synoviocytes obtained from the synovial fluid taken from RA patients were also easily observed (Fig. 2).

In the control group, the concentration of HEX activity without adding infliximab to the cell culture was mean 283.00 nkat/mL. The concentrations of HEX activity in the cultured synoviocytes were changed according to the time of incubation and doses of infliximab: 40 µg/mL, 60 µg/mL and 140 µg/mL. After 24 h of incubation with infliximab, the mean concentrations of HEX activity in the cell cultures were, respectively, 226.5 nkat/mL, 290.00 nkat/mL and 271.25 nkat/mL.

Table 1 shows a comparison of independent samples, i.e. a comparison of the levels of the concentration of HEX activity in the control and a study groups at different concentrations of infliximab.

<table>
<thead>
<tr>
<th>Concentration of INF</th>
<th>H</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 µg/mL</td>
<td>43,259</td>
<td>0.000</td>
</tr>
<tr>
<td>60 µg/mL</td>
<td>31,594</td>
<td>0.000</td>
</tr>
<tr>
<td>140 µg/mL</td>
<td>27,550</td>
<td>0.000</td>
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</table>

**Table 1.** Kruskal-Wallis Test. It is a comparison of independent samples, i.e. a comparison of levels of the concentration of HEX activity in the control and study groups at different concentrations of infliximab. “H” defines the test value of the Kruskal-Wallis test. P < 0.05 was considered significant.
After 48 h of incubation with infliximab, the mean concentrations of HEX activity in the cultured fibroblasts were, respectively, 243.00 nkat/mL, 266.00 nkat/mL and 289.50 nkat/mL, and after the next 72 h of incubation: 255.00 nkat/mL, 274.25 nkat/mL and 253.75 nkat/mL.

After the last 96 h of incubation with infliximab, the mean concentration of HEX activity of the cultured synoviocytes with infliximab were, respectively, 280.00 nkat/mL, 271.50 nkat/mL and 293.50 nkat/mL.

In the study group, the mean concentration of HEX activity without adding infliximab to the cell culture was 542.27 nkat/mL. As well as in the control group, the time of incubation and doses of infliximab: 40 µg/mL, 60 µg/mL and 140 µg/mL, had an influence on the concentration of HEX activity in the cultured fibroblasts. After 24 h of incubation with infliximab, the mean concentrations of HEX activity of the cultured cells were, respectively, 542.55 nkat/mL, 573.64 nkat/mL and 583.64 nkat/mL, and after the next 48 h of incubation with infliximab: 593.09 nkat/mL, 451.63 nkat/mL and 527.90 nkat/mL.

The mean concentrations of HEX activity of the cultured synoviocytes after 72 h of incubation with infliximab were, respectively, 515.18 nkat/mL, 546.72 nkat/mL and 543.45 nkat/mL.

At the end of the experiment, after 96 h of incubation with infliximab, the mean concentrations of HEX activity of the cell cultures were: 471.72 nkat/mL, 498.27 nkat/mL and 556.72 nkat/mL, respectively to infliximab doses: 40 µg/mL, 60 µg/mL and 140 µg/mL.

The Kruskal-Wallis test was used as a non-parametric method for testing whether the samples originate from the same distribution. At each level of infliximab concentration, the statistical significance was observed for the level of the concentration of HEX activity between the control and study group. A comparison between the control and study group, depending on the concentration of infliximab and the time of incubation, was performed using the Mann-Whitney test (Table 2, 3).

<table>
<thead>
<tr>
<th>Concentration of INF</th>
<th>Time of INF incubation</th>
<th>Control group mean concentration of HEX activity (min–max) in nkat/mL</th>
<th>Study group mean concentration of HEX activity (min–max) in nkat/mL</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 µg/mL</td>
<td>before INF administration</td>
<td>283 (110–510)</td>
<td>542 (300–892)</td>
<td>0.049</td>
</tr>
<tr>
<td></td>
<td>after 24 h</td>
<td>226.5 (115–424)</td>
<td>542.55 (300–812)</td>
<td>0.004</td>
</tr>
<tr>
<td></td>
<td>after 48 h</td>
<td>243 (110–390)</td>
<td>573.64 (305–940)</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>after 72 h</td>
<td>255 (190–352)</td>
<td>515.18 (302–775)</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>after 96 h</td>
<td>280 (180–388)</td>
<td>471.72 (275–737)</td>
<td>0.010</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Concentration of INF</th>
<th>Time of INF incubation</th>
<th>Control group mean concentration of HEX activity (min–max) in nkat/mL</th>
<th>Study group mean concentration of HEX activity (min–max) in nkat/mL</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 µg/mL</td>
<td>before INF administration</td>
<td>283 (110–510)</td>
<td>542 (300–892)</td>
<td>0.049</td>
</tr>
<tr>
<td></td>
<td>after 24 h</td>
<td>290 (201–446)</td>
<td>573.64 (295–787)</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>after 48 h</td>
<td>266 (165–459)</td>
<td>451.63 (269–632)</td>
<td>0.020</td>
</tr>
<tr>
<td></td>
<td>after 72 h</td>
<td>274.25 (120–415)</td>
<td>546.72 (345–775)</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>after 96 h</td>
<td>271.50 (169–351)</td>
<td>498.27 (145–840)</td>
<td>0.049</td>
</tr>
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</table>
The increase in lysosomal exoglycosidase activity has been observed in several inflammatory diseases. HEX has demonstrated higher activity in the joint fluid of the knees of patients with rheumatoid arthritis. The concentration of activity of HEX in blood serum obtained from patients with RA has been significantly higher compared to blood serum obtained from healthy human volunteers [17]. However the fibroblast-like synovial cell culture obtained from synovial fluid taken from RA patients has not been widely explained in the literature. This is also the first indication that infliximab could play an important role in decreasing the selected lysosomal exoglycosidase activity on fibroblast-like synovial cells in vitro, suggesting possibilities for an adjunctive local treatment in RA patients in the future.

The central role in the pathogenesis of RA is served by proinflammatory cytokines involved in the immune response. These include: IL-1α, IL-6, TNF-α, ICAM-1 (intercellular adhesion molecule-1), LFA-1 (lymphocyte functional antigen-1) and INFγ (IFN-γ) [18]. Cytokines stimulate the bone resorption process. The destruction of the joint leads to permanent damage to the cartilage, bone, tendons and ligaments, and subsequently to disability [1]. There is also a threat of intra- and outer-temporal complications. The process of bone destruction involves osteoclasts. They arise from macrophages and monocytes, and act directly on the bone matrix, causing bone erosion and remodeling through the secretion of enzymes such as proteases, glycosidases and exopeptidases. Although bone resorption occurs only beneath the ruffled border of osteoclasts, the effect of osteoclasts and their enzymes secreted in the bone adjacent surface is structural changes in the forms of discontinuous periosteum, irregular cementum line or eosinophilic bubbles appearing in the area between the joint surfaces [18].

Infliximab (INF) as an anti-TNF-α monoclonal antibody has emerged as a highly effective treatment in early and established RA. The present study was performed to investigate the effects of dose-related infliximab on the HEX activity of fibroblasts obtained from the synovial fluid taken from adult patients suffered from RA. We observed a significant difference between two groups, the control and the study, regarding the initial concentration of HEX activity without adding the drug, i.e. the concentration of HEX activity was almost twice as high in the study group as in the control. We confirmed that after infliximab administration, the concentration of HEX activity level was related to the drug dose and time of infliximab administration. The dose of 60 µg/mL infliximab decreases the concentration of HEX activity the most in the 48th h of culture growth. However the infliximab concentration results in no change in the concentration of HEX activity regarding the infliximab dose of 140 µg/mL and time of infliximab administration. Furthermore, we showed that the greatest decrease in HEX activity was observed when an infliximab dose of 60 µg/mL was used on the second day. Moreover, after subsequent drug administration at the same dose on the 3rd day of the cell culture, the HEX activity reached a higher level, and then in the 96th h it decreased again.

Schatteman et al., in a group of patients suffering from ankylosing spondylitis, proved the usefulness of intraarticular injection of INX in the treatment of refractory monoarthritis. The authors proved the effectiveness and safety of the intraarticular INX injection. Moreover, it is also possible to avoid parental TNF-blocking therapy in such cases [19]. Tse et al. emphasize that anti-TNFα therapy has potential for juvenile spondyloarthropathy treatment, however further prospective studies are required [20].

It is interesting that patients with early rheumatoid arthritis, initially treated with infliximab in combination with methotrexate, after achieving

### Table 4. U Mann-Whitney test. The comparison between the control and study groups depending on the concentration of infliximab: 140 µg/mL.

<table>
<thead>
<tr>
<th>Concentration of INF</th>
<th>Time of INF incubation</th>
<th>Control group: mean concentration of HEX activity (min–max) in nkat/mL</th>
<th>Study group: mean concentration of HEX activity (min–max) in nkat/mL</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>140 µg/mL</td>
<td>before INF administration</td>
<td>283 (110–510)</td>
<td>542 (300–892)</td>
<td>0.049</td>
</tr>
<tr>
<td></td>
<td>after 24 h</td>
<td>271.25 (148–401)</td>
<td>583.64 (320–783)</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>after 48 h</td>
<td>289.50 (127–452)</td>
<td>527.90 (274–710)</td>
<td>0.022</td>
</tr>
<tr>
<td></td>
<td>after 72 h</td>
<td>253.75 (141–401)</td>
<td>543.45 (225–887)</td>
<td>0.022</td>
</tr>
<tr>
<td></td>
<td>after 96 h</td>
<td>293.50 (132–454)</td>
<td>556.72 (125–880)</td>
<td>0.084</td>
</tr>
</tbody>
</table>
a disease activity score (DAS) ≤ 2.4, may even be excluded from the treatment of infliximab. However, it might work only for a low activity disease [21]. The same authors, two years later, presented data which showed that intraarticular infliximab injection was not effective in patients with a chronically inflamed knee joint [22].

Efficacy in patients with early RA is critically important, since it is understood now that the progression in inflammation severity and joint damage is slow in some patients and more rapid in others [23]. One of the good predictors of RA might be the level of HEX activity in synovial fluid. If our results have shown that infliximab decreases the enzyme activity in cell cultures, it might be possible to locally administer the drug into the inflamed knee joint. So far infliximab is used as multiple infusions combined with methotrexate [24]. Although TNF inhibitors such as infliximab are currently the gold standard of biologics for patients with inflammatory arthritis, there are still a number of questions regarding how to gain the maximum benefit from these agents. It seems crucial to provide more studies, especially clinical randomized ones, to obtain more data and to have a clear answer to Fish’s et al. question whether we should treat patients suffering from arthritis with intraarticular tumor necrosis factor blockers [25].

Because of that, further studies on fibroblast cell cultures obtained from the synovial fluid taken from patients suffering from RA, in the aspect of the efficacy of TNF-blocking agents such as infliximab, seem to be reasonable.

The authors concluded that infliximab has been shown to change the concentration of HEX activity depending on the drug dose and time of administration. Regarding its use in practice, further studies are needed to consider infliximab as a possible adjunctive local treatment against inflammatory rheumatic diseases.

References

Role of Infliximab in Rheumatoid Arthritis


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