Abstract

Objective. Because there is no known specific effective therapy for secondary amyloidosis at the present time, the aim of this study is to determine whether antirheumatic drugs such as methotrexate (MTX) and azathioprine (AZA) inhibit the development of experimental AA amyloidosis and to study or the time of drug administration effects this process.

Methods. C57BL/6 mice with amyloidosis induced by injections of casein and fibrin were treated with MTX and AZA. Two treatment regiments (prophylactic and therapeutic) were used. The drugs were administered through intragastric gavage: AZA – 5 times a week and MTX – 3 times a week for 5 or 4 weeks in doses of 1 mg/kg and 2.5 mg/kg respectively. At the end of experiment histological specimens of the spleen, liver and kidneys tissues were examined and the amyloid deposition was confirmed by positive tissue staining with Congo-red by using polarized microscopy. The average amount of amyloid deposition in various structures was visually evaluated on a 0 to 3 plus scale.

Results. The prophylactic treatment of amyloidosis with AZA and MTX showed a slight effect on amyloid deposition in the spleen and decreased it by 1.74% and 2.08%. Inflammatory reaction was decreased by 33.6% in AZA treated animals. MTX suppressed amyloid formation in the liver by 30.2%, while AZA increased it by 18.7%. Significant decrease of infiltration with PMN was found after the treatment with MTX and AZA and both drugs suppressed focal necrosis of hepatocytes. AZA also insignificantly suppressed the average of amyloid deposition in the kidneys by 50% and the lowest cases of glomerulonephritis were found (33.3%), in this group.

Therapeutic treatment with AZA significantly suppressed formation of amyloid deposits in the liver. MTX was found to inhibit their formation in the spleen and kidneys. A significantly decreased the areas of connective tissue and inflammatory reaction in the spleen were observed after the treatment with MTX. Decreased inflammatory reaction by 33.6% and the lower appearance of the multinuclear cells by 40.07% were found in AZA treated animals. No one animal treated with MTX had glomerulonephritis, while in AZA treated group it was revealed in 40% of mice. The treatment with MTX and AZA decreased inflammatory infiltration with PMN/MMN in the liver by 22.5% and 46%.

Conclusions. In this study MTX seemed to be more effective than AZA in the treatment of experimental amyloidosis. Its best effect was achieved under therapeutic treatment which lasted four weeks and significant suppression of amyloid deposits formation was found in the spleen and kidneys. AZA significantly suppressed amyloid deposits in the liver.
Keywords:
mice, experimental amyloidosis, antirheumatic drugs

Introduction

The classical systemic forms of primary and secondary amyloidosis and some familial forms continue to affect a few thousand patients each year and clinical significance of amyloid in aging populations is increased. Why amyloid deposits are more commonly found in older than younger people is not yet understood [1]. AA amyloidosis development in IL-6 transgenic mice also is found to be age-dependent [2]. Amyloid deposition plays a central role in several diseases which affect larger segments of the older population. It is shown, that the age of 45 years-old was a dangerous age limit for the development of secondary amyloidosis in rheumatoid arthritis (RA) patients [3]. Secondary amyloidosis, which develops secondarily to chronic inflammatory conditions such as RA, is now called amyloid-A (AA) amyloidosis because a major factor in the protein deposition process involves a cleaved product of the acute-phase protein, serum amyloid A (SAA) [4–6]. It is one of the main complications of RA with poor prognosis and as yet an undefined medical therapy. AA amyloidosis is a systemic disease, caused by the deposition of amloid fibrils in various organs and characterized by dysfunction and destruction of them [7, 8]. The pathogenesis of amyloidosis is unknown and therefore no specific management is available at present. The main treatment protocol for AA amyloidosis is management of the underlying inflammatory disease process, which usually focuses on the use of anti-inflammatory medications and immunosuppressive agents [8, 9]. The treatment of amyloidosis aims at reducing the stimuli from chronic inflammation, inhibiting the production and deposition of amyloid protein and promoting the lysis of amyloid protein. The most radical way to prevent or reduce tissue deposition of amyloid AA is to suppress production of serum-AA-protein. Strong association between RA activity and amyloidosis needs to use of immunosuppressive and combined therapies to prevent kidney injury and reduce risk of dialysis [9–11]. Different rate of development of AA amyloidosis in various immunomodulatory treatment groups of RA patients shows that the treatment have influence on deposition of amyloid in tissues [8 Nakamura, 2008] which depends on patients age and seropositivity of the disease [12].

Low doses of methotrexate (MTX) are used for the therapy of RA patients with amyloidosis [13] and authors suggest that MTX at these doses might be an alternative in the early treatment of amyloidosis secondary to RA in patients with preserved renal function.

Many of the lessons learned about amyloid have come from a study of rodent models of AA amyloid. Mouse models of AA amyloid are still the best animal models of amyloidogenesis available. Similarly as in humans, SAA1 and SAA2 isoforms in mice are highly homologous and the SAA1 of both human and mice is predominant in AA amyloid deposits [14].

In our previous investigations combined treatment with diclofenac and prednisolone (D/P) and monotherapy with supphasalazine (SSL) resulted in improvement of AA amyloidosis in mice and inhibited amyloid deposition in the internal organs [15].

In this study methotrexate (MTX), the standard for disease-modifying anti-rheumatic drugs in humans and azathioprine (AZA) – a purine analog with cytotoxic and immunosuppressive properties used in the treatment of refractory as well as active RA [16], were chosen to assess their pharmacological effects on the development of experimental amyloidosis in mice.

Materials and methods

Animals

70 adult (20–30 g body weight) C57BL/6 mice were obtained from the Institute of Immunology (Vilnius, Lithuania) and housed in air conditioned quarters with a 12 hr light-dark cycle and 20° C temperature. The animals were given standard laboratory food and water ad libitum. They were allowed to acclimatize for at least 5 days before testing. Throughout the study the animals were cared for in accordance with the European Convention and Guide for the Care and Use of Laboratory Animals and with Lithuanian laws. All the mice were used with the approval of the Lithuanian Laboratory Animal Use Ethics Committee under the State Food and Veterinary Service.

Substances and drugs

Experimental AA amyloidosis was induced by using the following inflammatory substances: vitamin-free casein (Sigma Chemical Co, Germany) and fibrin (Chemical Dynamics Corporation, USA). For the treatment of amyloidosis MTX (EBEWE Pharma Ges.m.b.H.Nfg.KG A-4866 Unterach, Austria), and AZA (The Wellcome Ltd London, England) were used.
The treatment of mice experimental AA amyloidosis with methotrexate and azathioprine

**Induction of amyloidosis**

The study model was that of casein- and fibrin-induced amyloidosis in C57BL/6 mice [17]. The animals received subcutaneous injections of 12% vitamin-free casein in a 0.02 N NaOH solution 5 days a week and injections of 5% fibrin once a week for a period of 5 or 6 weeks. All the injections were performed between 9 and 11 a.m. and had a total volume of 0.5 ml each.

**Groups of animals and the treatment schedules**

Two experiments were performed and two treatment regime protocols: prophylactic and therapeutic, were used. The animals in both experiments were divided into the three groups. The drugs were prepared ex tempore in saline solution and injected in a 0.5 ml solution into the stomach through a metal probe 3 times a week in the case of MTX (dose 2.5 mg/kg) and 5 times a week in the case of AZA (dose 1 mg/kg). The control group received the saline solution without any treatment. In the first experiment (30 C57BL/6 mice), the treatment was started simultaneously with the first casein injection (day 0) and lasted 5 weeks. In the second experiment (30 mice), the treatment was started after 2 weeks of stimulation with inflammatory substances and lasted 4 weeks. About one third of experimental mice died during the test period in both studies and they were excluded from experiment.

**Other investigations**

The body weight of the animals was determined once a week. The animals were sacrificed after the last drug application. The erythrocyte and leukocyte counts (made using a Picoscale, Hungary) and the erythrocyte sedimentation rate (ESR) were determined for the blood. The internal organs were examined macroscopically and weighed with kidney, spleen and liver samples being taken for histological analysis. The indices obtained were compared with the indices for normal (healthy) animals and control groups.

**Histopathology**

The formalin-fixed spleen, kidney and liver specimens were divided into two pieces and embedded in paraffin. Duplicate sets of 5 µm-thick sections from each piece of tissue were mounted on glass slides. One set was stained with haematoxylin-eosin and Brachet for light microscopic examination to determine the inflammation scores. The other set of slides was stained with Congo red according to Eastwood [18] and examined in polarized light with an Olympus BX51 microscope to assess the degree of amyloid deposition in the tissue. The method used to detect amyloid protein included the traditional Congo red staining, because the main method for diagnosing amyloid A (AA) amyloidosis is limited in animals because it requires a large array of animal specific anti-AA antibodies, which are not commercially available [19]. The histological grading of the amyloid was made semi-quantitatively using a scale of 0 to 3 according to the density of the amyloid masses seen under a microscope, where ‘−’ means amyloid was absent (0), ‘±’ traces of amyloid were observed (0.5), ‘+’ minimal (1), ‘++’ moderate (2), and ‘+++’ (3) heavy (abundant) amyloid deposits were present. Grading of the extent of staining was performed “blind” by at least two independent expert observers.

**Statistical analysis**

The results were expressed as mean values ± S.E.M. The differences between healthy animals, the control groups, and the test groups were statistically analyzed using Student’s test (where two means are compared). A value of P < 0.05 was defined as significant. Incidence of pathomorphological changes in different groups of mice and the histological amyloid grades were assessed by using the Mann-Whitney U test.

**Results**

**Animals, Organs, and Laboratory Features**

The body weight at the end of experiment in the tested groups was lower than in healthy animals (Table 1). A significant decrease in comparison with the control group was observed in the groups prophylactically treated with MTX (P < 0.001) and therapeutically treated with AZA (P < 0.02).

Splenomegaly in all the groups in contrast to the healthy animals (P < 0.001) was revealed after a post-mortem examination of the internal organs. The highest relative spleen weight was in the group of animals therapeutically treated with MTX, what significantly differed from the control (P < 0.001).

It should be noted that the relative weight of the liver in the control (P < 0.001) and both treated groups (P < 0.002–0.001) was significantly higher than in healthy animals, but no significant changes between the control and tested groups were observed.

The relative weight of kidneys in prophylactically treated groups was lower, but no significant changes in
Table 1. Weight of the body and organs in C57BL/6 mice with experimental amyloidosis treated with antirheumatic drugs

<table>
<thead>
<tr>
<th>Index</th>
<th>Prophylactic treatment</th>
<th>Therapeutic treatment</th>
<th>Healthy mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control (n = 5)</td>
<td>MTX (n = 7)</td>
<td>AZA (n = 6)</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>25.63±1.13</td>
<td>19.38±0.62***</td>
<td>22.50±1.12</td>
</tr>
<tr>
<td>Liver (g/kg-1)</td>
<td><strong>8.09±0.22</strong></td>
<td>8.10±0.47</td>
<td>8.65±0.39</td>
</tr>
<tr>
<td>Kidneys (g/kg-1)</td>
<td>1.24±0.10</td>
<td>1.31±0.13</td>
<td>1.35±0.07</td>
</tr>
<tr>
<td>Spleen (g/kg-1)</td>
<td><strong>3.19±0.30</strong></td>
<td><strong>2.96±0.19</strong></td>
<td><strong>2.63±0.26</strong></td>
</tr>
</tbody>
</table>

Note: Amyloidosis was induced by 0.5 ml subcutaneous injections of 12% casein solution 5 times a week and 5% fibrin solution once a week. Prophylactic treatment was started on day 0 and continued for five weeks. Therapeutic treatment was started after two weeks of stimulation with inflammatory substances and continued for four weeks. The drugs were administered by intragastric gavage: AZA – 5 times a week and MTX – 3 times a week for 5 or 4 weeks in doses of 1 mg/kg and 2.5 mg/kg respectively. Control group received 0.5 ml of saline. n – number of animals. Symbols on the left – the differences are significant between healthy mice and the test groups. Symbols on the right – the differences are significant between the control group and the other test groups. * P < 0.05, ** P < 0.01, + P < 0.02, ++ P < 0.002, *** P < 0.001.

Table 2. The frequency and extent of amyloid deposition in different tissue structures of mice with amyloidosis treated with methotrexate and azathioprine

<table>
<thead>
<tr>
<th>Drug</th>
<th>Tissue</th>
<th>Frequency of amyloid deposition (%)</th>
<th>Average of amyloid deposition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Prophylactic treatment</td>
<td>Therapeutic treatment</td>
</tr>
<tr>
<td>Control</td>
<td>Spleen</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Kidneys</td>
<td>87.5</td>
<td>87.5</td>
</tr>
<tr>
<td>MTX</td>
<td>Spleen</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Kidneys</td>
<td>71.4</td>
<td>28.6</td>
</tr>
<tr>
<td>AZA</td>
<td>Spleen</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Kidneys</td>
<td>50</td>
<td>40</td>
</tr>
</tbody>
</table>

Note: Prophylactic treatment was started on day 0 and continued for five weeks. Therapeutic treatment was started after two weeks of stimulation with inflammatory substances and continued for four weeks. MTX – methotrexate (dose 2.5 mg/kg 3 times a week), AZA – azathioprine (dose 1 mg/kg 5 times a week). % – percentage of animals with amyloid deposits. The differences are significant between the control group and the other test groups. * P < 0.05, + P < 0.02.

Comparison with the control and healthy animals were found. Only MTX significantly increased the relative weight of kidneys in therapeutically treated animals.

Changes of blood indices

The blood indices such as ESR and leukocytes increased, and the amount of erythrocytes decreased and differed significantly (except the count of leukocytes in the group with AZA) from the healthy animals (Fig. 1). The most expressed ESR was observed in the groups of animals that received MTX, although the significance with the control group was not revealed.

The significant decrease of leukocytes was found in the groups of animals prophylactically and therapeutically treated with AZA (P < 0.01–0.001). The amount of erythrocytes did not differ between the control and treated groups in prophylactic treated animals but the significant decrease was revealed after therapeutic treatment with MTX (P < 0.002) and AZA (P < 0.01).

Macroscopic and microscopic changes in internal organs

Macroscopic changes in the internal organs were found only in the liver (white spaces, spots, spotted surface) of 42.8% animals prophylactically treated with MTX. Investigation of macroscopic changes on day 42 after induction of amyloidosis and treatment with MTX and AZA that was started 2 weeks after the first injection of inflammatory substances revealed the lesion of the spleen (white furs) in 11.1% of mice treated with MTX and in 22.2% of control and AZA treated animals. In 11.1% of the control mice injury of the liver was also found (white spaces, spots).

An inflammatory reaction in the spleen was observed
The treatment of mice experimental AA amyloidosis with methotrexate and azathioprine in all the animals (Fig. 2). In the first experiment 87.5% of the animals of control group had multinuclear phagocytes and 100% of them — areas of eosinophilic connective tissue which were found around the follicles and covered 25–50% of the spleen. Treatment with MTX and AZA significantly expanded the areas of connective tissue in the spleen and had no positive effect on the appearance of multinuclear cells, and inflammatory reaction decreased by 33.6% in AZA received animals.

Although the areas of eosinophilic connective tissues in the spleen were found in all the tested animals of the second experiment, but their expression was not equal in the separate groups. In comparison with the control, a significant decreasing of the areas of connective tissue and inflammatory reaction was observed after the treatment with MTX (P < 0.05). Decreased inflammatory reaction by 33.6% and the lower appearance of the multinuclear cells by 40.07% (P < 0.01) were found in AZA treated animals.

Polymorphonuclear (PMN) infiltration of the liver in all the tested animals was observed. Significant decrease (P < 0.001) of infiltration with PMN was found after the prophylactic treatment with MTX and AZA (Fig. 2). Both drugs suppressed focal necrosis of hepatocytes.

Fig. 1. Blood indices of C57BL/6 mice with experimental amyloidosis treated with methotrexate (MTX) and azathioprine (AZA).

Amyloidosis was induced by 0.5 ml subcutaneous injections of 12% casein solution 5 times a week and 5% fibrin solution once a week. Prophylactic treatment was started on day 0 and continued for five weeks. Therapeutic treatment was started after two weeks of stimulation with inflammatory substances and continued for four weeks. Oral administration of drugs was made: MTX – 3 times per week in doses 2.5 mg/kg, AZA – 5 times per week in doses 1 mg/kg. + The differences are significant in comparison with healthy animals; * The differences are significant in comparison with the control group.
Therapeutic treatment with MTX and AZA decreased infiltration with PMN/MMN in comparison with the control group by 22.5% and 46% although the significant differences were found only by using AZA (P < 0.02).
Both drugs did not suppress the focal hepatocyte necrosis which was also observed in 88.9% of animals of the control group.

Glomerulonephritis in the first experiment developed in 87.5% of the control animals and in 100% of mice prophylactically treated with MTX (Fig. 2). Tubular edema and glomerular injury were also found in 100% of mice in 87.5% of the control animals and in 100% of mice prophylactically treated with AZA (33.3%), but fibrinoid necrosis of the cortex was observed in this group.

Glomerulonephritis and tubular edema developed in 37.5% of the control mice in the second experiment. Minimal glomerulonephritis was accompanied with glomerular lesions such as homogenization, thickening of capillary wells, complete obstruction, dystrophy, an enlarged mesangium, and decreased cellularity which were found in 50% of the control group animals (Fig. 2).

No one animal therapeutically treated with MTX had marked glomerulonephritis and in 85.7% of mice only a minimal glomerulonephritis and tubular edema was found. While in AZA treated group glomerulonephritis was revealed in 40% of mice, where slight enlarged mesangium and focal glomerular sclerosis were observed and the tubular edema was found in 100% of mice.

**Amyloid deposition**

The frequency and extent of the amyloid deposition in the various organs of the mice with experimental amyloidosis and treatment with MTX and AZA are summarized in the Table 2. Severe amyloidosis by using casein and fibrin injections during 5 weeks developed in the animals of the control group. Amyloid deposition was generally the most severe and obvious in the spleen of these mice and moderate (2+) and heavy (3+) deposits of perifollicular amyloid were observed in 12.5% and 87.5% of the animals respectively.

The majority of the mice in the control group had either 2+ (75%) or 3+ (25%) amyloid deposits in the liver and these deposits were also identified in the blood vessel walls (50% of animals) and pericollagenously (100%). Although amyloid was found in the kidneys in 87.5% of the mice in the control group, its deposition was lower: 75% of the animals had traces of amyloid and 12.5% minimal deposits. Amyloid was deposited predominantly in the tubular basement membrane (87.5%) but also occurred in blood vessel walls (25%).

Injections of inflammatory substances during 42 days induced a strong amyloidosis in the animals of the control group. Heavy (3+) amyloid deposits identified perifollicularly were revealed in 100% of the mice (Table 2). All the control animals had minimal (11.1%), moderate (44.4%), and heavy (44.4%) hepatic amyloid deposits in the blood vessel walls (77.8% of mice) and pericollagenously (100%). Although amyloid was found in the kidneys of 87.5% of the mice, its deposition was lower: 50% of the animals had traces of amyloid and 37.5% minimal deposits. In 50% of the cases amyloid was found in the blood vessel walls and in 62.5% pericollagenously.

The prophylactic treatment of experimental amyloidosis with MTX and AZA had only a slight insignificant effect on amyloid deposition in the spleen and decreased it by 1.74% and 2.08% in AZA and MTX treated animals compared with the control group (Table 2). Although therapeutic treatment of AA amyloidosis with MTX and AZA did not reduce the number of animals with amyloid deposition in the spleen, its manifestation in MTX treated group was lower (Table 2). Moderate (2+) amyloid deposits in 42.9% of the mice and heavy (3+) ones in 57.14% of the animals were found after treatment with MTX while in the control group only heavy amyloid deposits were revealed. Average amyloid deposition in the spleen was significantly lower than in the control group after treatment with MTX (P < 0.05) (Table 2). No positive effect on amyloid formation was observed in AZA treated group. Amyloid deposition was the same as in the control group and heavy amyloid deposits in 100% of animals identified perifollicularly were found.

Prophylactically used MTX suppressed amyloid formation in the liver by 30.2% while AZA increased it by 18.7% (Table 2). Under the influence of MTX, minimal (57.1%), moderate (28.6%) and high (14.3%) amyloid deposits were found while after prophylactic treatment with AZA high amyloid deposition were revealed in 66.7% of animals although the changes were not significant in comparison with the control group. A significant decrease in deposits in the liver was obtained by using AZA therapeutically (39.9% suppression; P < 0.02). Only 5 animals survived in this group and amyloid in the liver was found in all of them with minimal and moderate deposits being identified in 60% and 40% of the mice respectively. Generally amyloid was located pericollagenously, but in 40% of the animals it was found in the blood vessel walls.

Amyloid deposition in kidneys was not decreased after prophylactic treatment with MTX. Although AZA suppressed the average of amyloid deposition by 50%,
but significant changes were also not observed (Table 2). The marked inhibition of amyloid deposits after therapeutic treatment with MTX (66.7%; P < 0.05) was observed in the kidneys, where only traces of amyloid (14.28% of animals) and minimal (14.28% of mice) amyloid deposits were identified in tubular basement membranes and pericollagenously were found only in 28.6% of animals (i.e., in 2 of 7).

Although therapeutic treatment with AZA decreased the average amyloid deposition in the kidneys, but significant differences in comparison with the control group were not found (Table 2). Traces of amyloid and minimal deposits identified in the blood vessel walls and tubular basement membranes were found in 40% of the animals treated with AZA.

In summary, therapeutic treatment of experimental amyloidosis with AZA significantly suppressed amyloid deposits formation in the liver and MTX – in the spleen and kidneys.

Discussion

The pathogenesis of amyloidogenesis involves complex series of processes that result in the deposition of protein fibrils in various organs. During any inflammatory disorder, activated inflammatory cells such as macrophages secrete cytokines which serve as inducers of acute phase protein synthesis by the liver [20]. The synthesis of SAA is induced by several cytokines, such as IL-6, IL-1, TNF-α [21–23]. A sustained elevation of SAA is a prerequisite for the development of reactive AA amyloidosis. In the case of amyloidosis associated with RA, the fibrils are composed of amyloid A (AA protein) derived from serum amyloid A protein (SAA).

As model of amyloidosis, we selected casein and fibrin-induced amyloidosis in C57BL/6 mice on the basis of our earlier studies [17]. It is a suitable model for investigating and understanding the pathogenesis of amyloidosis, representing an equivalent to human secondary amyloidosis [14, 24]. The tests that were conducted showed that, induced in this way, the pathological process caused distinct amyloid deposition in the spleens and livers of the control mice. A longer induction of amyloidosis caused more distinct amyloid formation in the test organs.

The data presented here showed that the inflammatory substances caused an increase in spleen and liver weight and made the blood indices worse. It should be noted that the spleen had the strongest reaction to the pathological process due to the splenomegaly and heavy amyloid deposits that occurred in all the animals. The spleen is a primary target for AA fibril deposition in animals like mice [17, 25]. Although the formation of AA amyloid fibril deposits is not well understood, but amyloidogenic stimulation in casein-induced amyloidogenesis enhances the synthesis of proteoglycans, which is related to splenic murine reactive AA amyloid and precedes amyloid fibril formation [24, 26], and the largest AA deposits occurring around the splenic lymphoid follicles are shown [27].

The liver also distinctly reacted to the pathological process. Its absolute and relative weight was the highest in the control groups and the amyloid deposits were extensive. Amyloid deposits in the liver were associated with atrophy and necrosis of hepatocytes and were frequent and massive in the wall of blood vessels, and also located pericollagenously. Massive blood vessels and liver amyloidosis was accompanied with higher ESR and count of leukocytes.

Amyloid deposits in the kidneys were less marked and in the most cases their deposition was minimal and found in the wall of blood vessels and the tubular basal layers membranes. Glomerulonephritis and tubular edema were observed in the kidneys. The inflammatory process damages the glomerulus, resulting in a thickening of the glomerular basement membrane, cellular proliferation within the mesangium, hyalinization, sclerosis, and glomerular death [28]. The abundance of basement membrane glycosaminoglycan in the glomerulus is a main factor in renal AA deposition [29] since this component is important in forming the typical β-sheet when AA fibrils are encountered [30]. However, this does not exclude the possibility that increased apoptosis in renal T cells plays a causative role for renal amyloidosis [29].

Disease-modifying antirheumatic drugs (DMARDs) such as MTX and AZA used in RA therapy were studied for the treatment of experimental amyloidosis. The ability of antirheumatic drugs to lower levels of acute-phase proteins which are important for the development of amyloidosis have been studied by investigators. In our previous studies, treatment with SSL and D/P was shown to inhibit the development of experimental amyloidosis in mice [15]. Many antirheumatic drugs are capable of cytokine modulation [31–33].

MTX, the standard for disease-modifying anti-rheumatic drugs in humans and AZA were chosen to assess their pharmacological effects on the development of experimental amyloidosis in mice. MTX in low doses
was found to prevent the attacks of amyloidosis [13]. In our study MTX seemed to be more effective than AZA in the treatment of experimental amyloidosis. Amyloid deposition was different with respect to the time when the treatment was started. The prophylactic treatment of experimental amyloidosis with MTX showed only a slight effect on amyloid deposition in the spleen and suppressed amyloid formation in the liver by 30.2%. The best effect of MTX was achieved under therapeutic treatment which lasted four weeks and significant suppression of amyloid deposits formation in the spleen by 14.3% and kidneys by 66.7% was found. Treatment with AZA showed no effect on amyloid formation in the spleen and amyloid deposits did not differ from the control group by using both treatment protocols. Prophylactic treatment with AZA increased amyloid deposits in the liver by 18.7% while therapeutic treatment significantly decreased them by 39.9%. In the kidneys both treatment protocols suppressed amyloidogenesis although the significant difference in comparison with the control group was achieved by using prophylactic treatment. Although therapeutic treatment with AZA suppressed the average of amyloid deposition in the kidneys by 50%, but significant changes were also not observed. The disappearing of amyloid deposition after the 8-month course of the treatment with prednisolone and AZA was revealed by Kimura and coauthors [34] and AZA was found to prevent the nephritic syndrome in patients with RA and amyloidosis [35]. The hypothesis that AZA may enhance amyloid formation is shown by Yussin et al. [36]. Our findings did not completely support these observations where mentioned authors conclude, that AZA enhances experimental amyloidosis. But it should be noted, that the lethality of animals in the groups treated with AZA was the highest and maybe the mice with marked amyloidosis and toxic effects of AZA not survived. Treatment with DMARDs, including AZA and MTX has been associated with reduced levels of acute phase proteins [37] and IL-6, which is known to regulate hepatic production of many acute phase proteins [38]. Decreases in circulating IL-6, soluble IL-2R, IL-6R and TNF receptors and IL-1 levels have been reported with MTX, whereas AZA reduces IL-6 but not sIL-2R [31, 32]. So, the some beneficial effect on the amyloid deposition observed after the treatment with MTX and AZA may be linked with diminution of cytokine production and reduced levels of acute phase proteins.

Conclusions

Different rate of the development of AA amyloidosis in various treatment groups of mice showed that the antirheumatic drugs have influence on the deposition of amyloid in tissues and this process depends on the time of their application. The best effect of MTX was achieved under therapeutic treatment which lasted four weeks and significant suppression of amyloid deposits formation was found in the spleen and kidneys. Therapeutic use of AZA significantly suppressed amyloid deposits in the liver.

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