Effects of Antirheumatic Drugs on the Development of Experimental AA Amyloidosis in C57BL/6 Mice

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Summary

Because there is no known specific effective therapy for secondary amyloidosis at the present time, the aim of this study was to determine whether antirheumatic drugs inhibit the development of experimental AA amyloidosis, induced in a C57BL/6 mice by injections of casein and fibrin. Monotherapy with sulfasalazine (SSL) and diclofenac (D) and combined treatment with diclofenac and prednisolone (D/P) by using prophylactic and therapeutic treatment protocols were investigated. The drugs were administered through intragastric gavage 5 times a week for 5 or 6 weeks in the following doses: D - 1 mg/kg, P - 10 mg/kg, and SSL - 100 mg/kg. Histopathological examination of splenic, kidney and hepatic tissues of mice was performed. The amount of amyloid was assessed semi-quantitatively by polarizing microscopy after Congo Red staining.

Our study indicated that no positive effect from prophylactic treatment with D could be seen on amyloid deposition in investigated organs. Prophylactic combined treatment with D/P resulted in significant improvement of disease symptoms and markedly reduced amyloid deposits in the spleen, kidneys, and liver ($P < 0.02$-0.001). SSL therapy alone has been more successful in the prophylactic treatment of experimental amyloidosis: the decrease of amyloid deposits was statistically significant in all investigated organs ($P < 0.04 – 0.001$) and the most suppression of amyloid formation in the kidneys and liver was observed ($P < 0.004$-0.001). In therapeutic treatment of experimental amyloidosis, combined treatment with D/P showed the most inhibition of amyloid formation in the internal organs ($P < 0.006 – 0.001$). The highest suppression (by 86.7%; $P < 0.001$) of amyloid deposits was observed in the liver. Treatment of mice with D alone produced a significant reduction in amyloid deposition only in the liver ($P < 0.03$) and with SSL – only in the spleen ($P < 0.03$).

These findings suggest that D/P and SSL at relevant doses suppress amyloidogenesis and this suppression is possibly related to the anti-inflammatory effect of antirheumatic drugs. Although these drugs cannot completely inhibit the disease in this model, a possibility remains that they may be clinically useful in rheumatic diseases associated with the formation of amyloidogenic derivatives.

Introduction

Secondary (AA) amyloidosis is a systemic disease characterized by the dysfunction and destruction of organs through the deposition of amyloid protein. It can potentially complicate any disorder associated with sustained acute phase response *(Husby, 1992)* and the most frequent predisposing conditions in the developed world, idiopathic rheumatic diseases *(Hawkins, 2001)*.

AA amyloidosis was probably the first amyloid described clinically and the first for which animal models were established experimentally *(Kisilevsky & Ancsin, 2001)*. The induction and *in vivo* reversibility of AA amyloidogenesis have become power-

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ful tools for investigating the amyloid deposition mechanism and possible therapies *(Kisilevsky & Ancsin, 2001)*. Many of the lessons learnt about amyloid have come from the study of rodent models of amyloid A. Mouse models of AA amyloidosis are still the best animal models of amyloidogenesis available *(Kisilevsky, 1996)*.

The aims of amyloidosis treatment are suppressing chronic inflammation and inhibiting the production and deposition of amyloid protein. But the only, as well as the most practical, way to prevent the development or progression of reactive amyloidosis is to reduce inflammatory activity *(Yamada et al, 2001)*. It has been shown that some chemotherapeutic drugs such as melphalan, prednisone, and colchicine are effective in some patients *(Livneh et al, 1994)*. Other medications (terbutaline, aminophyllin, colchicine, and tenidap) are reported to inhibit experimental amyloidosis in mice *(Brandwein et al, 1994; Husebekk & Stenstad, 1996; Shtrasburg et al, 2001a)*.

In this study, we focused on therapy with sulfasalazine (SSL), diclofenac (D), and prednisolone (P), which have been widely used for the treatment of rheumatoid arthritis (RA) and examined whether these drugs showed an inhibitory potency against amyloid formation in internal organs and prevented the development of AA amyloidosis in mice.

Materials and Methods

Animals

A total 92 C57BL/6 male mice (approximately 10-12 weeks old), body weight 20-30 g, were obtained from the Institute of Immunology (Vilnius, Lithuania) and acclimated for 5 days.

They were maintained in plastic cages (5-8 per cage) with rodent chow and tap water *ad libitum*. During the experiment, the animals were housed at 20–22 °C temperature, at 50-60% relative humidity with a 12-hour light/dark cycle. 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 the following anti-inflammatory drugs were used: prednisolone (Gedeon Richter, Hungary), diclofenac (Glaxo Wellcome, Great Britain), and sulfasalazine (KRKA, Slovenia).

Induction of amyloidosis

Experimental AA amyloidosis was induced using casein and fibrin solutions: 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 *(Leonaviciene et al, 2005)*. 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 drugs were prepared *ex tempore* in saline solution and injected in a 0.5 ml solution into the stomach through a metal probe 5 times a week. The animals in both experiments were divided into four groups. The control group (1st group) received the saline solution without any treatment. The test groups were treated with diclofenac (dose: 1 mg/kg) and prednisolone (10 mg/kg) [2nd group; D/P], diclofenac alone (1 mg/kg) [3rd group; D], and sulfasalazine (100 mg/kg) [4th group; SSL].

In the first experiment (40 C57BL/6 mice), the treatment was started simultaneously with the first casein injection (day 0) and lasted 5 weeks. In the second experiment (52 mice), the treatment was started after 2 weeks of stimulation with inflammatory substances and lasted 4 weeks.

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 morphological 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 (general inflammatory reaction, inflammatory cell infiltration) and hepatocytes necrosis. Each parameter was scored on a 0 to 3 point scale. Tubular edema, glomerulonephritis, and connective tissue areas (the latter was evaluated in percentages) were observed by microscope. The other set of slides was stained with Congo red according to Eastwood *(Eastwood & Cole, 1971)* 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 *(Shtrasburg et al, 2001b)*. 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. Two histopathologists independently analysed all the specimen sections.

Statistical analysis

All data were expressed as mean ± SEM. Statistical analysis was done using SPSS/PC software version 8.0 using *t* test statistics for continuous variables and P values less than 0.05 were considered to be significant. A nonparametric Mann-Whitney U statistical test was applied to analyse histologically observed differences and amyloid deposits in the internal organs. The effects of treatments were compared with those of controls.

Results

1. Prophylactic treatment of experimental amyloidosis with antirheumatic drugs

Animals, Organs, and Laboratory Features

The total weight of the animals varied between 20 and 30 g. No animals were lost in the group treated with D/P and two (20%) each in the control group and in the groups which received SSL and only D.

A post-mortem examination of the internal organs revealed splenomegaly ($P < 0.001$) in all the groups in contrast to the healthy animals (Table 1). The highest absolute and relative spleen weight was in the control group and the lowest in the group treated with D/P. The absolute and relative weight of the liver also markedly increased in the control group and significantly differed from the healthy group and the group of animals treated with D/P. In the group which received D the relative weight of the liver increased and was significantly higher than in the control group ($P < 0.05$).

The blood indices (ESR, leukocytes, and erythrocytes) in all the groups were almost the same and only differed significantly from the healthy animals (Table 2).

Histological examination

The frequency and extent of the amyloid deposition and inflammatory lesions in the various organs of the mice with experimental amyloidosis and treat-

Table 1. Weight of the body and organs in C57BL/6 mice with experimental amyloidosis treated with antirheumatic drugs

Organ		Prophylactic treatment				Therapeutic treatment				Healthy mice (n=5)
		Groups				Groups				
		1st Control (n=8)	2nd D/P (n=10)	3rd D (n=8)	4th SSL (n=8)	1st Control (n=9)	2nd D/P (n=9)	3rd D (n=8)	4th SSL (n=9)	
Body weight (g)		25.63±1.13	21.00±0.67**	22.20±1.20	24.38±1.13	22.55±1.09	$^{+}$21.11±1.18	**19.62±1.08	*21.78±1.19	26.67±1.67
Liver	Absolute (g)	$^{++}$2.06±0.07	1.32±0.10***	2.12±0.13	1.89±0.17	**2.11±0.13	1.76±0.14	1.86±0.14	*1.93±0.13	1.57±0.09
	Relative (g/kg^{-1})	***8.09±0.22	6.30±0.43^{++}	9.68±0.70*	7.72±0.52	***9.32±0.19	*8.31±0.33^{++}	***9.62±0.78	***8.86±0.32	5.89±0.25
Kidneys	Absolute (g)	0.31±0.02	0.26±0.02	0.31±0.01	0.27±0.02	0.34±0.02	0.34±0.02	0.36±0.03	0.38±0.03	0.37±0.03
	Relative (g/kg^{-1})	1.24±0.10	1.23±0.08	1.43±0.09	1.14±0.07	1.53±0.05	1.64±0.07	**1.85±0.09**	1.76±0.17	1.38±0.12
Spleen	Absolute (g)	***0.80±0.06	***0.49±0.04***	***0.57±0.05^{+}	***0.65±0.04	***0.64±0.03	***0.62±0.04	***0.73±0.02***	***0.65±0.05	0.10±0
	Relative (g/kg^{-1})	***3.19±0.30	***2.34±0.17*	***2.61±0.27	***2.72±0.23	***2.87±0.12	***3.02±0.26	***3.38±0.27	***3.00±0.10	0.376±0.02

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 5 times a week. The 1st (control) group received 0.5 ml of saline solution, the 2nd diclofenac (dose: 1 mg/kg) and prednisolone (dose: 10 mg/kg) [D/P], the 3rd diclofenac [D] (dose 1: mg/kg), and the 4th sulfasalazine [SSL] (dose: 100 mg/kg). n – number of animals. Symbols on the left – the differences are significant between normal 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. Effect of therapy with antirheumatic drugs on the blood indices of C57BL/6 mice with experimental amyloidosis

Index	Prophylactic treatment				Therapeutic treatment				Healthy mice (n=5)
	Groups				Groups				
	1st Control (n=8)	2nd D/P (n=10)	3rd D (n=8)	4th SSL (n=8)	1st Control (n=9)	2nd D/P (n=9)	3rd D (n=8)	4th SSL (n=9)	
ESR (mm/h)	**3.50±0.33	$^{++}$3.30±0.39	**3.87±0.58	*3.00±0.38	***4.22±0.49	**3.55±0.50	$^{++}$3.71±0.47	$^{+}$4.00±0.90	1.33±0.33
Leukocytes (10^9 L)	$^{++}$24.48±4.09	***20.29±2.21	**21.69±4.09	***18.55±2.52	***24.76±2.83	**15.37±2.58*	***23.24±2.42	$^{++}$20.08±3.36	6.40±0.15
Erythrocytes (10^{12} L)	***4.75±0.31	***5.02±0.28	**5.18±0.26	$^{++}$5.15±0.23	***4.84±0.09	***5.17±0.16	***4.49±0.22	***4.93±0.16	6.74±0.29

Note: D/P - diclofenac (1 mg/kg) and prednisolone (10 mg/kg), D – diclofenac (1 mg/kg), SSL – sulfasalazine (100 mg/kg). n – number of animals. Symbols on the left – the differences are significant between normal 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 3. Pathomorphological changes and amyloid deposits (%) in the spleen, kidneys, and liver of C57BL/6 mice with experimental amyloidosis treated with antirheumatic drugs

Organ				Prophylactic treatment				Therapeutic treatment			
				Groups				Groups			
				1st Control	2nd D/P	3rd D	4th SSL	1st Control	2nd D/P	3rd D	4th SSL
Spleen	Connective tissue areas		n/n	8/8	10/10	8/8	8/8	9/9	9/9	8/8	9/9
			%	100	100	100	100	100	100	100	100
	Multinuclear phagocytes		n/n	7/8	10/10	8/8	8/8	9/9	9/9	8/8	9/9
			%	87.5	100	100	100	100	100	100	100
	Inflammatory reaction		n/n	8/8	10/10	8/8	8/8	9/9	9/9	8/8	9/9
			%	100	100	100	100	100	100	100	100
	Amyloid	Perifollicularly	n/n	8/8	4/10	8/8	8/8	9/9	6/9	8/8	9/9
			%	100	40.0	100	100	100	66.7	100	100
		Blood vessel walls	n/n	-	3/10	-	-	-	-	-	-
			%		30.0						
Kidneys	Minimal glomerulonephritis		n/n	1/8	2/10	-	-	4/8***	7/9+	8/8¨++	1/9˙++
			%	12.5	20.0			50	77.8	100	11.1
	Glomerulonephritis		n/n	7/8	4/10	5/8	-	3/8	-	-	1/9
			%	87.5	40.0	62.5		37.5			11.1
	Tubular edema		n/n	-	10/10	5/8	7/8**	3/8	3/9	1/8	8/9
			%		100	62.5	87.5	37.5	33.3	12.5	88.9
	Amyloid deposits		n/n	7/8	3/10	8/8	1/8	7/8	2/9	5/8	3/9
			%	87.5	30.0	100	12.5	87.5	22.2	62.5	33.3
	Amyloid deposit location	Blood vessel walls	n/n	2/8	1/10	-	-	4/8	1/9	-	2/9
			%	25	10.0			50	11.1		22.2
		Tubular base-ment membrane	n/n	7/8	-	8/8	1/8	-	2/9	1/8	1/9
			%	87.5		100	12.5		22.2	12.5	11.1
		Pericollagenous	n/n	-	2/10	-	-	5/8	-	5/8	-
			%		20.0			62.5		62.5	
Liver	Inflammatory reaction (PMN/MMN)		n/n	8/8	7/10*	8/8	8/8	9/9	9/9	8/8	9/9
			%	100	70.0	100	100	100	100	100	100
	Hepatocyte necrosis		n/n	8/8	8/10*	8/8	8/8	9/9	7/9	8/8	9/9
			%	100	80.0	100	100	100	77.8	100	100
	Amyloid deposits		n/n	8/8	8/10	8/8	7/8	9/9	5/8	7/8	9/9
			%	100	80.0	100	87.5	100	62.5	87.5	100
	Amyloid deposit location	Blood vessel walls	n/n	4/8	8/10	8/8	3/8	7/9	1/8	6/8	5/7
			%	50	80.0	100	37.5	77.78	12.5	75	55.5
		Pericollagenously	n/n	8/8	5/10	8/8	7/8	9/9	5/8	7/8	9/9
			%	100	50.0	100	87.5	100	62.5	87.5	100

Note: D/P – diclofenac (1 mg/kg) and prednisolone (10 mg/kg), D - diclofenac (1 mg/kg), SSL – sulfasalazine (100 mg/kg). PMN – polymorphonuclear infiltrates, MMN – monomorphonuclear infiltrates (lymphocytes, plasma cells, and macrophages). n/n – number of animals with organ changes / total number of animals investigated. % - percentage of animals with changes in organs and with amyloid deposits. Prophylactic treatment: * - very small focal PMN and very small necrotic focuses; + -amyloid in glomerulus; ** - 50% slight tubular edema, 25% moderate tubular edema, and 12.5% heavy tubular edema. Therapeutic treatment: + - very slight glomerular changes, ˙ - very slight increase in the mesangium, ¨ - a slight increase in the mesangium, ++ - focal glomerular sclerosis, *** - damage to the glomerulus (homogenization, thinning of the capillary walls, partial obstruction, dystrophy, an enlarged mesangium, and decreased cellularity).

Table 4. Pathomorphological changes and average amyloid deposits in the spleen, kidneys, and liver of C57BL/6 mice with experimental amyloidosis treated with antirheumatic drugs

Organ		Prophylactic treatment				Therapeutic treatment			
		Groups				Groups			
		1st Control (n=8)	2nd D/P (n=10)	3rd D (n=8)	4th SSL (n=8)	1st Control (n=9)	2nd D/P (n=9)	3rd D (n=8)	4th SSL (n=9)
Spleen	Connective tissue areas (%)	29.38±1.85	20.80±1.69**	34.63±3.76	38.38±3.54	39.67±3.37	26.56±1.75**	33.63±3.95	27.44±1.87*
	Multinuclear phagocytes	1.25±0.16	1.67±0.17	1.50±0.19	1.63±0.18	2.67±0.17	2.44±0.17	2.75±0.16	2.33±0.17
	Inflammatory reaction	1.13±0.13	1.10±0.10	1.00±0	0.81±0.09	2.00±0.17	1.33±0.17+	1.38±0.18*	1.22±0.15**
	Amyloid deposit average	2.88±0.12	0.78±0.22***	3.00±0	2.25±0.25*	3.00±0	1.67±0.23***	2.88±0.12	2.56±0.17*
Kidneys	Amyloid deposit average	0.50±0.09	0.17±0.08+	1.00±0***	0.063±0.06++	0.63±0.12	0.11±0.07**	0.44±0.15	0.28±0.14
Liver	PMN/MMN infiltration	2.00±0	0.65±0.11***	1.69±0.30	0.81±0.16***	1.11±0.16	0.78±0.12	1.25±0.21	0.61±0.07**
	Hepatocyte necrosis	2.13±0.12	0.55±0.09***	1.63±0.24	1.12±0.08***	1.39±0.20	1.00±0.22	1.94±0.20	1.55±0.13
	Amyloid deposit average	2.25±0.16	0.78±0.17***	2.83±0.17*	0.44±0.06***	2.33±0.23	0.31±0.09***	1.38±0.32*	1.89±0.309

Note: D/P – diclofenac (1 mg/kg) and prednisolone (10 mg/kg), D - diclofenac (1 mg/kg), SSL – sulfasalazine (100 mg/kg). 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. PMN/MMN –polymorphonuclear / monomorphonuclear infiltrates (lymphocytes, plasma cells, and macrophages). n – number of animals. Inflammation scores (general inflammatory reaction, inflammatory cell infiltration) and hepatocytes necrosis were scored on a 0 to 3 point scale. Connective tissue areas evaluated in percentages were observed by microscope. The nonparametric Mann-Whitney U test was applied to analyze differences for all parameters examined. The effects of treatment were compared with those of controls. * - $P < 0.05$, ** - $P < 0.01$, + - $P < 0.02$, ++ -$P < 0.002$, *** - $P < 0.001$.

ment are summarized in the Tables 3 and 4 and shown in Figures 1 and 2.

The amount of amyloid deposited in the spleen was significant in the animals of the control group (Tables 3 and 4). Moderate (2+) and heavy (3+) deposits of perifollicular amyloid were observed in 12.5% and 87.5% of the animals respectively (Fig. 1C, Table 5). An inflammatory reaction was observed in all the animals (Table 3). 87.5% of animals in the control group had multinuclear phagocytes and 100% of the animals had areas of eosinophilic connective tissue which were around the follicles and covered 25-50% of the spleen.

The majority of the mice in the control group had either 2+ (75%) or 3+ (25%) amyloid deposits in the liver (Fig. 1D, Table 5) and these deposits were also identified in the blood vessel walls (50% of animals) and pericollagenously (100%) (Table 3). Polymorphonuclear (PMN) infiltration of the liver was observed in all the tested animals.

Table 5. Amyloid induction in the spleen, kidneys, and liver of C57BL/6 mice with experimental amyloidosis treated with antirheumatic drugs

Organ			Prophylactic treatment				Therapeutic treatment			
			Groups				Groups			
			1st Control	2nd D/P	3rd D	4th SSL	1st Control	2nd D/P	3rd D	4th SSL
Spleen	Traces (±)	n/n %	-	-	-	-	-	-	-	-
	Minimal (+)	n/n %	-	5/10 50.0	-	1/8 12.5	-	4/9 44.4	-	-
	Moderate (++)	n/n %	1/8 12.5	1/10 10.0	-	4/8 50	-	1/9 11.1	1/8 12.5	4/9 44.4
	Heavy (+++)	n/n %	7/8 87.5	-	8/8 100	3/8 37.5	9/9 100	1/9 11.1	7/8 87.5	5/9 55.6
Kidneys	Traces (±)	n/n %	6/8 75	3/9 30.0	-	1/8 12.5	4/8 50	2/9 22.2	3/8 37.5	1/9 11.1
	Minimal (+)	n/n %	1/8 12.5	-	8/8 100	-	3/8 37.5	-	2/8 25	2/9 22.2
	Moderate (++)	n/n %	-	-	-	-	-	-	-	-
	Heavy (+++)	n/n %	-	-	-	-	-	-	-	-
Liver	Traces (±)	n/n %	-	6/10 60.0	-	7/8 87.5	-	5/8 62.5	-	-
	Minimal (+)	n/n %	-	2/10 20.0	-	-	1/9 11.1	-	4/8 50	4/9 44.4
	Moderate (++)	n/n %	6/8 75	1/10 10.0	1/8 12.5	-	4/9 44.4	-	2/8 25	2/9 22.2
	Heavy (+++)	n/n %	2/8 25	-	7/8 87.5	-	4/9 44.4	-	1/8 12.5	3/9 33.3

Note: D/P – diclofenac (1 mg/kg) and prednisolone (10 mg/kg), D - diclofenac (1 mg/kg), SSL – sulfasalazine (100 mg/kg). n/n – number of animals with amyloid deposits / total number of animals investigated. % - percentage of animals with amyloid deposits.

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 (Table 5). Amyloid was deposited predominantly in the tubular basement membrane (87.5%) but also occurred in blood vessel walls (25%). Chronic renal lesions with glomerulonephritis were revealed in 87.5% of the mice and 12.5% of animals had minimal glomerulonephritis (Table 3).

No positive effects from treatment with D could be seen on amyloid deposition in the spleen, kidneys, and liver (Tables 3, 4, and 5).

But the combination of D with P not only decreased the number of animals with amyloid deposits but also significantly ($P < 0.001$) suppressed (by 72.9%) amyloid formation in the spleen (Table 4). Only minimal (5 of 10 (50%) of the animals) and moderate (10%) amyloid deposits (Fig. 1A, Table 5) were found perifollicularly (40% of mice) and in blood

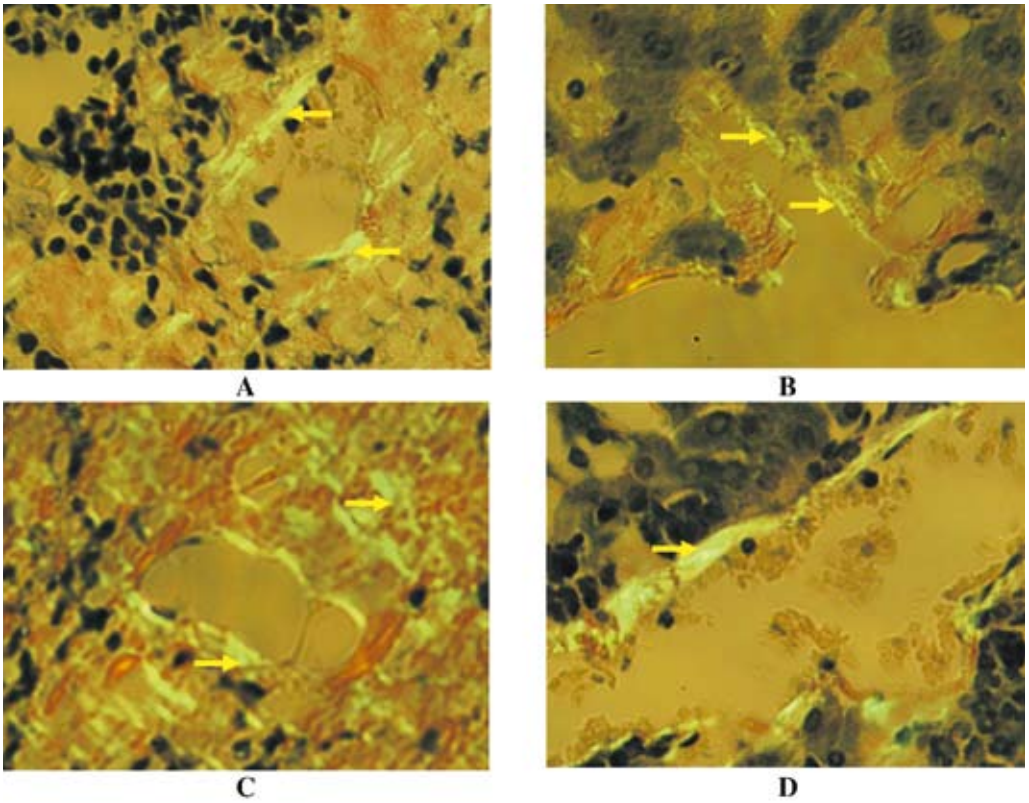

Figure 1. Amyloid deposits in C57BL/6 mice with experimental AA amyloidosis prophylactically treated with diclofenac and prednisolone (D/P).
Minimal amyloid deposits in the spleen (**A**) and liver (**B**) of mice treated with D/P. Heavy deposits in the spleen (**C**) and liver (**D**) of the control group mice. Stained with Congo red acid solution, x200.

vessel walls (30%). Treatment with SSL also significantly suppressed amyloid formation by 21.9% in the spleen ($P < 0.05$) (Table 4).
The same effect after prophylactic treatment in the kidneys was observed, where amyloid was absent in most of the animals of the groups which received D/P and SSL, or only traces of amyloid were found (30% and 12.5% respectively), deposited in blood vessel walls (10%) and pericollagenously (20%) in the first case, and in the tubular basement membrane (12.5%) in the second case. The D/P combination suppressed amyloid deposits in the kidneys by 66.0% ($P < 0.02$) and SSL by 87.4% (Table 4). The pathological process in the kidneys was also lower in these groups than in the control group (Tables 3 and 4).
Although the number of animals with amyloid deposits in the liver did not decrease, the amyloid deposition was lower (by 65.3%; Table 4) after the treatment with D/P: 70% of the animals had traces of amyloid, 20% minimal deposits, and 10% moderate deposits (Fig. 1B, Table 5). Amyloid was identified in blood vessel walls (80%) and pericollagenously (50%) (Table 3).
A more pronounced inhibitory effect (77.8% in comparison to the control group) on amyloid depo-

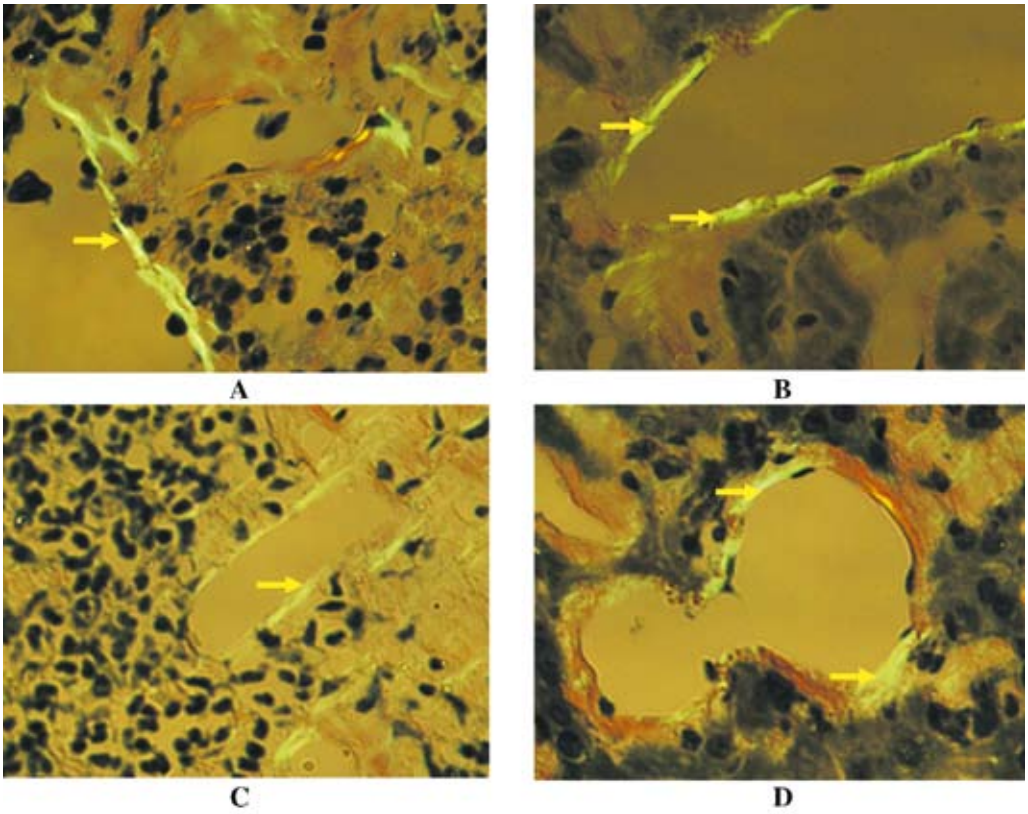

Figure 2. Amyloid deposits in C57BL/6 mice with experimental AA amyloidosis following therapeutic treatment with D/P and sulfasalazine (SSL).
Moderate and minimal deposits in the spleen and liver of the mice treated with SSL (**A**, **B** respectively) and D/P (**C**, **D**). Stained with Congo red acid solution, x200.

sition in the liver was obtained by using SSL ($P < 0.001$; Table 4). Only traces of amyloid were found in 87.5% of animals (Table 5).

The D/P combination significantly decreased the connective tissue areas in the spleen ($P < 0.007$), the polymorphonuclear (PMN) infiltration of the liver ($P < 0.001$), and hepatocyte necrosis (by 72.4%; $P < 0.001$; Table 4). Only very small focal PMN (Table 3) and very small necrotic focuses were found in the hepatocytes using this treatment (Table 3).

In the groups treated with D/P and D alone, glomerulonephritis was found in 40% and 66.7% of the animals respectively.

Although treatment with SSL increased the connective tissue areas in the spleen ($P < 0.04$), it markedly decreased PMN infiltration of the liver (by 59.5%; $P < 0.001$) and hepatocyte necrosis (by 47.4%; $P < 0.001$) (Table 4). Glomerulonephritis was not found in any animal of this group but tubular edema developed in 87.5% of the animals (50% very slight, 25% moderate, and 12.5% marked) (Table 3).

So, both the D/P combination and SSL seem to be efficacious in the prophylactic treatment of experimental AA amyloidosis. D/P was more effective than D alone and more effective than SSL in inhibiting amyloid deposition in the spleen but the latter

(SSL) was more effective with amyloid formation in the kidneys and liver. Prophylactic treatment of AA amyloidosis with D/P and SSL significantly improves of this disorder and did not produce any side effects during the whole experiment.

2. Therapeutic treatment of experimental amyloidosis with antirheumatic drugs

Animals, Organs, and Laboratory Features

30.8% of the animals (4 of 13) in the control group and the groups treated with D/P and SSL were lost during the experiment. The mortality of the animals (5 of 13) in the D group was 38.5%.

The average body weight at the end of the experiment was significantly lower in all the test groups in comparison to the healthy animals (Table 1). The absolute and relative liver weight was the highest in the control group and significantly differed from the healthy animals ($P < 0.01$ and $P < 0.001$). The relative weight of the liver and the absolute and relative weight of the spleen of all the test groups were also significantly higher than that of the healthy animals but treatment with D/P decreased the relative weight of the liver ($P < 0.002$) in comparison to the control group.

The absolute weight of the kidneys in the test groups did not differ from the control group and the healthy animals but the relative weight increased markedly in the group of animals treated with D ($P < 0.01$).

The changes in the blood indices, such as the ESR and the leukocyte and erythrocyte counts, were worse compared to the healthy animals (Table 2). The highest ESR and leukocyte counts were observed in the control group. Treatment with D/P significantly reduced the leukocyte count ($P < 0.05$) in comparison to this group.

Histological examination

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 (Tables 3-5). An inflammatory reaction as well as areas of connective tissue and multinuclear cells in the spleen was found in all the animals of this group (Table 3).

All the control animals had minimal (11.1%), moderate (44.4%) or heavy (44.4%) hepatic amyloid deposits in the blood vessel walls (77.8% of mice) and pericollagenously (100%). Inflammatory polymorphonuclear and monomorphonuclear infiltration (PMN/MMN) as well as hepatocyte necrosis were seen in 100% of the animals.

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 (Table 5). In 50% of the cases amyloid was found in the blood vessel walls and in 62.5% pericollagenously.

Glomerulonephritis and tubular edema developed in 37.5% of the mice and minimal glomerulonephritis accompanied with glomerular lesions (homogenization, thickening of capillary walls, complete obstruction, dystrophy, an enlarged mesangium, and decreased cellularity) were found in 50% of the control group animals (Table 3).

Although treatment of experimental AA amyloidosis with D and SSL did not reduce the number of animals with amyloid deposition in the spleen, its manifestation was lower (Tables 3-5). Moderate (2+) amyloid deposits in 12.5% and 44.4% of the mice and heavy (3+) deposits in 87.5% and 55.6% of the animals were found after treatment with D and SSL respectively.

Combined therapy with D/P decreased the manifestation of amyloid and the number of animals with perifollicular amyloid deposits. Amyloid was identified in 66.7% of the animals. Moderate and heavy deposits of amyloid were observed in 11.1% and minimal in 44.4% of the mice treated with D/P (Fig. 2C; Table 5). Average amyloid deposition in the spleen was significantly lower than in the control group after treatment with D/P ($P < 0.001$) and SSL (Fig. 2B; $P < 0.03$), which was especially obvious in the D/P group (44.3% suppression) (Fig. 2A, C; Table 4).

All the tested drugs significantly reduced the inflam-

matory reaction in the spleen ($P < 0.05$-0.01) while D/P and SSL markedly decreased the areas of connective tissue in comparison to the control group ($P < 0.006$ and $P < 0.012$ respectively).

The same inhibition of amyloid deposits after treatment was observed in the kidneys, where D/P suppressed the average formation of amyloid by 82.5% ($P < 0.006$) (Table 4). In most cases amyloid was absent (7 of 9 mice) or only traces of amyloid (22.2%) were identified in the blood vessel walls (11.1% of the mice) and tubular basement membranes (22.2% of mice). SSL inhibited amyloid formation by 55.5% with such formations being found in 33.3% of the mice (11.1% traces and 22.2% minimal deposits) (Tables 4 and 5).

D insignificantly reduced amyloid formation in the kidneys. Traces of amyloid and minimal deposits in the blood vessel walls and tubular basement membranes were found in 62.5% of the mice treated with D.

Glomerulonephritis was absent in the animals treated with D/P and D but minimal glomerulonephritis with a slight enlargement of the mesangium, focal glomerular necrosis, and tubular edema were found respectively in 100% and 12.5% of the mice treated with D (Table 3).

Minimal glomerulonephritis with slight glomerular changes in the first case and focal glomerular necrosis in the second was observed respectively in 77.8% and 11.1% of the D/P and SSL treated groups. 11.1% of the mice (1 of 9) treated with SSL had glomerulonephritis and 88.9% tubular edema. The latter was found in only 33.3% of the animals treated with D/P (Table 3).

The highest suppression (by 86.7%; $P < 0.001$) of amyloid deposits in the liver was observed after treatment with D/P (Table 4; Fig. 2D). Traces of pericollagenous amyloid were found in 62.5% of the animals (Table 5).

A significant decrease in deposits in the liver was also obtained using D (40.8% suppression; $P < 0.03$) but their manifestation was much stronger than in the D/P group. 87.5% of the animals had amyloid in the blood vessel walls (75%) and pericollagenously (87.5%) with minimal, moderate, and heavy deposits being identified in 50%, 25%, and 12.5% of the mice respectively (Tables 3-5).

Treatment with D/P and SSL decreased inflammatory PMN/MMN infiltration (by 33.6% and 44.2%) but significant changes were observed after treatment with SSL ($P < 0.01$) (Table 4). Only combined D/P treatment suppressed focal hepatocyte necrosis by 28% in comparison to the control group.

Thus, therapeutic treatment of experimental amyloidosis with a D/P combination showed the most expressed inhibitory effect on amyloid formation in all the tested organs. D alone significantly decreased amyloid deposits in only the liver. The positive suppression effect was also observed by using SSL, especially on amyloid formation in the spleen.

Discussion

The AA amyloidosis associated with chronic inflammatory diseases is relatively rare but important because diagnosis is often difficult, the prognosis is poor, and no known specific effective therapy for the disease exists at the present time *(Hawkins, 2001)*. It can potentially complicate any disorder associated with a sustained acute phase response but in the developed world, chronic rheumatic diseases have been asserted to be the most frequent predisposing conditions for the development of AA amyloidosis *(Hawkins, 2001; Wakhlu et al, 2003)*.

We selected casein and fibrin-induced amyloidosis in C57BL/6 mice on the basis of our earlier studies *(Leonaviciene et al, 2005)*. It is a suitable model for investigating and understanding the pathogenesis of amyloidosis, representing an equivalent to human secondary amyloidosis *(Stenstad et al, 1994)*.

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 spenomegality and heavy amyloid deposits that occurred in all the animals. The spleen is a primary target for AA fibril deposition in animals like mice *(Wien et al., 2001)*. Although the formation of AA amyloid fibril deposits is not well understood, in the murine model of amyloidosis the deposits increase in various organs with the largest AA deposits occurring around the splenic lymphoid follicles *(Huchinson et al, 2001)*, which our data also show *(Leonaviciene et al, 2005)*. 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 *(Snow et al, 1991; Stenstad et al, 1994)*.

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.

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 *(Grauer, 2002)*.

The abundance of basement membrane glycosaminoglycan in the glomerulus is a main factor in renal AA deposition *(Mountz & Hsu, 1997)* since this component is important in forming the typical ß-sheet when AA fibrils are encountered *(Kisilevsky, 1992)*. However, this does not exclude the possibility that increased apoptosis in renal T cells plays a causative role for renal amyloidosis *(Mountz & Hsu, 1997)*.

The three categories of medications used in rheumatoid arthritis (RA) therapy were studied for the treatment of experimental amyloidosis: nonsteroidal anti-inflammatory drugs (NSAIDs), disease-modifying antirheumatic drugs (DMARDs), and corticosteroids. The NSAID group was represented by diclofenac (D), corticosteroid preparations by prednisolone (P), and the DMARD group by sulfasalazine (SSL). In our experiments, one group of mice with experimental amyloidosis received a combination of D and P, because in practice, many patients with RA are treated with low dose corticosteroids, often in combination with other antirheumatic drugs.

It is known that the mechanism for NSAID action inhibits cyclooxygenase (COX-1 and COX-2) *(Bakowsk &, Hanly, 2000; McKenna, 1999)*. COX-2 expression is induced, particularly during the inflammatory process *(Pairet & Engelhardt, 1996)*. Diclofenac, one of the most widely used NSAIDs, is genuinely broad spectrum, having a similar inhibitory action on both the enzyme's isoforms *(McGeer, 2000)*. Relative selectivity for COX-2 vs COX-1 for D is 0.45:1.43 *(Vane & Botting, 1995)*. Among standard NSAIDs, it has the most favourable profile *(Paire & van Ryn, 1998)* and produces its analgesic effect by actions at the inflammation locus.

But in our study single, D therapy was not satisfactory in the treatment of experimental amyloidosis. Administration of D at 1 mg/kg during the five weeks produced an increase in pathomorphological changes in the spleen and showed no positive effect on amyloid deposition in all the investigated organs. Shorter D treatment suppressed the splenic inflammatory reaction and significantly decreased only hepatic amyloid formation. But it should be pointed out that the abnormalities observed macroscopically and histologically in the kidneys and liver were more frequently revealed in the D group than in the other treated groups.

Although NSAIDs are generally well tolerated, they are associated with a spectrum of potential clinical toxicities *(Bakowsky & Hanly, 2000; Langenegger & Michel, 1999; Singh et al., 1994)*. Nephrotoxicity is a clinically important NSAID side effect *(Khan et al., 1998; Sandler et al, 1991).* Some alterations of renal function are COX-2-related mechanism-based effects *(Crofford, 2000)*. Besides interstitial nephritis as well as nephrotic and end stage renal disease, which all occur rarely *(Perneger et al., 1994; Schlondorff, 1993)*, the most common side effect is a decrease in renal function, which is caused by a reduction in renal blood flow. It was shown that chronic treatment with NSAIDs may result in

COX-2 mobilization where COX-2 is either not translocated efficiently into the lumen of the nuclear envelope (endoplasmic reticulum) or loses its high affinity for the membrane *(Simmons et al., 1999)*. COX-2, but not COX-1, was highly induced by diclofenac *(Simmons et al, 2000)* and this induction was dose dependent *(Simmons et al., 1999)*.

Although the administration of D had no effect on the development of experimental amyloidosis, the D/P combination was active in suppressing this process. Both treatment protocols caused the most significant inhibitory effect on splenic amyloid formation. But the prophylactic treatment showed the greatest reducing effect on spleen weight and was more effective in decreasing amyloid deposits (by 72.9% whereas therapeutic treatment reduced the deposits 44.3% compared to the control group). Both treatments decreased the areas of eosinophilic connective tissue and inflammatory reaction in the spleen and improved the blood indices.

The same effect was observed in the kidneys and liver but a distinct inhibitory effect on amyloid formation was revealed with the therapeutic treatment. Both treatments significantly reduced the relative weight of the liver and suppressed inflammatory PMN infiltration and hepatocyte necrosis. The inhibitory effect on the latter was distinctly observed with the prophylactic treatment. The cases of glomerulonephritis also decreased after the treatment. All these events showed the positive effect of D/P.

It should be pointed out that steroid treatment has been tested on human and animal AA amyloidosis but the results were contradictory and the success limited *(Cohen et al, 1962; Fields et al, 1973; Grayzel et al, 1956; Maxwell et al, 1964)*. But Shtrasburg et al *(2005)* showed that hydrocortisone suppressed the second phase of murine amyloidosis. It could be related to our results, where the combination of P and D suppressed the amyloid deposits in the internal organs of the mice. Corticosteroids are also known to be effective inhibitors of COX-2 *(Masferrer et al, 1994)*.

The ability of antirheumatic drugs to lower levels of acute-phase proteins which are important for the development of amyloidosis have been studied by variousinvestigators. In animal models and in humans it has been shown that treatment with NSAIDs, corticosteroids, and sulfasalazine have been associated with reduced levels of acute-phase proteins *(Cush et al, 1990; Danis et al, 1992; Geiger et al, 1993)* and levels of certain cytokines which stimulate hepatocytes to synthesize C reactive protein (CRP) as well as serum amyloid protein A (SAA) *(Husebekk & Stenstad, 1996; Littman et al, 1995; Loose et al, 1993)*, the precursor for protein AA in secondary amyloid fibrils *(Husebekk et al, 1985)*. The production of acute-phase proteins by the liver is regulated by cytokines including IL-6, IL-1, and TNFα *(Richards et al, 1991)*. The relationship between IL-6 levels and the levels of the acute-phase proteins is of interest since IL-6 is known to regulate the hepatic production of many acute-phase proteins *(Kordula et al., 1991)*. Some cytokines such as IL-1 also increased COX-2 activity *(Fu et al., 1990; Ristimaki et al, 1994)*.

Many antirheumatic drugs are capable of cytokine modulation *(Barrera et al, 1996; Franke et al, 1997; Dessein & Joffe, 2006)*. Cytokine over-production, which is thought to be responsible for the acute-phase response in mice with amyloidosis, can be down-regulated by prednisolone and other immunosuppressive drugs. Prednisolone reduced the expression of TNF-α, IL-1ß, and IL-6 *(Rioja et al., 2004; Patten et al, 2004)*. Both IL-6 and IL-1ß increase the production of hyperalgesic prostaglandins, the former by mobilising arachidonic acid and the latter by inducing the expression of the cyclo-oxygenase-2 (COX-2) gene *(Bottin &, Botting, 2000)*.

Prophylactic and therapeutic treatment of experimental amyloidosis with SSL also had a positive effect and significantly suppressed amyloid deposits in the spleen although to a lesser degree than D/P. Prophylactic treatment reduced its deposition by 21.9% and therapeutic treatment by 15% in comparison to the control group. A distinct suppression of amyloid deposits was found in the kidneys (87.4%) and liver (80.4%) with the prophylactic treatment and by 55.6% and 18.9% respectively with the ther-

apeutic treatment.

In respect to SSL, it is a slow acting antirheumatic drug *(Tett, 1993)*. Its action is associated with low toxicity and SSL is commonly used in Europe as the DMARD of choice *(Boers et al., 1997)* in early and mild disease *(Jackson & Williams, 1998)*. The metabolism of SSL is complex and, to some extent, genetically determined. The drug's action mechanism is not well understood but involves decreased production of cytokines and a decreased proliferative response by the lymphocytes *(Gardner & Furst, 1995)*. Treatment with SSL has been associated with a reduction in IL-1α, IL-1ß, and TNFα *(Danis et al., 1991, 1992; Remvig & Andersen, 1990)* but not in sIL-2R *(Crilly et al., 1993)* or IL-6 *(Danis et al., 1992)* concentrations although the latter was not corroborated in another study *(Watson et al., 1992)*. SSL also inhibits the binding of TNFα to its receptor *(Shanahan et al., 1990)*.

A beneficial effect by salazosulfapyridine (SASP) in a patient with secondary renal amyloidosis was observed by Hidaka et al *(Hidaka et al, 1998)*. SASP was evidently effective for arthritis and the improvement of renal function. It might have a beneficial effect on AA amyloidosis by suppressing inflammatory cytokines. AA protein is derived from SAA which is synthesized by inflammatory cytokine stimulation *(Ganapathi et al, 1991; Gottenberg et al, 2003; McNiff, 1995; Mihara et al, 2004)* where IL-6 is a key cytokine for the induction of AA amyloidosis *(Mihara et al, 2004)*.

The results of our study indicate that treatment with SSL and D/P can significantly suppress amyloid deposition in a murine amyloidosis model. This therapy has been successful in the prophylactic and therapeutic treatment of murine amyloidosis. We suggest that the treatment causes a reduction in acute-phase proteins and this reduction is associated with a decrease in plasma levels of proinflammatory cytokines. Thus one clue to the clinical effect of the investigated drugs may be their ability to reduce the levels of proinflammatory cytokines and another clue, a reduction in COX-2 expression.

In conclusion, our experiments indicated a different development rate for experimental amyloidosis in various treatment groups of mice. Prophylactic and therapeutic combined treatment with D/P resulted in significant improvement of disease symptoms and markedly reduced amyloid deposits in the spleen, kidneys, and liver. SSL therapy alone has been more successful in the prophylactic treatment of experimental amyloidosis where it suppressed amyloid formation in the kidneys and liver more effectively than D/P. The information gleaned from such studies may have applicability in the prevention and treatment of disorders associated with pathological amyloid deposition such as found in patients with rheumatoid arthritis.

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