Influence of Dextran Sulphate, Fibrin, and Ubiquitin on the Development of Casein-Induced Experimental AA Amyloidosis in C57BL/6 mice

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Summary

The influence of subcutaneous injections of dextran sulphate (DS) and fibrin (F), as well as of an intraperitoneal injection of ubiquitin (Ub), was investigated on 48 male C57BL/6 mice subjected to conventional casein (C) induced amyloidosis. Histopathological examination of spleen and kidney tissue 3 and 5 weeks after termination of the amyloidogenic stimulus showed that the amount of amyloid deposited (rated trace, minimal, moderate or heavy) increased progressively with the duration of the amyloidogenic stimulus. After 3 weeks of stimulation, 16.7% of mice injected with C had some perifollicular amyloid deposits in the spleen while all had traces of amyloid in the kidney. Some amyloid was detected in the spleen of 33.3% of the mice treated with C+DS and C+Ub and 83.3% treated with C+F. Half the latter group also showed traces and half minimal amyloid deposits in their kidneys. In the other test groups, the incidence of kidney amyloidosis was less.

The most extensive tissue deposits were seen at 5 weeks postinjection (p.i.) with most in the C+F-treated animals, all showing significantly more than the control C-treated group. Thus half the C+F-treated animals had moderate and half heavy deposits throughout their spleens. Glomerulonephritis, kidney tubular edema and some amyloid deposits were present in all of the animals. C+Ub resulted in a similar incidence of amyloid accumulation in the spleen but in the kidneys 66.7% of animals had only traces of amyloid and 33.3%, minimal amyloid deposits. Amyloid was deposited in the mouse kidneys predominantly in the arterial walls but also occurred in the basement membrane and interstitial tissues. A post-mortem examination of the internal organs revealed splenomegaly in all the test groups and increased liver weight in the C-, C+F-, and C+Ub-treated groups. The leukocyte count and ESR (erythrocyte sedimentation rate) were also higher in all the experimental groups.

Thus, the results indicated that F and Ub play a role in the amyloid deposition process in the experimentally induced disorder in C57BL/6 mice and could enhance this pathological process.

Introduction

Amyloidosis is a general term for a group of diseases characterized by the excessive deposition of ultrastructurally identical but biochemically distinct protein fibrils either focally or multifocally in body tissue *(Hubbard et al, 2001)*. It is generally a chronic, progressive, insidious disease of unknown cause, which may not become clinically apparent until major organ dysfunction occurs because of the displacement, atrophy, or death of normal cells *(Aielle, 1998; Berkow, 1992)*.

There are at least 16 types of human diseases associated with the deposition of protein fibrils resulting in tissue damage and degeneration (Pepys, 1996). Amyloid fibrils are formed by polymerization of abnormal states of normally soluble proteins or peptides *(Kelly, 1998)*.

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Approximately 45% of all generalized amyloidoses are secondary or AA amyloidosis *(Hoshii et al, 1994; Rocken et al, 1996; Strege et al, 1998)*. It is associated with chronic inflammation and results from systemic deposition of the acute phase reactant, serum amyloid A (SAA) protein, in a fibrillar structure *(Husby et al, 1994; Hazenberg & Rijswijk, 2000)*.

Although AA amyloidosis associated with chronic inflammatory diseases is rare, it is important to keep in mind because diagnosis is often difficult, the prognosis is poor and there is no known specific effective therapy for the disease at the present time.

AA amyloidosis can potentially complicate any disorder associated with a sustained acute phase response. In the developed world, chronic rheumatic and connective tissue diseases have been supposed to be the most frequent predisposing conditions to this type of systemic amyloidosis in man, with the predominant involvement of the spleen, liver, kidneys and other parenchymal organs *(McNiff et al, 1995; Kobayashi et al, 1996; Hawkins, 2001; Ishii et al, 2003; Wakhlu et al, 2003)*.

In vivo each amyloid is composed of two classes of components *(Kisilevsky & Fraser, 1997)*: the first is a defining protein, the second is a set of common structural constituents *(Kisilevsky & Szarek, 2002)*, which include serum amyloid P, proteoglycans, usually perlecan (the basement membrane form of heparan sulphate [HS]), proteoglycan [HSPG], laminin, collagen IV, and apolipoprotein E *(Pepys et al, 1997; Kisilevsky & Fraser, 1996; Ancsin & Kisilevsky, 1999; Kisilevsky & Ancsin, 2001)*. Although the protein fibrils are the main component of the amyloid substance, the importance of other components in amyloid pathogenesis is not well established *(Westermark, 1998)*. Glycosaminoglycans, mainly in proteoglycan form, are ubiquitous ingredients of the amyloid substance but basement membrane heparan sulfate proteoglycan may be the most important and may promote protein aggregation into amyloid fibrils *(Snow et al, 1991)*.

The interactions between common components and the amyloidogenic protein play a role in amyloidogenesis *(Kisilevsky & Fraser, 1997)*.

The pathogenesis of secondary amyloidosis *in vivo* is not well understood. Much of our knowledge of the pathophysiology of this disease is derived from animal models *(Gruys & Snel, 1994)* because reactive amyloidosis is the most common form in animals *(Berkow, 1992)*.

Abnormal synthesis and degradation of SAA are the two main mechanisms underlying murine AA amyloidosis. In the mouse model, AA amyloidosis develops after approximately 25 days of inflammatory challenge. This lag phase for amyloid deposition can be shortened dramatically by administration of a small amount of amyloid extract containing an amyloid-enhancing factor (AEF) *(Gruys & Snel, 1994; Kindy et al, 1995; Johan et al, 1998, Shtrasburg et al, 2001)*. AEF, a proteolysing and ubiquitin-like factor, appears to promote AA amyloidosis during sustained high levels of circulating SAA *(Chronopoulos et al, 1991,1992; Alizadeh-Khiavi et al, 1992)*.

In the most prevalent experimental approach, amyloidosis is elicited after 2 to 3 weeks, during which susceptible mice are injected repeatedly, usually with azocasein or casein *(Gruys, Snel, 1994; Dwulet & Benson, 1987; Gervais et al, 1998; Ham et al, 1997)*.

We have developed an experimental model of AA amyloidosis that involves C57BL/6 mice with casein-induced amyloidosis and tried to enhance it by injections of dextran sulphate, fibrin, and ubiquitin.

Materials and Methods

Animals

48 adult male (20-25 g body weight) C57BL/6 mice were obtained from the Institute of Immunology (Vilnius, Lithuania) and housed 6 per cage in air-conditioned quarters with a 12 hr light-dark cycle. The animals were given standard laboratory food and water *ad libitum*. They were allowed to acclimatise 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
Experimental amyloidosis was induced by using the following inflammatory substances: vitamin-free casein (Sigma Chemical Co, Germany), dextran sulphate m.w. T 500 (Loba-Chemie, Austria), fibrin (Chemical Dynamics Corporation, USA), and ubiquitin (Sigma-Aldrich Chemie, Germany).

Induction of amyloidosis
The animals were subjected to a conventional amyloid induction protocol receiving 0.5 ml subcutaneous injections of 12% vitamin-free casein in a 0.02 N NaOH solution for 5 days a week for a period of 3 or 5 weeks. The animals were divided into four equal groups of 12 mice each. A control group (1st group) was injected with casein (C) only. In addition to C, the 2nd and 3rd groups received subcutaneous 1% dextran sulphate (DS) and 5% fibrin (F) injections respectively once a week and the 4th group, a single intraperitoneal 1 μg/ml injection of ubiquitin (Ub). All the injections were performed between 9 and 11 a.m. and had a total volume of 0.5 ml each.

The body weight of the animals was determined once a week. The animals were decapitated at 3 (half of the mice) and 5 weeks (the other half) postinjection (p.i.). The erythrocyte and leukocyte counts (made using a Picoscale, Hungary) and the ESR were determined in the blood. The internal organs were examined macroscopically and weighed while kidney and spleen samples were taken for morphological analysis. The indices obtained were compared with the indices for normal (healthy) animals.

Histopathology
The formalin-fixed spleen and kidney 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 for light microscopic examination to determine inflammation scores. The other set of slides was stained with Congo red according to Eastwood *(Eastwood & Cole, 1971)* (because acid Congo red staining in our previous investigations was superior to the classical alkaline Congo red staining with respect to the detection of AA amyloid deposits in tissue specimens) and examined in polarized light with an Olympus BX51 microscope to assess the degree of amyloid deposition in the tissue. The histological grading of the amyloid was made semiquantitavely using a scale of 0 to 3 according to the density of amyloid masses seen under the microscope, where '–' means amyloid was absent (0), '±' – traces of amyloid were observed (0.5), and '+' – minimal (1), '++' – moderate (2), and '+++' (3) heavy (abundant) amyloid deposits were present. Two histopathologists independently analysed all the specimen sections.

Statistical analysis
The results were expressed as mean values ± S.E.M. The differences between healthy animals, the control group, and the test groups were statistically analysed by Student's t test (where two means are compared). A value of $P < 0.05$ was defined as significant.

Results
Animals, Organs, and Laboratory Features
No animals were lost during the experiment. The total weight of the amyloidotic animals varied between 25 and 30 g.

The average weight of the organs at different stages of the experiment is shown in Table 1. A postmortem examination of the internal organs revealed splenomegaly ($P < 0.001$) in all the test groups and increased liver weight in the C- ($P < 0.01$), C+F- ($P

Table 1. Effect of dextran sulphate, fibrin, and ubiquitin on the body and the weight of organs in C57BL/6 mice with casein-induced experimental amyloidosis

Groups	Duration of experiment	n	Body weight	Liver		Kidneys		Spleen	
				Absolute (g)	Relative (g/kg^{-1})	Absolute (g)	Relative (g/kg^{-1})	Absolute (g)	Relative (g/kg^{-1})
1st (C)	3 weeks	6	21.67±1.05*	1.43±0.08	6.70±0.50	0.32±0.017	***1.47±0.05	0.62±0.03***	*2.87±0.16***
	5 weeks	6	28.33±1.05	*2.05±0.09**	7.25±0.21*	0.35±0.02	1.23±0.05	*0.68±0.04***	2.42±0.14***
2nd (C+DS)	3 weeks	6	24.0±1.00	1.46±0.13	6.08±0.46	0.34±0.04	1.42±0.15	0.56±0.05***	*2.32±0.15***
	5 weeks	6	25.0±1.82	*1.7±0.12	6.82±0.22*	0.32±0.01	1.30±0.4	*0.50±0.06***	2.06±0.27***
3rd (C+F)	3 weeks	6	21.67±2.11	1.63±0.10	7.72±0.51^{+}	0.30±0.026	1.39±0.05	0.57±0.02***	2.71±0.20***
	5 weeks	6	27.5±1.12	1.98±0.04**	7.26±0.25**	0.32±0.01	1.16±0.05	0.83±0.07***	3.10±0.38***
4th (C+Ub)	3 weeks	6	25.0±1.82	1.53±0.08	6.25±0.41	0.28±0.03	***1.12±0.06	0.55±0.13***	2.27±0.23***
	5 weeks	6	28.33±1.05	1.98±0.12*	7.06±0.50	0.32±0.01	1.12±0.06	0.63±0.04***	2.27±0.21***
Healthy mice		5	26.67±1.67	1.57±0.09	5.89±0.25	0.37±0.03	1.38±0.12	0.10±0	0.376±0.02

Note: C – casein, DS – dextran sulphate, F – fibrin, Ub – ubiquitin. Symbols on the right - The differences are significant between normal mice and the test groups. Symbols on the left - The differences are significant between the C-treated group and the other test groups after 3 and 5 weeks post-injection respectively. * – $P < 0.05$; ** – $P < 0.01$; + – $P < 0.02$; *** – $P < 0.001$

< 0.01), and C+Ub-treated (P < 0.05) groups after 5 of weeks stimulation in comparison with healthy animals. The relative weight of the liver markedly increased in the 1st (C) and the 2nd (C+DS) groups (P < 0.05) whereas in the 3rd (C+F) group it was significantly higher at both 3 (P < 0.02) and 5 (P < 0.01) weeks p.i. At the end of the experiment the absolute weight of the liver and spleen and the relative weight of the spleen at 3 weeks p.i. in the 2nd group significantly differed from the control C-treated group (P < 0.05). The relative weight of the kidneys was the lowest in the 4th group receiving C+Ub and also significantly differed from the control group (P < 0.001).

The changes in the blood indices are shown in Table 2. After 3 weeks of stimulation, the highest ESR was observed in the 3rd (C+F-treated) group which significantly differed from the 1st (C-treated) group.

Table 2. Effect of dextran sulphate, fibrin, and ubiquitin on the blood indices of C57BL/6 mice in casein-induced experimental amyloidosis

Groups	Duration of experiment	n	ESR (mm/h)	Leukocytes (10^9 L)	Erythrocytes (10^{12} L)
1st (C)	3 weeks	6	$^{+}$4.00±0.45***	14.50±1.05***	4.44±0.10***
	5 weeks	6	$^{+}$*2.67±0.21**	15.83±2.35**	4.86±0.15***
2nd (C+DS)	3 weeks	6	5.20±0.49***	17.60±2.31^{++}	4.65±0.16***
	5 weeks	6	5.60±1.43^{+}	19.40±3.29**	4.42±0.26***
3rd (C+F)	3 weeks	6	$^{+}$6.00±0.45***	18.47±1.52***	4.75±0.23***
	5 weeks	6	$^{+}$3.83±0.31***	18.92±1.92***	4.69±0.22***
4th (C+Ub)	3 weeks	6	5.00±0.45***	19.30±3.59**	4.50±0.24***
	5 weeks	6	*3.67±0.33***	18.57±3.34**	4.86±0.23***
Healthy mice		5	1.33±0.33	6.40±0.15	6.74±0.29

Note: C – casein, DS – dextran sulphate, F – fibrin, Ub – ubiquitin. Symbols on the right – The differences are significant between normal mice and the test groups. Symbols on the left – The differences are significant between the C-treated group and the other test groups after 3 and 5 weeks post-injection respectively. * – $P < 0.05$; ** – $P < 0.01$; + – $P < 0.02$; ++ - $P < 0.002$; *** – $P < 0.001$

This difference between the groups also continued until the end of the experiment (5 weeks p.i.) (P < 0.02). The same was found between the 1st (C) and the 4th (C+Ub) groups (P < 0.05).

Thus, the use of other inflammatory substances along with C impaired such blood indices as the ESR and the leukocytes count.

Histological examination

The amount of amyloid deposited increased progressively with the duration of amyloidogenic stimulus. After 3 weeks of stimulation, amyloid could be detected in the spleen and kidneys (Tables 3, and 4). Mice injected with C had only rare (1 of the 6) minimal (1+) perifollicular amyloid deposits in the marginal zone of the spleen follicles (16.7%) and traces of amyloid in the kidneys (6 of 6 or 100%).

The use of other inflammatory substances enhanced the development of the pathological process (Tables 3 and 4). Traces and minimal deposits of amyloid were observed in the spleen in 33.3% of the mice treated with C+DS and C+Ub and in 83.3% of the animals treated with C+F (at 3 weeks p.i.). 50% of the mice in the last two groups also had traces of amyloid deposits in the kidneys. In the other test groups the incidence of kidney amyloidosis was far less. Traces of amyloid deposits were observed in the C+DS (83.3% of the animals) and C+Ub groups (66.6%) but in the latter, 16.7% of the mice also had minimal amyloid deposits.

At the end of the experiment (5 weeks p.i.) 100% of the animals of the 1st (C) group were observed to have multinuclear phagocytes (Table 3) and areas of eosinophylic connective tissue around the follicles (Fig. 1, A), which covered 1/3-1/4 of the spleen. The most extensive amyloid deposits in this organ were also seen at 5 weeks p.i. Amyloid deposition, identified by green birefrigence following Congo red staining, was revealed in 100% of the mice treated with C: moderate amyloid deposits were identified in 83.3% of the mice and heavy deposits in 16.7%. 33.3% of the animals had focus glomerulonephritis and tubular edema, 66.7% had diffuse glomerulonephritis, and 50% tubular fibrinoid necrosis.

Table 3. Effect of dextran sulphate, fibrin, and ubiquitin on the pathomorphological changes in the spleen and kidneys of C57BL/6 mice with casein-induced amyloidosis

Groups	Duration of experiment (weeks)	Spleen				Kidneys										
		Haematoxylin-eozin				Congo red		Haematoxylin-eozin								Congo red
		Areas of connective tissue		Multinuclear phagocytes		Amyloid deposits (perifollicularly)		Minimal glomerulonephritis		Glomerulonephritis		Tubular edema		Fibrinoid necrosis		Amyloid deposits
		n/n	%	n/n	%	n/n	%	n/n	%	n/n	%	n/n	%	n/n	%	n/n	%
1st (C)	3	1/6	16.7	1/6	16.7	1/6	16.7	5/6	83.3	4/6	-	2/6	33.3	-	-	6/6	100
	5	6/6	100	-	-	6/6	100	2/6	33.3	4/6	66.7	2/6	33.3	3/6	50	6/6	100
2nd (C+DS)	3	-	-	-	-	2/6	33.3	1/6	16.7	-	-	5/6	83.3	-	-	5/6	83.3
	5	3/6	50	2/6	33.3	6/6	100	-	-	6/6	100	5/6	83.3	-	-	6/6	100
3rd (C+F)	3	-	-	2/6	33.3	5/6	83.3	3/6	50	3/6	50	1/6	16.7	2/6	33.3	6/6	100
	5	6/6	100	4/6	66.7	6/6	100	-	-	6/6	100	6/6	100	-	-	6/6	100
4th (C+Ub)	3	1/6	16.7	2/6	33.3	2/6	33.3	2/6	33.3	-	-	4/6	66.7	-	-	4/6	66.7
	5	6/6	100	-	-	5/6	83.3	-	-	5/6	83.3	1/6	16.7	3/6	50	6/6	100

Table 3 (continued) — Kidneys, Congo red, Locality of amyloid deposits

Groups	Duration of experiment (weeks)	Arterial walls		Tubular basement membrane		Interstitial tissues	
		n/n	%	n/n	%	n/n	%
1st (C)	3	6/6	100	-	-	-	-
	5	2/6	33.3	-	-	4/6	66.7
2nd (C+DS)	3	4/6	66.7	2/6	33.3	-	-
	5	4/6	66.7	-	-	2/6	33.3
3rd (C+F)	3	6/6	100	-	-	2/6	33.3
	5	4/6	66.7	-	-	6/6	100
4th (C+Ub)	3	4/6	66.7	-	-	2/6	33.3
	5	3/6	50	-	-	4/6	66.7

Note: C – casein, DS – dextran sulphate, F – fibrin, Ub – ubiquitin. n/n – number of animals with changes in organs / total number of animals investigated. % - percentage of animals with changes in the spleen and kidneys.
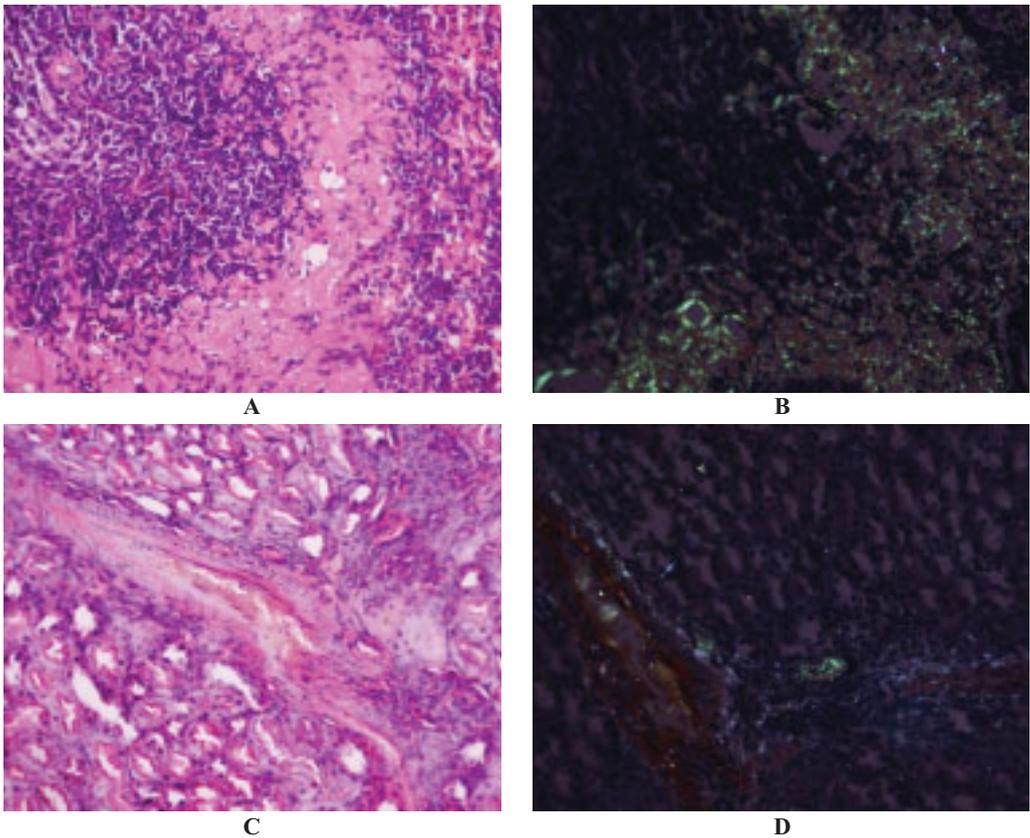
Figure 1. C57BL/6 mouse with casein-induced amyloidosis (5 weeks p.i.). Areas of eosinophylic connective tissue in the marginal zone of the spleen follicles (**A**, haematoxylin-eosin, x200). Heavy amyloid deposits in spleen (**B**) and minimal deposits in the kidney tubular basement membrane (**D**, stained with Congo red acid solution, x200). Kidney tubular edema (**C**, haematoxylin-eosin, x200).

Figure 1 shows areas of eosinophylic connective tissue in the marginal zone of the follicles (A), heavy amyloid deposits in the spleen (B), tubular edema (C), and minimal amyloid deposits in kidney tubular basement membrane (D) in the mice treated with C. The same line of events is observed in Figures 2. There we can see moderate (Fig. 2, A and B), and heavy (Fig. 2, C) amyloid deposits in the spleen under the treatment with C+DS (A), C+F (B), and C+Ub (C) and minimal amyloid deposits in the kidney (Fig. 2, D) when using C+F.

Eosinophylic connective tissue areas and multinuclear phagocytes in the spleen were found in 50%

and 33.3% respectively of the animals treated with C+DS (2nd group). DS intensified the pathological process in the kidneys, where glomerulonephritis was found in 100% of the animals and tubular edema in 83.3% (Table 3).

Although amyloid was found in 100% of the mice, its deposition was lower: 16.7% of the animals had traces of amyloid, 16.7% minimal deposits, and 66.6% moderate deposits (Table 4). Amyloid was identified in blood vessel walls (66.7%) and interstitial tissues (33.3%).

100% of the mice treated with C+F (3rd group) had eosinophylic connective tissue areas and 66.7%
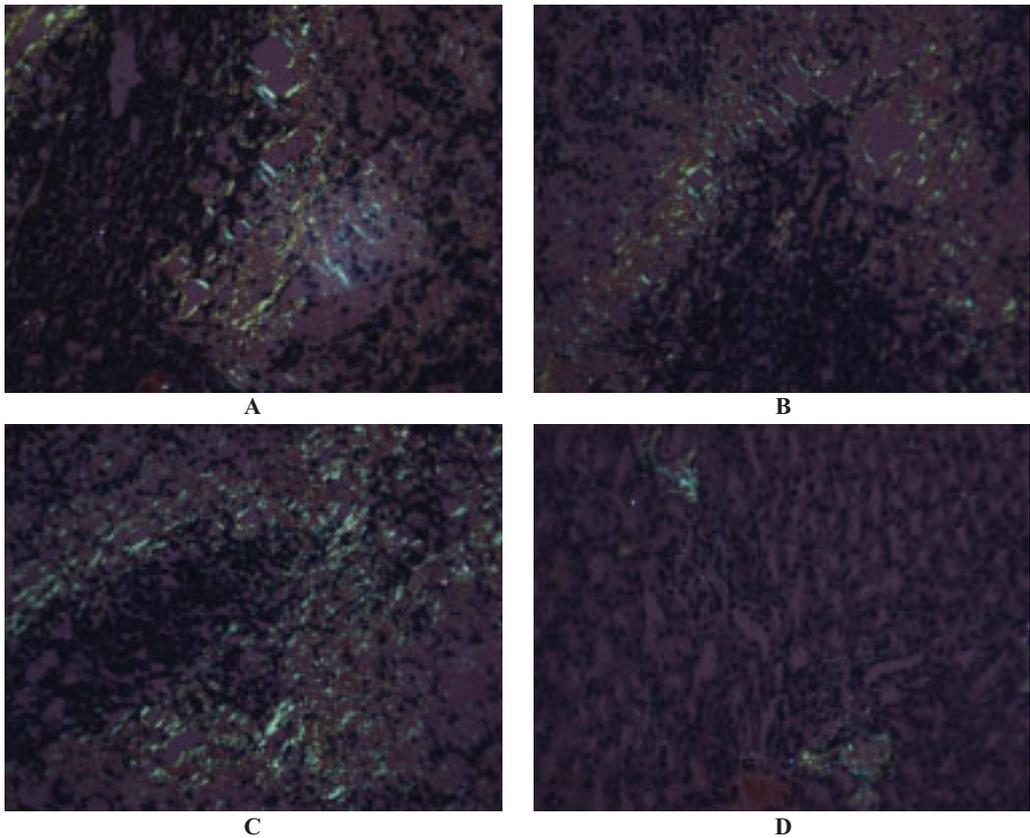
Figure 2. C57BL/6 mouse with casein and dextran sulphate- (**A**), casein and fibrin- (**B**, **D**) and casein and ubiquitin-induced (**C**) amyloidosis (5 weeks p.i.). Moderate (**A**, **B**) and heavy (**C**) amyloid deposits in the spleen (**A** and **B**, stained with Congo red acid solution, x200) and minimal amyloid deposits in the kidney (**D**, stained with Congo red acid solution, x200).

multinuclear phagocytes. Markedly increased amyloid deposition was observed in these animals: moderate (2+) (50% of animals) and heavy (3+) (50%) deposits throughout the spleens were revealed in all the animals (6 of 6) of this group. 100% of the mice showed predominantly chronic renal lesions with glomerulonephritis and kidney tubular edema. 33.3% also had tubular fibrinoid necrosis. Amyloid was deposited in the mouse kidneys predominantly in the arterial walls (66.7% of the animals) but also occurred in the basement membrane and interstitial tissues (100%) (Table 3). Only 16.7% of the mice had traces of amyloid,

66.6% had minimal deposits, and 16.7% moderate deposits (Table 4). Average amyloid deposition was significantly higher than in the control (C-treated) group (P < 0.02) (Table 5).

C+Ub resulted in a similar incidence of amyloid accumulation in all the spleens (6 of 6) but in the kidneys, 66.6% of the animals had only traces of amyloid and 33.3% minimal deposits. Amyloid was identified in the blood vessel walls (50%) and interstitial tissues (66.7%). Glomerulonephritis, tubular edema, and tubular fibrinoid necrosis were found in 100%, 16.7% and 50% of the animals respectively. Summarizing the results obtained, we can ascertain

Table 4. Effect of dextran sulphate, fibrin and ubiquitin on amyloid deposition in the spleen and kidneys of C57BL/6 mice with casein-induced amyloidosis

Groups	Duration of experiment (weeks)	Spleen (amyloid deposits)								Kidneys (amyloid deposits)							
		Traces (±)		Minimal (+)		Moderate (++)		Heavy (+++)		Traces (±)		Minimal (+)		Moderate (++)		Heavy (+++)	
		n/n	%	n/n	%	n/n	%	n/n	%	n/n	%	n/n	%	n/n	%	n/n	%
1st (C)	3	-	-	1/6	16.7	-	-	-	-	6/6	100	-	-	-	-	-	-
	5	-	-	-	-	5/6	83.3	1/6	16.7	6/6	100	-	-	-	-	-	-
2nd (C+DS)	3	1/6	16.7	1/6	16.7	-	-	-	-	5/6	83.3	-	-	-	-	-	-
	5	1/6	16.7	1/6	16.7	4/6	66.6	-	-	4/6	66.7	2/6	33.3	-	-	-	-
3rd (C+F)	3	3/6	50	2/6	33.3	-	-	-	-	3/6	50	3/6	50	-	-	-	-
	5	-	-	-	-	3/6	50	3/6	50	1/6	16.7	4/6	66.6	1/6	16.7	-	-
4th (C+Ub)	3	1/6	16.7	1/6	16.7	-	-	-	-	4/6	66.6	1/6	16.7	-	-	-	-
	5	-	-	-	-	3/6	50	3/6	50	4/6	66.7	2/6	33.3	-	-	-	-

Note: C – casein, DS – dextran sulphate, F – fibrin, Ub – ubiquitin. n/n – number of animals with amyloid deposits / total number of animals investigated. % - percentage of animals with amyloid deposits.

that the usage of F and Ub in addition to C to some degree enhances the formation of amyloid. Also, the higher pathology of the spleen and kidneys under treatment with F possibly reflects the formation of amyloid in these organs.

Discussion

Protein destruction, like protein synthesis, is a fundamental mechanism by which organisms control diverse cellular functions *(Hershko & Ciechanover, 1998; Hochstrasser, 1996; Varshavsky, 1997)* and the stability (and hence abundance) of critical regulatory proteins in the cell is often dynamically controlled in response to external or internal stimuli *(Tyers & Willems, 1999)*.

Amyloidosis is a protein metabolism disorder. Proteins that are normally soluble are transformed into insoluble fibrillar structures, which are deposited in the extracellular space of organs and tissues, thereby resulting in clinical disease *(Xing & Higuchi, 2002)*.

As a model of amyloidosis, we have selected the casein-induced amyloidosis in C57BL/6 mice, which are susceptible to this pathological process, and tried to enhance it by using DS, F, and Ub. The data presented here showed that all the inflammatory substances caused an increase in spleen and liver weight and made the blood indices worse. During this period the ESR was found to be significantly elevated.

It was shown that F and Ub appeared to play a role in the process of amyloid deposition in the experimentally induced disorder in the animals. Histopathological examination of the spleen and kidney tissues of mice killed at different times after the termination of the amyloidogenic stimulus showed increased amyloid deposition in these animals compared to the controls.

Examination of the organs was done at 3 and 5 weeks p.i., because it was known that in C57BL/6 mice, which have a high level of AEF, amyloidosis developed after 15-21 injections of casein *(Hebert et al, 1990)*. The formation of AA amyloid fibril deposits is not well-understood but in the murine

Table 5. Effect of dextran sulphate, fibrin, and ubiquitin on average amyloid induction in the spleen and kidneys of C57BL/6 mice with casein-induced amyloidosis

Groups	Duration of experiment	Amyloidotic animals/total		Amyloid deposit average	
		Spleen (n/n)	Kidneys (n/n)	Spleen	Kidneys
1st (C)	3 weeks	1/6	6/6	0.17±0.17	*0.5±0
	5 weeks	6/6	6/6	2.17±0.16***	+0.5±0
2nd (C+DS)	3 weeks	2/6	5/6	0.25±0.17	0.42±0.08
	5 weeks	6/6	6/6	1.58±0.27++	0.67±0.10
3rd (C+F)	3 weeks	5/6	6/6	0.58±0.15	*0.75±0.11
	5 weeks	6/6	6/6	2.5±0.22***	+1.08±0.20
4th (C+Ub)	3 weeks	2/6	6/6	0.25±0.17	0.67±0.10
	5 weeks	5/6	6/6	2.0±0.45**	0.67±0.10

Note: C – casein, DS – dextran sulphate, F – fibrin, Ub – ubiquitin. n/n – number of animals with amyloid deposits / total number of animals investigated. Symbols on the right – The differences are significant between the average induction of amyloid deposition in the groups after 3 weeks post-injection (p.i.) and the average induction of amyloid deposition after 5 weeks p.i.. Symbols on the left – The differences are significant between the C-treated group and other test groups after 3 and 5 weeks post-injection respectively. * – $P < 0.05$; ** – $P < 0.01$; + – $P < 0.02$; ++ - $P < 0.002$; *** – $P < 0.001$

model of amyloidosis, deposits increase and are variable in respect to their extent and localization. The highest AA deposits are around the spleen lymphoid follicles *(Huchinson et al, 2001)*, which our data also show. There is a greater increase in deposition when F and Ub are used together with casein. After 3 weeks of stimulation, however, minimal perifollicular amyloid had developed, representing early-phase amyloidosis. After 5 weeks, moderate and heavy amyloid deposits were found in a developed phase of amyloidosis created by using casein injections as the background for F and Ub. Extensive AA amyloidosis was observed in 100% of the spleens whereas only minimal AA deposition was found in the kidneys. But although the amyloid deposits were the highest in the spleens of the animals treated with C+F, a significant increase in amyloid content, compared to the controls (casein-treated animals), was detected in the kidneys after the end of the administration of the inflammatory substances.

It is known that amyloidogenic stimulation in casein-induced amyloidosis enhances the synthesis of proteoglycans, which are related to murine spleen reactive AA amyloid. This increase of proteoglycan expression precedes amyloid fibril formation *(Stenstad et al, 1994)*. The authors suggest that free glycosaminoglycans may be a specific feature of amyloidosis and that different proteoglycans (heparan sulphate proteoglycan, chondroitinsulphate proteoglycan, and dermatansulphate chains) and glycosaminoglycans play a role in the formation and stabilization of amyloid fibrils *in vivo*. Since Ub demonstrates AEF activity *in vivo* and binds non-covalently to AA amyloid, some authors *(Chronopulos et al, 1991; Alizadeh-Khiavi et al, 1992)* suggest that Ub may indeed be 'fibril-AEF' and may play a crucial role in the pathogenesis of amyloidosis.

Fibrin split products and fibrinopeptides are the factors which enhance the permeability of the blood vessels and chemotaxis of the polymorphonuclear cells. Our findings showed that the use of F and Ub during casein-induced amyloidosis enhanced amyloid deposition in the kidneys, although more distinct intensification was observed through the use of F.

In addition, glomerulonephritis, when the coagulation system is activated and F deposition occurs,

developed in all the animals receiving the additional inflammatory substances. The inflammatory process damages the glomerulus, resulting in the thickening of the glomerular basement membrane, cellular proliferation within the mesangium, hyalinization, sclerosis, and glomerular death *(Grauer, 2002)*.

Mountz and Hsu *(1997)* speculate that the abundance of basement membrane glycosaminoglycan in the glomeruli is a main factor for renal AA deposition since this component is important in forming the typical ß-sheet when encountering AA fibrils *(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)*.

Injections of casein as a background for DS did not intensify the formation of amyloid deposits in the mouse spleens. At the end of the experiment they were even higher in the group receiving only casein injections, where moderate amyloid deposits were found in 83.3% of the mice and heavy deposits in 16.7% (in the C+DS group, 66.6% of the mice had moderate deposits). The changes in the kidneys were slight in both these groups but there were some large changes in the mice receiving C and DS injections, where traces of amyloid were found in 66.7% and minimal deposits in 33.3%.

DS belongs to the class of compounds known as polyanions. It is closely analogous to the group of sulphate polysaccharides designated as mucopolysaccharides, which are abundant in connective tissue and the skin. There are many reports in the literature describing the antiviral effects of DS. It seems to be optimal for inhibiting *herpes simplex virus, human cytomegalo virus, and vesicular stomatitis virus* as well as protecting T-lymphocytes from HIV *in vitro (Mitsuya et al, 1988)*. DS blocks the binding of virions to lymphocyte membranes.

Extensive studies on the chronic toxicity of DS have revealed undesirable long-term effects from long-term infusions, including hair loss, painful joints, and brittle bones.

In 1984, B. Ehlers and H. Diringer from the Robert Koch Institute in Berlin showed that DS could prolong the incubation period for scrapie in mice infected with the disease *(Ehlers & Diringer, 1984)*. According to the authors' data, 1 mg of DS 500 administered intraperitoneally as a single dose before the infection effectively delays and sometimes even prevents clinical scrapie in mice *(Ehlers & Diringer, 1984)*. Because DS is present in mononuclear phagocytes *in vivo* for more than 7 months after administration, the authors thought that it directly impairs early agent replication in the mononuclear phagocytes of the spleen and lymph nodes *(Ehlers et al, 1984)*. Following intraperitoneal administration of DS, a peripheral blood leukocyte population rich in macrophages is stimulated in mice *(Colditz, 1988; Golemboski et al, 1990)*.

In conclusion, our studies demonstrated that AA amyloid deposition in the spleen and kidneys of mice increased progressively with the duration of the amyloidogenic stimulus. Besides the casein which may be capable of forming amyloid fibrils, other inflammatory substances such as F and Ub can enhance its deposition in C57BL/6 mice. But future studies of amyloidogenesis, especially the mechanism of amyloid fibril formation, are important.

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