EXPERIMENTAL SYSTEMIC AMYLOIDOSIS INDUCED BY IMMUNIZATION WITH SYNGENEIC ORGAN EXTRACTS IN MICE

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The exact pathogenesis of amyloidosis is not yet understood, and a number of attempts have been made to produce amyloidosis in animals. Casein was the first substance found capable of inducing amyloidosis in animals (1), and subsequently several other animal models have been developed (2–7). In the induction of amyloidosis in these animal models, certain agents have been investigated as accelerating factors for deposition of amyloid (8–11), or as blocking agents (12). More recently, reactions of cellular immunity involving T, B, and other immunological cells have been evaluated in the mechanism of amyloid deposition in animals (13–19). Transfer studies have also been reported using a model of experimental amyloidosis in animals (20, 21).

In these studies, exogenous immunogens were used; syngeneic tissue substances have not as yet been used. We have been able to produce systemic amyloidosis in mice by immunizing with syngeneic organ (liver or kidney) extracts and CFA. This model will throw some light on the pathogenesis of amyloidosis.

Materials and Methods

Induction of Systemic Amyloidosis. 6-wk-old male mice of strains C57BL/6 (B6, H-2^b), BALB/c (H-2^d), and C3H/He (H-2^k) were used. Fresh liver or kidney of each strain was homogenized and a 100,000 *g* supernatant was used as an immunogen. Immunization was performed within 24 h of preparation. 20 male mice of each strain were intramuscularly immunized six times at weekly intervals with 0.1 ml of the supernatant containing 10 mg of protein and an equal volume of CFA. As the control, another 20 mice of each strain were given syngeneic mouse sera (10 mg of protein) and CFA, or CFA alone, six times at weekly intervals. Another 20 mice of each strain were given the liver or kidney extract alone every second day for 6 wk, and histological examination was performed 7 d after the last injection.

Histological Examination. At 7 d, 14 d, 1 mo, 2 mo, and 3 mo after the final injection, animals were killed, and specimens of the liver, kidney, spleen, brain, heart, stomach, and intestine were taken for histological examination. These specimens were checked for deposition of amyloid by hematoxylin and eosin staining, Congo-red birefringence and electron microscopy. The grade of amyloid deposit was scored as follows. In liver: −, negative; ±, mild amyloid deposit; +, quantity of amyloid deposit in the hepatocyte is <50% of the average size of the hepatocyte in section; ++, quantity of amyloid deposit is between 50% and 100% of the size of hepatocytes in section; +++, quantity of amyloid deposit is about equal to the size of hepatocytes; and ++++, quantity of amyloid deposit

J. EXP. MED. © The Rockefeller University Press · 0022-1007/86/06/1553/13 $1.00 1553
Volume 163 June 1986 1553–1565

is larger than the average size of hepatocytes. In kidney: −, negative; ±, mild amyloid deposit; +, <10% of glomeruli containing amyloid deposit; ++, 10–50% of glomeruli containing amyloid; +++, 50% of glomeruli containing amyloid, some having marked amyloid deposition; ++++, all of glomeruli having marked amyloid deposition. The grade of amyloid deposition in the spleen was judged in the same way; it was graded by the number (area on a section) of affected spleen cells and the amount of amyloid deposit.

Characterization of Amyloid Deposit. Amyloid deposit in each organ obtained from amyloidotic B6 mice was checked using an anti-mouse amyloid protein (AA)[1] antibody. A piece of tissue was removed from the liver, kidney, spleen, and other organs of B6 mice immunized with liver extracts and CFA six times at weekly intervals. These fresh, frozen organ tissues were checked by indirect immunofluorescence using monoclonal rat IgG culture supernatant antibody against mouse AA and anti-rat IgG goat IgG conjugated with FITC. As a control, anti-rat IgG goat IgG conjugated with FITC was used on each specimen by the direct immunofluorescence study. Monoclonal anti-mouse AA rat IgG culture supernatant was kindly provided by Prof. Shunsuke Migita, Cancer Research Institute, Kanazawa University, Kanazawa. AA was purified from B6 mice immunized with syngeneic liver extract and CFA six times at weekly intervals by the method of Pras et al. (22) and Isersky et al. (23). Native amyloid fibril was prepared from amyloidotic mice by the combination of homogenization and centrifugation. These materials were denatured with 6 M guanidine-HCl and used as denatured amyloid protein (DAP). Antigenic reactivity of DAP derived from B6 mice immunized with liver extracts and CFA was checked with monoclonal anti-mouse AA antibody by the immunodiffusion method.

Fractionation of Sensitized and Nonsensitized Spleen Cells from Donor Mice and Their Transfer. Immediately after killing, spleen cells of donor mice, in which systemic amyloidosis had been induced by injecting liver extracts and CFA, or of control animals were separated using a stainless steel mesh (No. 200, Abe Science Co., Chiba, Japan). A portion of them was passed through a nylon-wool column and separated into nylon-wool column nonadherent (T-enriched) cells and nylon-wool column adherent cells, using RPMI 1640 (Gibco Laboratories, Grand Island, NY) with 10% FCS calf serum (Gibco Laboratories). After separation, these spleen cells were resuspended in HBSS. $1–5 \times 10^7$ cells of each fraction of sensitized and control spleen cells were injected into the tail veins of untreated recipient mice. To assess the quantities of amyloid deposit in the organs of recipient mice, histological examination of the liver, kidney, and spleen was carried out by killing them at 48 h, 7 d, 14 d, 1 mo, 2 mo, and 3 mo after the injection.

Treatment of Sensitized Spleen Cells with Anti-Thy-1,2; Anti-Ly-1; and Anti-Ly-2 Antibody. Nylon-wool column adherent spleen cells obtained from donor mice were treated with anti-Thy-1,2 mAb (clone F7D5; Olac 1976 Ltd., Blackthon Bicester Oxon, England) and Low-Tox-M rabbit complement (Olac). $1–5 \times 10^7$ of the sensitized nylon-wool adherent cells were treated with anti-Thy-1,2 antibody, placed on ice for 45 min, and washed three times with HBSS. The cells were then treated with rabbit complement for 45 min at 37°C. After washing three times, $1–5 \times 10^7$ of treated cells were injected into 20 normal B6 recipient mice. As a control, another set of sensitized nylon-wool adherent cells was treated with either anti-Thy-1,2 antibody or rabbit complement alone, and injected into 20 normal B6 mice. These animals were killed 2 mo after injection for histological examination. Furthermore, $1–5 \times 10^7$ adherent cells of donor mice were treated by the combination of anti-mouse Ly-1 or Ly-2 rat γ-globulin (Fab fraction), anti-rat mouse γ-globulin (MAR), and rabbit complement. These cells were transferred to 20 normal recipient mice, and histological examination was performed 2 mo after the injection. These anti-Ly-1, anti-Ly-2, and MAR were kindly provided by Dr. Takemori, Department of Immunology, Institute of Environmental Health, School of Medicine, Chiba University, Chiba.

Pathogenetic Investigation of Liver Extract with Various Treatments. Liver extract of B6 mice was treated by various methods, then the treated immunogen was checked for its ability to induce systemic amyloidosis. Each of these immunogens containing 10 mg of

[1] *Abbreviations used in this paper:* AA, amyloid protein; DAP, denatured amyloid protein.

TABLE I

Induction of Systemic Amyloidosis in Mice Immunized with Liver Extract and CFA

Exp.	Pathogen	Number examined	Histological findings*					
			–	±	+	++	+++	++++
1	Liver extract‡ + CFA:							
	BALB/c	20	0	4	5	6	5	—
	C3H/He	20	0	2	8	6	4	—
	C57BL/6	20	0	0	0	4	11	5
2	Normal serum from mice of each strain‡ + CFA:							
	BALB/c	20	13	5	2	—	—	—
	C3H/He	20	11	5	3	1	—	—
	C57BL/6	20	12	4	2	2	—	—
3	FCA alone:							
	BALB/c	20	20	—	—	—	—	—
	C3H/H	20	14	5	1	—	—	—
	C57BL/6	20	19	1	—	—	—	—

* Grade of amyloid deposit in liver was determined as described in Materials and Methods.
‡ 10 mg protein/mouse.

protein was injected with CFA six times at weekly intervals, and histology was examined 7 d after the final injection. The grade of amyloidosis in each group was determined by the quantities of amyloid deposited in the liver.

The liver extract was separated into a microsomal fraction and a supernatant after 100,000 *g* centrifugation for 60 min, and an amount of each fraction that contained 10 mg of protein was injected with CFA into normal B6 mice six times at weekly intervals, and histological changes were studied 7 d after the last injection. Since this supernatant had a greater capability of inducing systemic amyloidosis than the microsomal fraction, further study was carried out using 100,000 *g* supernatant. The supernatant was treated either by freezing and thawing three times, then was lyophilized or digested by 10% trypsin (from bovine pancreas, Sigma Chemical Co., St. Louis, MO) at 37°C for 30 min. After these treatments the immunogen or the treated supernatant was intramuscularly injected with CFA six times, and histological examination was done 7 d after the last injection.

Results

Systemic Amyloidosis Induced by Syngeneic Organ Extracts. Table I shows histological findings regarding amyloid deposition in the liver of mice injected with 100,000 *g* supernatant of syngeneic liver extract and CFA. The grade of amyloidosis was determined by the amount of deposit in the liver. C57BL/6 (B6) mice injected with liver extract and CFA revealed the most prominent amyloid deposition in their organs, followed by BALB/c and C3H/He mice. Fig. 1 *a* shows liver histology in a B6 mouse injected with liver extract and CFA, using marked deposition of amyloid in the lobule. The quantity of amyloid deposition was larger than the average size of hepatocytes in section. Fig. 1 *b* shows birefringence of liver sections stained with Congo red in a B6 injected once with liver extract. An electron micrograph of the liver with amyloid deposition as shown in Fig. 2, clearly shows amyloid fibrils. Fig. 3 shows deposition of amyloid in the spleen of mice injected with liver extract. As controls, normal serum of each

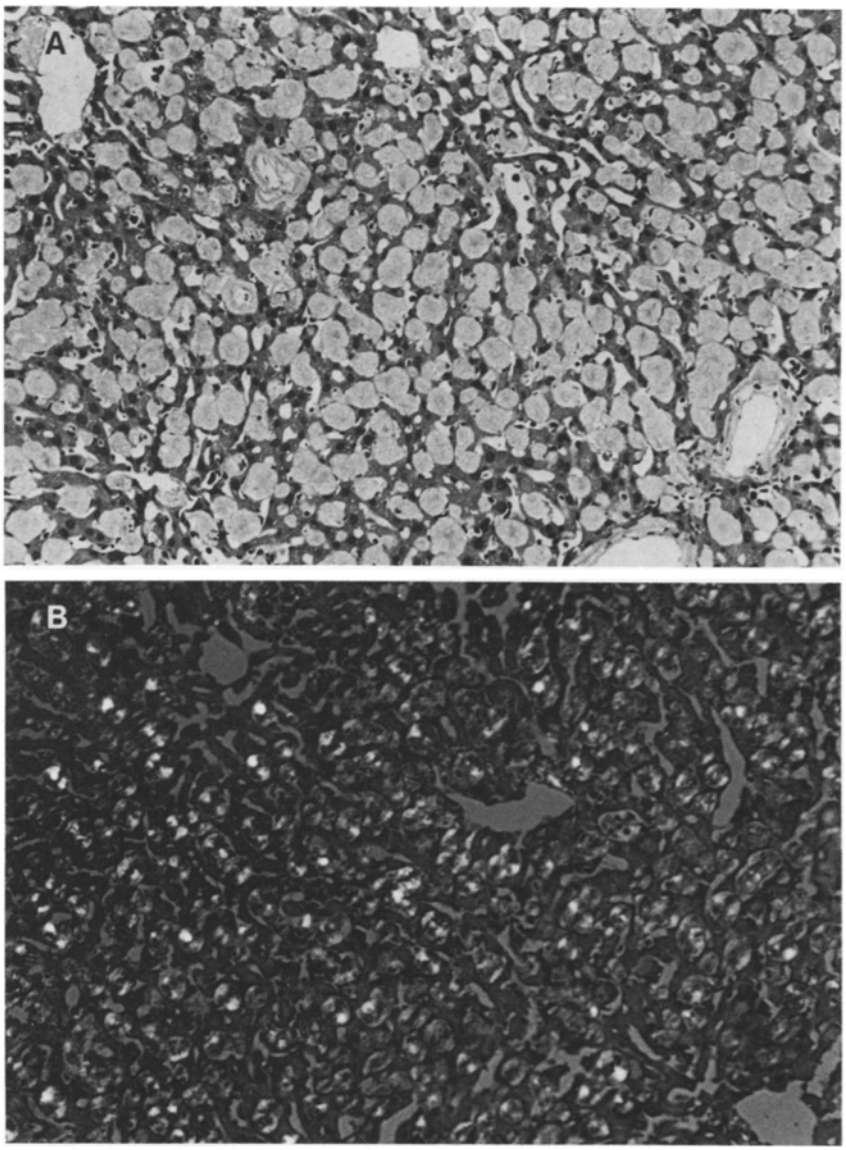

FIGURE 1. (*A*) Liver of C57BL/6 (B6) mouse immunized six times with a liver extract and CFA (H & E, × 250). Marked amyloid deposition is seen within the liver lobule. (*B*) Liver of B6 mouse immunized with liver extract and CFA (Congo-red birefringence, × 250).

strain with CFA or CFA alone was injected in the same way, and histology was examined 7 d after the final injection. In the organs of control animals, amyloid deposition was negative or minimal compared with those injected with liver extract mixed with CFA.

A 100,000 *g* supernatant of B6 kidney extract mixed with CFA was also injected to 20 B6 mice in exactly the same way and histological examination was made. As shown in Table II, deposition of amyloid in the liver of B6 mice injected with a liver extract was more prominent compared with the liver of

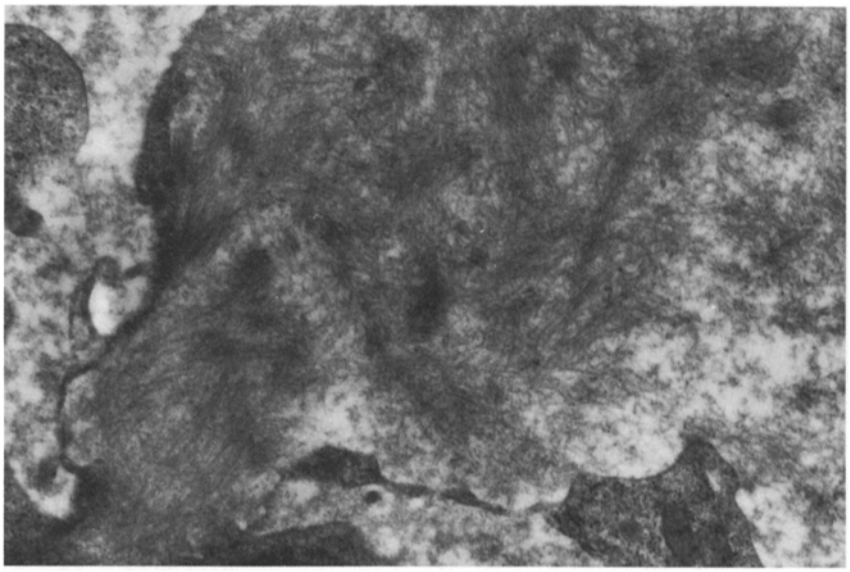

FIGURE 2. Electron micrograph of the liver of C57BL/6 (B6) mouse showing amyloid fibrils (× 40,000).

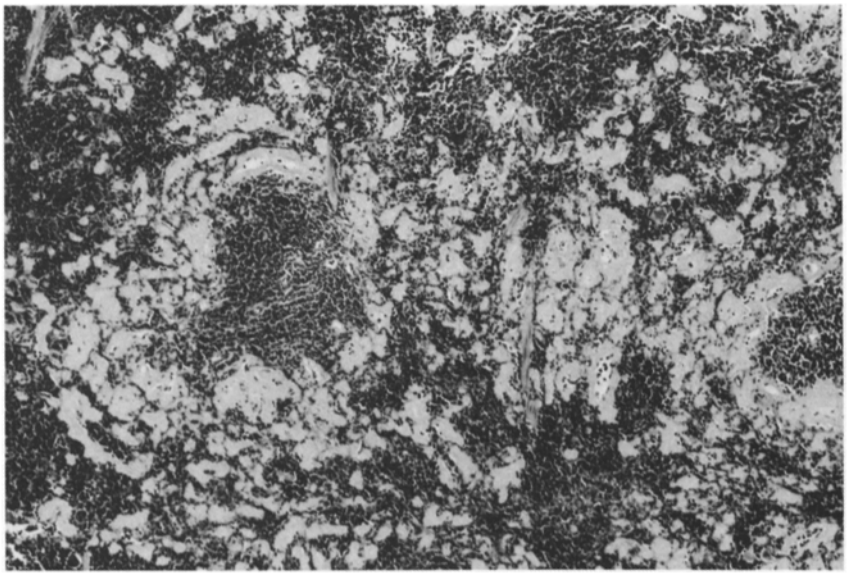

FIGURE 3. Spleen of C57BL/6 (B6) mouse immunized six times with a liver extract and CFA (H & E, × 250). Marked amyloid deposition is seen.

mice given a kidney extract and CFA. Similarly, deposition of amyloid in the kidney of mice injected with kidney extract was more marked compared with mice injected with a liver extract and CFA. This seems to suggest partial organ specificity in the production of amyloidosis in the liver and kidney. Fig. 4*a* shows a hematoxylin-eosin stain of the kidney of B6 mice given a 100,000 *g* supernatant of the kidney extract and CFA six times. Prominent amyloid deposition is seen

TABLE II

Induction of Systemic Amyloidosis in C57BL/6 Mice Injected with Syngeneic Liver or Kidney Extract and CFA

Exp.	Immunogen	Histology of:	Number examined	Histological findings*			
				+	++	+++	++++
1	Liver extract‡ + CFA	Liver	20	—	5	12	3
		Kidney	20	5	10	5	—
		Spleen	20	—	12	8	—
2	Kidney extract‡ + CFA	Liver	20	1	14	5	—
		Kidney	20	6	2	6	6
		Spleen	20	5	14	1	—

* Grade of amyloid deposit was determined as described in Materials and Methods.
‡ 10 mg protein/mouse.

particularly in all glomeruli of the immunized mice. Fig. 4*b* shows birefringence of Congo-red stain in the kidney. The liver or kidney extract alone was capable of inducing amyloidosis without CFA, although the deposition was much less pronounced in degree (Table III). Quick-frozen sections of organs of B6 mouse with amyloid deposit was checked with a monoclonal anti-mouse AA antibody. Fig. 5, *a* and *b* show positive immunofluorescence of AA in liver and kidney specimens, respectively. In an immunodiffusion study, DAP extracted from the liver of amyloidotic B6 mice reacted with monoclonal anti-mouse AA antibody.

Transfer of Spleen Cells of Donor Mice to Recipient Mice. Using a nylon-wool column, spleen cells of immunized donor mice and control animals were separated into adherent and nonadherent cells. Each fraction of spleen cells and unseparated whole spleen cells were injected into normal recipient B6 mice via a tail vein, and histology was examined at intervals. Although no changes were seen in the organs of recipient mice until 1 mo after the transfer, systemic amyloidosis became apparent in the recipient mice 2 mo after the injection. The most prominent histological changes were found in the organs of recipient mice injected with nylon-wool adherent spleen cells (Table IV). As shown in Table IV, no significant histological change occurred in the recipient mice injected with spleen cells of control animals.

Treatment with Anti-Thy-1,2, Anti-Ly-1, and Anti-Ly-2 Antibodies and Complement. As the data in Table IV show, the column adherent spleen cells of B6 mice injected with liver extract and CFA induced the most prominent amyloidosis in recipient mice 2 mo after injection. Those primed adherent spleen cells were treated with anti-Thy-1,2 monoclonal antibody and complement to deplete T cells, and treated adherent cells were injected into normal B6 mice followed by histological examination. As shown in Table V, amyloidosis was much less severe in mice injected with spleen cells treated by the combination of anti-Thy-1,2 antibody and complement. Further study using anti-Ly-1 and Ly-2 antibodies showed that this treatment completely abolished the amyloidosis inducing effect of primed adherent spleen cells.

Pathogenetic Study of Immunogen in Liver. A study was carried out with fractions of the liver extract to determine the active factor in the immunogen.

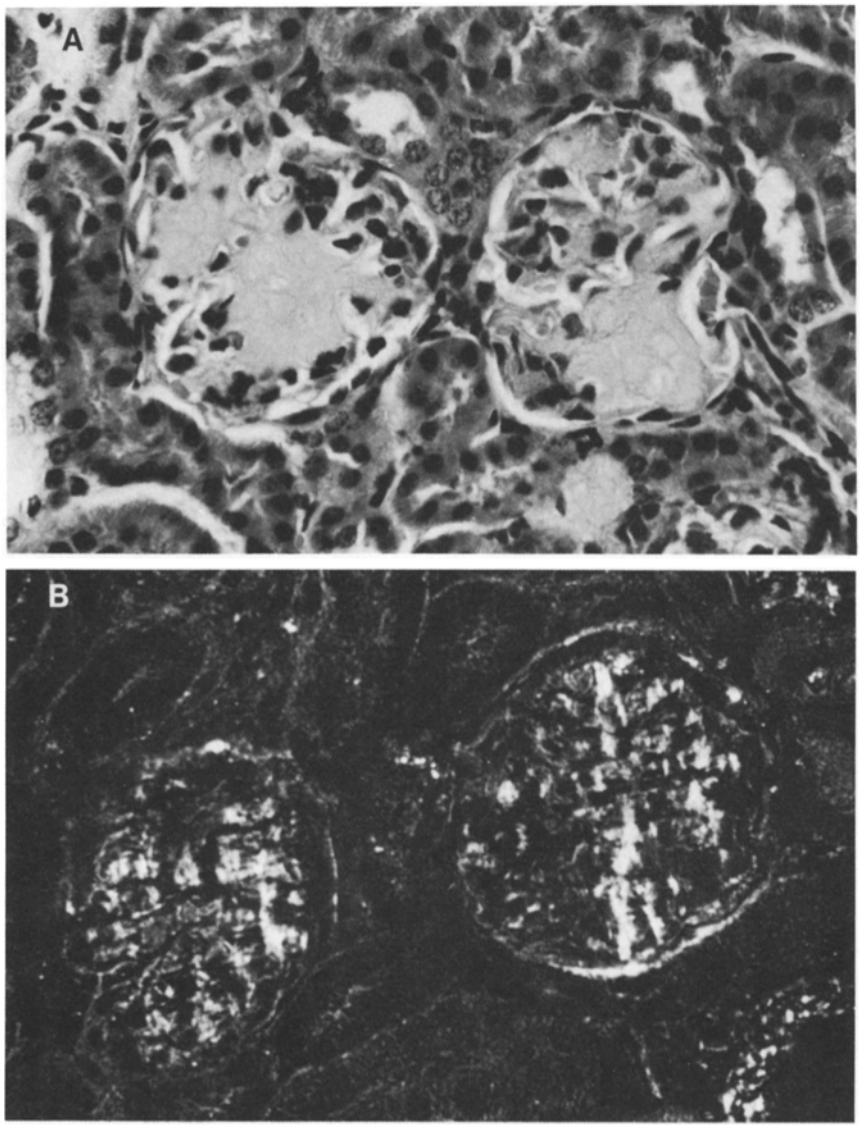

FIGURE 4. (*A*) Kidney of C57BL/6 (B6) mouse immunized six times with kidney extract and CFA (H & E, × 250). Marked amyloid deposition is seen in the glomeruli. (*B*) Kidney of B6 mouse immunized with liver extract and CFA (Congo-red birefringence, × 250).

As shown in Table VI, the supernatant of liver extract and a microsomal fraction in an amount equivalent to 10 mg protein was used as the immunogen six times at weekly intervals, and histological examination was performed 7 d after the final injection. The ability to induce amyloidosis was stronger with the soluble liver fraction (100,000 *g* supernatant) than with the microsomal fraction. The 100,000 *g* supernatant of liver extract was further treated by various methods, as given in Table VI. When the immunogen was stored at 4°C, its amyloidosis-inducing activity was maintained for 24 h, but was nearly lost after 72 h. The

TABLE III
Induction of Systemic Amyloidosis in C57BL/6 Mice Injected with Syngeneic Kidney or Liver Extract Alone

Exp.	Immunogen	Histology of:	Number examined	Histological findings*			
				−	±	+	++
1	Liver extract‡	Liver	20	2	9	9	—
		Kidney	20	10	10	—	—
		Spleen	20	—	2	12	6
2	Kidney extract‡	Liver	20	4	1	15	—
		Kidney	20	5	0	15	—
		Spleen	20	4	1	1	14

* Grade of amyloid deposit was determined as described in Materials and Methods.
‡ 10 mg protein/mouse.

activity of the supernatant was lost after freezing and thawing more than three times; both lyophilization and trypsin digestion destroyed the activity almost completely.

Discussion

Experimental amyloidosis has been induced in mice with casein, but 15–30 injections are required (1–3, 5, 7, 8, 15–17, 20, 21, and 24). Other investigators produced amyloidosis by injecting CFA (4), bacteria (25), endotoxin of *Escherichia coli* (5 and 6), or by radiation (8). In these animal models, amyloidosis is induced by a substance that does not exist in animal body, or by a physical means. Our study is the first demonstration of systemic amyloidosis produced by substances contained in the animal organs, namely, syngeneic liver or kidney extract, together with CFA given only six times at weekly intervals. All mice injected with these syngeneic organ extracts and CFA showed a marked deposition of amyloid, mainly in the liver and kidney followed by the spleen. Furthermore, partial organ specificity was shown with these extracts. Using a monoclonal anti-mouse AA antibody, it was shown that the deposited material was mouse AA. In this animal model, CFA may act as an enhancing factor like irradiation (9), or an amyloid-enhancing factor described by Axelrad et al. in animal tissues (11), since deposition of amyloid was much less pronounced in degree in the animals injected with liver or kidney extract without CFA. Control animals similarly given sera of syngeneic animals and CFA or CFA alone showed negative results or only a minimal histological change. These data seem to indicate that pathogenetic substances are contained in these organs, and that they are partially organ-specific. Analysis of the putative pathogenetic substance contained in a 100,000 *g* supernatant of the liver extract suggested that its characteristics are: (*a*) easily degraded by freezing and thawing, or lyophilization; (*b*) stable at 4°C for 24 h; and (*c*) digested by trypsin. The amyloidosis inducing substance in liver extract might be composed of unstable proteins or protein-bound substances.

In the mouse strains (C57BL/6, C3H/He, and BALB/c) used in this study, C57BL/6 (B6) showed the most prominent systemic amyloidosis, followed by C3H/He and BALB/c. Strain differences in the occurrence of systemic amyloi-

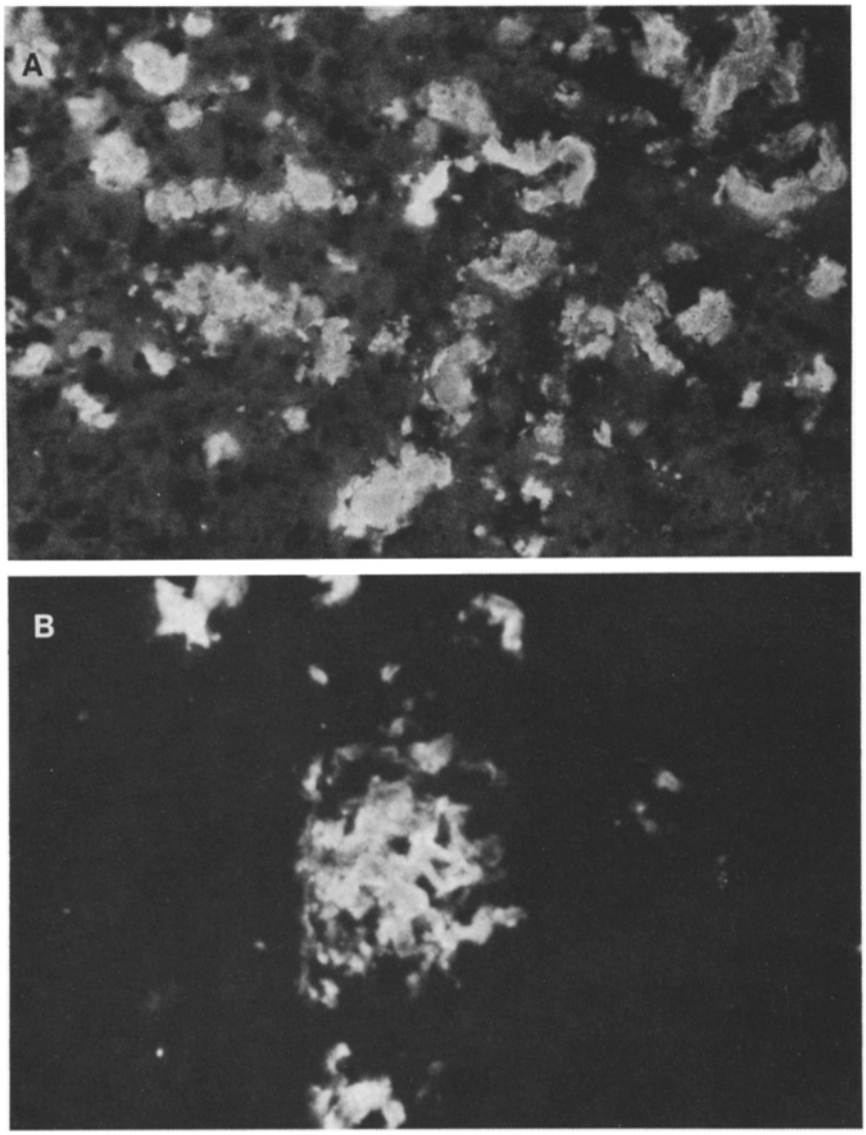

FIGURE 5. Indirect immunofluorescence study using a monoclonal anti-mouse AA antibody on liver (*A*) and kidney (*B*) specimens. Positive immunofluorescence of AA is seen.

dosis have been reported in mice. Hardt et al. reported that amyloid deposit induced by casein injection was more marked in C3H/He and C57BL/6 mice (21). Amyloid synthesis and accumulation in situ was studied, and involvement of mucoprotein, glycoprotein-producing periodic acid/Schiff-positive cells (26) or impaired Kupffer cells (27) has been suggested. Synthesis of serum amyloid protein (SAA) in hepatocytes has been demonstrated in casein-induced amyloid animals in vivo and in vitro (24, 28). It is well known that an immunological mechanism is involved in the pathogenesis of secondary amyloidosis. In the pathogenesis of amyloidosis, a role of plasma cells or their precursors (16), B

TABLE IV

Induction of Systemic Amyloidosis in the Organs of Mice Injected with Primed Spleen Cells at 8 wk After Transfer

Exp.	Fraction of spleen cells	Number examined	Histological findings*			
			−	±	+	++
1	Transfer of spleen cells of donor mice injected with liver extract + CFA:					
	Whole spleen cells	20	16	2	2	—
	Nylon wool column nonadherent cells	20	17	1	1	1
	Nylon wool column adherent cells	20	3	1	2	14
2	Transfer of spleen cells of control mice injected with CFA alone:					
	Whole spleen cells	20	20	—	—	—
	Nylon-wool column nonadherent cells	20	20	—	—	—
	Nylon-wool column adherent cells	20	20	—	—	—

* Grade of amyloid deposit was determined as described in Materials and Methods.

TABLE V

Induction of Systemic Amyloidosis in the Organs of Recipient Mice Injected with Primed Nylon/Wool Column Adherent Spleen Cells Treated with Anti-Thy-1,2; Ly-1; Ly-2; and/or Complement

Treatment	Number examined	Histological findings*			
		−	±	+	++
No treatment	20	3	1	2	14
Complement alone	20	8	2	5	5
Anti-Thy-1,2 mAb alone	20	5	4	8	3
Anti-Thy-1,2 mAb + complement	20	17	3	—	—
Anti-Ly-1 rat anti-mouse γ-globulin (Fab) + mouse anti-rat γ-globulin (MAR)	20	18	2	—	—
Anti-Ly-2 rat anti-mouse γ-globulin (Fab) + MAR + complement	20	19	1	—	—

* Grade of amyloid deposition in liver was determined by the criteria in Materials and Methods.

cell–macrophage interaction (17), and depletion of T cell population (18, 19) have been reported. However, it is not known which set of cells is responsible for the induction of amyloidosis.

In our transfer study, most prominent systemic amyloidosis occurred in recipient mice 2 mo after the transfer of nylon-wool adherent spleen cells of donor mice. Treatment of these adherent cells with anti-Thy-1,2; Ly-1; and Ly-2 antibodies and complement abolished deposition of amyloid, but a single antibody or complement alone did not. These data seem to indicate that nylon-wool adherent T cells with Ly-1^+,2^+,3^+ phenotype of donor mice play an important role in the induction of systemic amyloidosis, and that these T cells may work as helper or amplifier cells. In some reports, it was suggested that interactions between T cells with Ly-1^+,2^+,3^+ and Ly-1^+ cells was necessary for the manifestation of the delayed-time hypersensitivity (DTH) response (29, 30). This may

TABLE VI

Systemic Amyloidosis Induced by Variously Treated Liver Extracts of C57BL/6 (B6) Mice

Exp.	Immunogen*	Number examined	Histological findings in liver‡					
			–	±	+	++	+++	++++
1	Fraction of B6 liver extract:							
	Supernatant of 100,000 *g* centrifugation§	20	0	0	0	5	12	3
	Microsomal fraction§	20	0	0	8	6	6	—
2	Treatment of supernatant of 100,000 *g* centrifugation of liver extract§:							
	4°C for 24 h	20	0	0	0	4	13	3
	4°C for 72 h	20	4	4	12	—	—	—
	Freezing and thawing‖	20	18	2	—	—	—	—
	Lyophilization	20	3	5	12	—	—	—
	10% trypsin digestion	20	15	3	2	—	—	—

* Given with CFA six times at weekly intervals.
‡ Grade of amyloid deposition was determined by the criteria in Materials and Methods.
§ 10 mg protein/mouse.
‖ Over three times.

suggest that induction of amyloidosis in recipient mice may be related to a cell to cell interaction of a delayed-type immunological reaction under our conditions. In another study, amyloid deposition was seen in an early stage in B6 mice treated by 300 rad radiation to deplete suppressor cells (data not shown). This phenomenon was found in both donor and recipient mice. These data seem to indicate significant involvement of suppressor T cells in the induction of amyloidosis in our model. Further studies would hopefully clarify the role of cellular immunity in the induction of amyloidosis in animals.

Summary

Systemic amyloidosis was induced consistently in mice by intramuscular injection of syngeneic organ (liver and kidney) extracts mixed with CFA six times at weekly intervals. Syngeneic organ extract with CFA also induced amyloidosis of a lesser degree. All three strains of mice (C57BL/6, C3H/He, and BALB/c) injected with a syngeneic liver extract mixed with CFA developed systemic amyloidosis; the most prominent amyloid deposition occurred in C57BL/6 (B6) mice, followed by C3H/He and BALB/c. The amyloid substance deposited in these animals was identified as mouse amyloid A protein (AA). Furthermore, an organ specificity of the immunogen in inducing amyloidosis was suggested with liver and kidney extracts.

Primed spleen cells of the immunized B6 mice were fractionated by a nylon-wool column and injected to normal recipient mice via the tail vein. Organs of the recipient mice developed systemic amyloidosis 8 wk after the transfer, and the most prominent histological changes occurred in the recipient mice given nylon-wool column adherent spleen cells. Using anti-Thy-1,2; Ly-1; Ly-2, antibody and complement, it was suggested that T cells, especially Ly-1,2,3$^+$ T cell populations in the primed nylon-wool adherent cells, play an important role in the induction of systemic amyloidosis. It was shown further that the amyloidosis-

inducing substance in liver extract was composed of unstable proteins or protein-bound substance.

Received for publication 30 October 1985 and in revised form 12 March 1986.

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