

SOME FACTORS IN THE DEVELOPMENT, LOCALIZATION AND REABSORPTION OF EXPERIMENTAL AMYLOIDOSIS IN THE RABBIT*

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Certain aspects of the problem of amyloidosis have engaged our attention for a number of years. This paper is a summary of our findings, and is dedicated to a great teacher and investigator of pathology whose manifold interests also include experimental amyloidosis.

METHODS

Healthy rabbits, weighing between 1500 and 2000 gm. and kept on an adequate mixed diet, were injected intravenously with strains of hemolytic streptococci freshly isolated from the upper respiratory passages of patients with acute glomerulonephritis. Scarlet fever streptococci, green-producing streptococci from the respiratory passages or urines of patients with chronic nephritis and systemic lupus erythematosus, pneumococci of types I and III, and a Friedländer bacillus were also used, along with some other strains, including a hemolytic streptococcus of canine tonsillar and endocardial origin. All organisms were grown in blood broth.

A series of rabbits was also injected with scarlet fever anti-toxin, horse serum, rabbit serum, rabbit plasma albumin and rabbit plasma globulin.

Material for histologic study was fixed in a 10 per cent aqueous solution of formaldehyde and in Zenker's solution and stained with Congo red for amyloid and with hematoxylin and eosin for general purposes. Ordinarily, kidney, liver, spleen, adrenal and myocardium were sectioned; occasionally, other organs.

Determinations of plasma proteins, blood urea and nonprotein nitrogen, and plasma cholesterol were made by the usual clinical microchemical methods. Albuminuria was estimated with the heat and acetic acid test and at times by quantitative chemical analysis.

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RESULTS

Amyloid was found in the kidneys, liver or spleen (or in several of these organs) in 104 of the 181 rabbits injected with bacteria, or 57 per cent. In general, rabbits injected with strains of virulent hemolytic streptococci, Friedländer's bacilli or pneumococci developed amyloid sooner and more extensively than animals given the relatively avirulent green-producing streptococci or other organisms long after their isolation from human cases. In the case of staphylococci, septicopyemia killed the animals in too short a period to permit development of amyloidosis. The dosage used may have been too large, for other investigators¹⁻³ have produced amyloidosis in rabbits with this organism. No other organism employed failed to bring about the deposition of amyloid in some rabbits.

The histology of experimental amyloidosis has been repeatedly described and we have alluded to it in earlier preliminary reports.^{4,5} In a large majority of rabbits coming to autopsy before amyloidosis has had time to develop, marked lymphoid and reticular cell hyperplasia of the splenic follicles has been observed, often with numerous mitotic figures. The amyloid was always extracellular.

Factors in the Distribution of Amyloid

The localization of amyloid was characteristic. In the kidney, amyloid was found only in the glomerular tuft and along the medullary capillaries. In the spleen, amyloid was usually deposited in the periphery of the follicle, extending inward and outward as it increased. The follicular arterioles were only slightly involved. In the liver, amyloid was observed about the peripheral sinusoids, extending centrally and producing the typical atrophy. The adrenal gland exhibited amyloid around capillaries in the zona reticularis near the medulla, spreading later toward the zona fasciculata. In no case was the entire adrenal cortex infiltrated nor extensive atrophy of tissue produced. Amyloid was never found in the myocardium, lungs, thymus or aorta. It was sometimes seen in the tail of the pancreas, involving the capillaries of both the ordinary and islet parenchyma. A few observations of the stomach and intestines showed amyloid about the capillaries of the mucosa.

The relative distribution of amyloid in the main sites of occurrence—kidney, spleen, liver and adrenal—was determined largely by the total length of the experimental period and the duration of survival after the injection period (Table I). Thus, grading the amount of amyloidosis on a basis of 4 plus as a maximum in each organ and classifying the rabbits into groups, the following distribution was obtained: (a) No amyloid in 80 rabbits living less than 7 weeks. There were a few animals without amyloid living longer periods, up to 38 months. (b) Moderate to considerable amyloid in the spleen (or liver) with little or none in the kidneys in 24 rabbits, of which 9 died in 4 to 8 weeks, 12 in 3 to 5 months, none in 6 to 11 months and 3 in 12 to 18 months. (c) Moderate to considerable amyloid in the spleen, kidneys and often the liver, less often in the adrenal, in 26 rabbits, of which none died prior to 8 weeks, 13 in 2½ to 6 months, 4 in 7 to 11 months and 9 in 12 to 24 months. (d) Moderate to considerable amyloid in the kidneys and often the adrenals, with little or none in the spleen or liver in 36 animals, of which 1 died in 2½ months, 1 in 5 months, 14 in 7 to 11 months and 20 in 12 to 30 months.

TABLE I
Distribution of Amyloid According to Duration of the Experimental Period

Period	Total No. of rabbits used	No. without amyloid	With generalized amyloidosis	With amyloid chiefly splenic, little or none in kidneys	With amyloid chiefly renal, little or none in spleen
1-2 mo.	77	68	0	9	0
3-6 mo.	31	4	13	12	2
7-11 mo.	20	2	4	0	14
12-30 mo.	35	3*	9†	3‡	20

Eighteen rabbits used in the experiment are not included in this table since, while they showed slight amounts of amyloid, there was too little to consider organ-distribution significant.

* 12-38 months.

† 12-24 months.

‡ 12-18 months.

|| 12-30 months.

Apparently, bacterial amyloidosis develops first in the spleen and liver and later in the kidneys with subsequent diminution or disappearance of splenic and hepatic amyloid but an increase in renal and, probably, in adrenal amyloid.

In 10 rabbits bacteria were injected in two separate courses, 8 to 10 months apart in 6 animals and from 1½ to 3½ months apart in 4 animals. In 2 out of the 3 rabbits in the group (b)

showing predominance of splenic amyloid in spite of a total experimental period of 12 to 14 months, the time elapsed from the beginning of the second injection course to death was $4\frac{3}{4}$ and 2 months, respectively, or long enough to permit the deposition of amyloid. In the 4 rabbits in group (c), 3 had total experimental periods of 10 to 15 months, with the second experimental periods of 1, $4\frac{1}{2}$ and 5 months, respectively. In this group either the first or the second injection courses could have produced the amyloid in 2 of the 3 rabbits. On the other hand, 3 rabbits in group (d) had total experimental periods of 5, 17 and 28 months, respectively. In the latter 2 rabbits there was no amyloid in the spleen, but the second experimental periods lasted 10 and 16 months, respectively, or long enough to explain the predominantly renal amyloid.

Factors in the Development of Amyloidosis

The duration of bacterial injections seems to have little relation to the development of amyloidosis except for the longer period required in the case of avirulent strains. In our earlier experiments,⁴ it was found that freshly isolated and virulent strains of hemolytic streptococci from cases of acute nephritis produced albuminuria (renal amyloid) much sooner than did the less virulent or older strains. Without frequent biopsies on the spleen and in the absence of albuminuria it is impossible to determine the exact onset of amyloidosis. However, in 17 rabbits with well developed amyloidosis the entire injection period extended over 3 weeks or less, in 6 animals for only 3 to 4 days. Yet in this group the total experimental period was $1\frac{1}{2}$ to 3 months in 4 out of 5 rabbits injected with freshly isolated strains of "nephritic" hemolytic streptococci, and from 12 to 26 months in all 9 rabbits injected with freshly isolated strains of *Streptococcus viridans* or other avirulent organisms.

The rôle of chronic infection and suppuration was not clearly significant in our series of positive animals. Disregarding terminal infections in the lung, only a small percentage of our rabbits had vegetative endocarditis, suppurative arthritis, cholecystitis, pericholecystitis or hepatitis, or chronic pneumonitis. The rabbits without amyloid were infected to about the same extent as the positive animals. The etiologic significance of small infarcts in

the kidney seemed dubious. Rabbits with suppuration due to staphylococci died too soon to develop amyloidosis.

Reabsorption of Amyloid

Apart from direct biopsy evidence available in a small series of rabbits,⁵ strong indirect support of the reabsorption theory is given by the data on the distribution of amyloid in the kidneys, spleen, liver and adrenals in relation to the total experimental period (Table I). In the spleen, the process of reabsorption seems to be largely the result of invasion of leukocytes, polyblasts and capillaries from the adjacent pulp. The amyloid loses its staining power, is broken up into fragments and gradually disappears. Participation of foreign body giant cells in this process^{2, 6-8} was limited to 3 animals although a few more showed giant cells, presumably megakaryocytes, unrelated to the amyloid masses. Following the reabsorption of splenic amyloid the follicles remained atrophic and at times definitely fibrotic. The liver showed little or no residual portal fibrosis. No clear instance of absorption of amyloid in the adrenal has been found in our series.

Evidence for reabsorption of renal amyloid in the rabbit has not appeared in our extensive study. Once the glomeruli and medullary capillaries have become moderately involved, tubular dilatation and degenerative changes set in with later atrophy and ultimate fibrosis. The kidneys usually remain large in spite of fibrosis because of the persistent amyloid, and weights two or three times the normal are a regular occurrence even in the late stages of renal disorganization. The rôle of tubular obstruction in parenchymal atrophy is important.

Experimental Hyperglobulinemia and Amyloidosis

This aspect of the problem has been studied in several ways. First of all, data have been obtained on the variations in the plasma proteins during and after the injection of bacteria in rabbits with or without amyloid. The average control plasma albumin in 73 rabbits was 4.28 ± 0.40 gm. per cent, with 11 values below 3.88 and 17 above 4.68 gm. per cent. The mean control plasma globulin in this series was 1.70 ± 0.41 gm. per cent, with 14 values below 1.29 gm. per cent and 11 values above 2.11 gm. per

cent, 5 being over 2.93 gm. per cent. These globulin figures are slightly lower than those we⁵ reported on another series of 46 rabbits with an average control level of 1.86 ± 0.37 gm. per cent.

During the period of bacterial injection, if not less than 3 weeks, the plasma globulin rose to 3 gm. per cent or higher in 34 out of 40 rabbits studied. Elevated plasma globulin was maintained during the longer injection courses.

The plasma globulin values after the period of bacterial injection varied considerably in different rabbits and often in the same rabbit. In 15 animals, the plasma globulin exceeded 2.5 gm. per cent during 2 months or longer, often reaching levels of 3.5 to 4 gm. per cent. However, in 14 rabbits the plasma globulin remained for several months below 2.5 gm., often below 2.0 gm. per cent. No clear difference in the degree or distribution of amyloidosis was found in these two groups, but 5 out of 8 rabbits without amyloid had low plasma globulins. Finally, a number of animals showed irregular fluctuations over a period of months. In the rabbits receiving two courses of bacteria, the second injection period usually was followed by a sharper rise in plasma globulin than the first. Albuminuria, sufficient to lower the plasma albumin to 2.5 gm. per cent or less, was usually associated with an elevation of plasma globulin to 2.5 gm. per cent or more, apart from the possible influence of bacterial injections. The total plasma protein usually reached 7 to 8 gm. per cent when the globulin fraction increased, except in cases of low plasma albumin secondary to albuminuria, when the plasma proteins fell to 5 or 4 gm. per cent.

The plasma globulin was also elevated by the repeated intravenous injection of scarlet fever antitoxin (horse). Of 5 rabbits injected over a maximum period of $1\frac{1}{2}$ months, 3 died of pulmonary edema, 1 lived 10 months and 1 for 2 years (after a single injection). None showed amyloid but all had marked hyperplasia of the spleen. The plasma globulin values in the 4 rabbits studied reached 3.36 to 5.94 gm. per cent during the period of injection, the rise beginning within a week. In the 2 rabbits that survived the injection period, the plasma globulin figures remained within normal levels.

Direct elevation of the rabbit's plasma globulin was made by intravenous injection of rabbit whole serum and rabbit plasma

globulin prepared in various ways. Five rabbits were given serum for periods up to 7 months. Three animals with a total of 1160 to 3000 cc. of serum during 5 to 7 months, with total experimental periods of 9, 16 and 19 months, showed slight to moderate amyloidosis in the kidneys at autopsy, although no amyloid was detected in either the kidney or portion of spleen removed at 5, 1½ and 5 months, respectively. Hyperplasia of the splenic follicles was found in the biopsy specimens. The plasma globulin in the 4 rabbits studied was maintained at 3 to 4 gm. per cent during the period of injection except when the amount of serum was reduced. The plasma albumin also rose so that the total protein reached 8 to 9 gm. per cent. When the serum was discontinued, the plasma globulin promptly fell to normal.

Rabbit plasma globulin was injected intravenously for periods of 1½ to 4 months in 6 rabbits. Considerable splenic or renal amyloid developed in 4 animals, a trace in 1 and none in 1. The definitely positive results were obtained with the more denatured globulin preparations. The plasma globulin rose during the injection period to 3 to 4 gm. per cent in all the rabbits, and persisted at a high level as long as globulin was given in adequate amounts. The highest plasma globulins, 4.6 to 5.9 gm. per cent, for periods of 1½ and 2½ months, were observed in the 2 animals with no amyloid. The plasma globulin fell to normal within a few weeks after the injection period except in the rabbits with persistent albuminuria due to renal amyloidosis. In 2 rabbits given the heat-denatured globulin many giant cells containing amyloid were seen in the spleen.

Dietary Experiments

A few experiments involving the injection of bacteria into rabbits kept on diets free from ascorbic acid or consisting of hay alone revealed no definite influence upon the production or course of amyloidosis. However, in 1 rabbit kept as a control on a hay diet for 10 months and surviving another 7 months on the regular diet, considerable amyloid was present in the kidneys without an obvious focus of inflammation. No amyloid has been observed in any control rabbit on the regular diet up to 3 years nor in rabbits used as blood donors.

Proteinuria

Albuminuria, if persistent, practically always indicated renal amyloidosis.^{4,5} In general, the degree and duration of proteinuria were proportional to the amount of glomerular amyloid. No albuminuria was found in rabbits with considerable amyloid in the spleen or liver but without renal amyloid. However, the absence of proteinuria did not exclude the presence of traces or small amounts of amyloid in the kidney, as was demonstrated in 7 rabbits. Following proteinuria, the plasma albumin fell to 2 gm. per cent or less, the plasma globulin often rose to 2.5 gm. per cent or more, the weight went down and the plasma cholesterol increased temporarily. In several animals the hypo-albuminemia caused transudation into the serous cavities.

COMMENT

Our experiments on some 200 rabbits have demonstrated the facility of production of amyloidosis in this animal by means of intravenous injection of bacteria. This is in agreement with the scattered results of previous investigators.^{1,2,10-12} The most rapid and most extensive amyloidosis has followed the use of hemolytic streptococci freshly isolated from inflamed upper respiratory passages and tonsils of individuals with acute, recurrent subacute, or active chronic glomerulonephritis. Other organisms have also proved effective in initiating amyloidosis when freshly isolated. After ageing on laboratory media, larger doses and more prolonged injections are necessary. In a great majority of our positive experiments, no focus of chronic suppuration or inflammation was found to account for the apparently progressive evolution of amyloidosis long after injections of bacteria had ceased and long after they must have disappeared from the animal body. In several rabbits, only three or four daily injections of bacteria sufficed to produce amyloidosis.

A constant and striking feature in this study was the change in distribution of amyloid with the lapse of experimental time. Splenic amyloid predominated markedly over renal amyloid in practically all of the rabbits coming to autopsy within 2 months of the onset of the experiments. Animals with periods of 2 to 6 months were very likely to show more or less uniform amyloidosis in the spleen, kidneys and liver. After 7 to 11 months the

tendency increased for marked renal and adrenal amyloid with little or none in the spleen and liver. The apparent exceptions of behavior could be reasonably explained as the deposition of amyloid in response to a second, or more recent, course of injection of bacteria. In several instances biopsies of the spleen and kidney gave direct confirmation of the later change from predominantly splenic to predominantly renal amyloid.

These observations on the reabsorption of amyloid in the spleen and liver confirmed previous reports on reversibility of amyloidosis in these organs in the mouse,⁸ rabbit,^{2,3} horse⁷ and man.^{2,6,13,14} However, in spite of discontinuance of bacterial injections and the disappearance of splenic amyloid, renal amyloid not only remained but actually increased, ultimately causing disorganization of parenchyma and varying degrees of fibrosis and functional impairment. While the situation may be different in human renal amyloidosis, the literature is not convincing.^{6,13,14} Physiologically, conditions are more favorable for the reabsorption of amyloid in the spleen and liver than in the kidney.

There has been much speculation concerning the chemical nature of amyloid, the mechanism of its precipitation in the walls of arterioles and capillaries, and the relation of the processes of immunity, including hyperglobulinemia, to the pathogenesis of amyloidosis. A recent excellent study of the physical chemistry of human amyloid¹⁵ illustrates the difficulties involved. The rôle of chondroitin-sulfuric acid is still an intriguing problem.¹⁶ Such factors as hyperglobulinemia, the circulating or local precursors of amyloid, antigen-antibody precipitation and others have been discussed at some length by previous investigators.¹⁷⁻²¹ It is generally agreed that any type of prolonged cellular stimulation by foreign protein of external or internal origin leads to both hyperglobulinemia and amyloidosis in a highly susceptible animal, like the mouse or the rabbit, or the horse used for the production of immune sera.⁷ The apparent rarity of amyloidosis in individuals with kala-azar or lymphogranuloma inguinale, in which very high plasma globulin levels often occur, is difficult to understand.

Our direct attempt to test the theory of hyperglobulinemia as a cause of amyloidosis by injecting whole rabbit serum or concentrated rabbit plasma globulin in 10 rabbits yielded conflicting

results difficult to interpret, unless we assumed that the method of preparation of the serum proteins was such as to denature them for the rabbit. In that event the absence of amyloid in the 2 animals given the best globulin preparation is strong evidence against the simple assumption of hyperglobulinemia as a cause of amyloidosis. It has been reported,¹⁸ without details, that electro-ultrafiltered rabbit globulin produced neither amyloid nor precipitins in rabbits. More experiments are necessary, including identification of the plasma globulin during the formation of amyloid.

The more indirect attempts to correlate hyperglobulinemia and amyloidosis^{5,17,21} cannot, in our experience, lead to a conclusive decision. Whether amyloid is formed or not, the parenteral injection of antigen stimulates a rise in plasma globulin, relative or absolute. The plasma globulin level may be markedly increased or normal for months after the injection period in rabbits with or without amyloidosis. The low figures cannot be explained, as has been suggested,²¹ on the basis of proteinuria or hepatic amyloidosis. A second course of injections of bacteria or other antigen usually leads to a rapid rise in the plasma globulin level even in rabbits which failed to respond to the first series.

Some miscellaneous observations are of interest. The incidence of gross aortic medial necrosis, calcification and atheromatosis⁹ in the amyloid rabbits was 12 per cent, six times as high as in the nonamyloid and control groups. Anemia and loss of weight were frequent concomitants of the process of amyloidosis and were intensified during periods of considerable proteinuria. Anemia itself did not cause amyloidosis in a series of blood-donor rabbits. The spleen often contained many erythrocyte-laden and blood-pigment-laden macrophages. In rabbits with experimental periods of a year or longer, renal amyloidosis was usually associated with marked tubular atrophy and obstruction, fibrosis of the parenchyma and hyalinization of the glomeruli containing amyloid. In spite of this "contraction" of the kidneys, the weight still exceeded the normal because of the amyloid content. Amyloid was practically never found in any renal vessels other than the intraglomerular arterioles.

SUMMARY

1. Amyloidosis has been produced in a large series of rabbits by the injection of various bacteria from human sources.
2. Splenic and hepatic amyloid appear early, but can also disappear in time. Evidence of active reabsorption of amyloid is presented.
3. Renal amyloid develops later but tends to increase with time to the point of extreme disorganization and fibrosis of the parenchyma, and functional insufficiency. There is no evidence of absorption of renal amyloid in the rabbit.
4. Hyperglobulinemia, relative or absolute, is a constant finding during the longer periods of bacterial injection, but may or may not persist in the after period. Albuminuria may elevate the plasma globulin relatively.
5. Artificial hyperglobulinemia, the result of injections of rabbit serum or globulin, does not regularly produce amyloidosis. The positive results may be secondary to denaturation of the serum proteins. Presumably, other factors than hyperglobulinemia are necessary for the development of amyloidosis.
6. Amyloidosis may appear and progress in the absence of ordinary signs of inflammation or suppuration in the rabbit.
7. Gross aortic disease in the form of medial necrosis and calcification or atheroma is six times as prevalent in rabbits with amyloidosis as in the nonamyloid series. Atheroma is always associated with persistent hypercholesterolemia.
8. The pathogenesis of amyloidosis is not adequately explained by the prevailing theories and requires further investigation.

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DESCRIPTION OF PLATE

PLATE 118

- FIG. 1. Moderate amyloidosis in the kidney (biopsy) of rabbit No. 9, injected with a scarlatinal streptococcus for 10 months and showing albuminuria for 3 months. The glomeruli contained amyloid in amounts varying from 1 to 4 plus. The proximal convoluted tubules are atrophic and the distal tubules markedly dilated. There is some increase in interstitial tissue. Hematoxylin and eosin stain. $\times 170$.
- FIG. 2. Marked amyloidosis in glomeruli, and degeneration of tubules in rabbit No. 3, injected with a "nephritic" hemolytic streptococcus for $3\frac{1}{2}$ months in two courses, with a total experimental period of $6\frac{1}{2}$ months and persistent albuminuria in the last $1\frac{1}{2}$ months. Congo red stain. $\times 170$.
- FIG. 3. Marked glomerular amyloidosis, tubular atrophy and obstruction, and interstitial fibrosis in rabbit No. 11, injected with a type I pneumococcus for 6 months. Albuminuria appeared at $2\frac{1}{2}$ months and persisted during the 5 months after the injection period. Hematoxylin and eosin stain. $\times 63$.
- FIG. 4. Marked glomerular amyloidosis and fibrosis of parenchyma in rabbit No. 312; injected with *Streptococcus viridans* for 4 months and surviving another 14 months, with albuminuria during most of this period. The spleen and liver showed only traces of amyloid. Van Gieson's stain. $\times 75$.
- FIG. 5. Medial necrosis and calcification, and intimal fibrosis in aorta of rabbit No. 535, injected with a "nephritic" hemolytic streptococcus for 7 weeks in two courses, with a total experimental period of 10 months. There was generalized amyloidosis. The aorta was calcified as far as the origin of the renal arteries. Hematoxylin and eosin stain. $\times 18$.
- FIG. 6. Atheromatous plaque in ascending aorta of rabbit No. 360, injected with a Friedländer bacillus during $1\frac{1}{2}$ months and surviving another 14 months. There was marked generalized amyloidosis, with fibrotic kidneys. The plasma cholesterol ranged between 179 and 352 mg. per cent for a year. Van Gieson's stain. $\times 250$.

