IMMUNOMORPHOLOGICAL STUDY OF THE EARLY STAGES OF AMYLOIDOGENESIS

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The early stages of amyloidogenesis were studied in experiments on mice. Frozen sections through the spleen and other organs were incubated by the indirect Coons' method initially with pure rabbit antibodies against fibrillary protein of mouse amyloid, later with pure fluorescent goat antibodies against rabbit IgG. In the early stages, after one or two injections of casein, amyloid protein was observed to appear in the intercellular space and in the blood filling the lumen of the sinuses of the spleen and liver. The formation of typical deposits of amyloid in the spleen was complete by the 5th-7th day of the experiment after three to five injections of casein.

KEY WORDS: amyloidogenesis; amyloid antigen; immunofluorescence.

The origin of amyloid protein in secondary amyloidosis in man and in experimental amyloidosis of animals is unknown. Besides the fibrillary protein of the tissue amyloid, relatively recently a soluble amyloid protein has been found in the blood of patients and animals with common antigenic determinants with fibrillary protein [11]. Most investigators who have studied the formation of specific amyloid fibrils in experimental amyloidosis have used electron microscopy. Some workers have concluded that the amyloid fibrils are formed intracellularly, specifically within the lysosomes [13], whereas others conclude that they are formed extracellularly [3]. However, it is impossible by electron microscopy to determine whether the cell is the producer of the amyloid protein or whether it simply participates somehow in its conversion into fibrils. The most reliable method of determination of the cells synthesizing amyloid protein is the immunomorphological method, using specific antisera against the required antigen proteins.

The object of this investigation was to study amyloid formation in the initial stages of amyloidogenesis in mice.

EXPERIMENTAL METHOD

For the immunomorphological study 80 mice of strains CB, C57BL, and BALB/c were given daily subcutaneous injections of 1 ml 13% sodium caseinate. The animals were killed after 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 injections respectively, on the 2nd, 3rd, 4th, 5th, 7th, 8th, 9th, and 10th days of the experiment (group 1). Twenty BALB/c mice received a daily injection of 0.5 ml of 13% sodium caseinate and were killed at the same times (group 2). Serial frozen sections through the spleen, liver, kidney, heart, and adrenals of each mouse were stained with thioflavin T and Congo red and tested by the indirect Coons' method. In the first stage the sections were incubated with pure rabbit antibodies against mouse amyloid fibril protein (AFP), isolated from the corresponding antiserum with the aid of a solid adsorbent [6]. The antiserum was obtained after prolonged (8-9 months) immunization of rabbits with hydrolyzed pure mouse AFP [2] in Freund's complete adjuvant, and before isolation of the antibodies it was absorbed with hydrolyzed homogenate of normal mouse organs. In the second stage the sections were incubated with goat antibodies against rabbit immunoglobulin G, labeled with fluorescein isothiocyanate. As a control of specificity of the antibodies to mouse AFP, normal mouse organs were treated with them and amyloid organs were incubated with nonimmune rabbit γ globulin or with antibodies of other specificity. In both cases the specific fluorescence was completely absent.

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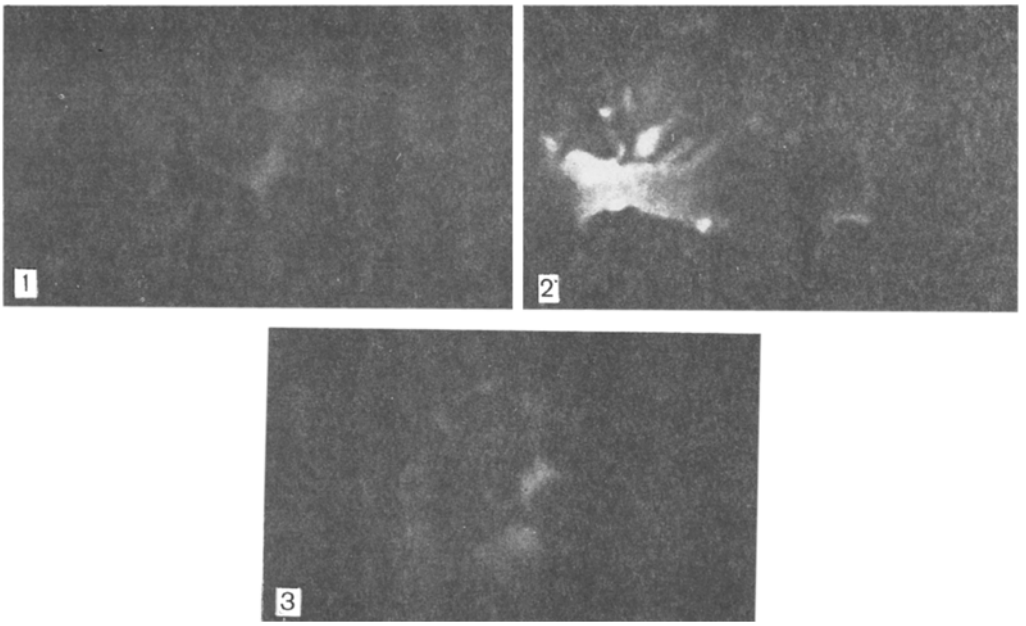

Fig. 1. Spleen of mouse receiving two injections of casein; palely fluorescent amorphous material visible extracellularly in section treated with pure antibodies against AFP by the indirect Coons' method; 450×.

Fig. 2. Fluorescence of blood in lumen of splenic sinus of mouse after three injections of casein. Section treated as in Fig. 1; 270×.

Fig. 3. Particles of amyloid deposited in marginal zone of spleen of mouse after four injections of casein. Section treated as in Fig. 1; 100×.

EXPERIMENTAL RESULTS

By the use of pure antibodies against mouse AFP the time and place of appearance of the amyloid protein could be established. After only one or two injections (the difference in time was due to the individual reaction of the animals and to genetic differences) weak fluorescence of amorphous extracellular material, visible only under high power, was recorded in the marginal zone of the spleen in the mice of group 1 (Fig. 1). No fluorescence was yet present in the other organs. The histological stains thioflavin T and Congo red did not reveal these structures. Subsequent injections of casein (the second or third) caused a sharp increase in the mass of amorphous material in the marginal zone and its appearance between the cells of the red pulp. Protein reacting with antibodies against mouse AFP also was present in the blood filling the lumen of the sinuses of the spleen and liver (Fig. 2). Parallel with the quantitative changes, qualitative changes also were observed: Against the palely fluorescent mass, deposits of material with very bright fluorescence could be distinguished. These first bright particles were formed in the space adjacent to the sinuses of the spleen, and the walls of the sinuses themselves also gave intense fluorescence. Besides the particulate deposits, amyloid sometimes formed reticular structures in the perifollicular zone, resembling an argyrophilic carcass. At this stage it was still impossible to detect the amyloid histologically. After three or four injections the amyloid forming in the spleen and perisinusoidal space of the liver now stained with Congo red and thioflavin T. However, by treating the sections with antibodies against AFP a much more complete and detailed picture was obtained of the distribution of amyloid in the organs. By this time nearly all the palely fluorescent amorphous "preamyloid" material had condensed into particles (Fig. 3). Later, only an increase in the volume of the amyloid mass without any further qualitative changes was observed. By the 7th day of the experiment bright fluorescence had spread to all the marginal zone of the spleen and into the red pulp; discrete particles could be seen among the cells of the white pulp. The quantity of amyloid in the liver was increased. After 10 days (seven or eight injections) treatment with antibodies against AFP revealed amyloid in the kidneys between the cells of the tubular epithelium and in individual glomeruli, and also in the adrenals (mainly in the cortex) and in the heart, as a thin band between the contractile fibers (separate areas in the subepicardial layer). Thioflavin T and Congo red did not reveal this "early" amyloid in the heart and kidneys, by contrast with the amyloid masses in the spleen and liver of the same mouse.

In the mice of group 2 amyloid production began later, after five injections; apart from differences in time, no other differences were observed between the two groups.

The data described above are evidence of the unusually rapid formation of amyloid in mice following injection of large doses of protein (about 100 mg casein per injection). This process took place in three stages. The first stage is the formation of "preamyloid" – a protein binding antibodies against mouse AFP but not detectable by histological stains. Pale luminescence of this protein was found in the intercellular spaces of the marginal zone and red pulp and in blood filling the lumen of the sinuses of the spleen and liver (one or two injections). The second stage is the formation of "immature" amyloid. In sections treated with antibodies against AFP the immature amyloid gives bright fluorescence but still cannot be detected by the specific stains – thioflavin T and Congo red (two to four injections). The third stage is the formation of mature amyloid, possessing the typical staining properties and binding Congo red and thioflavin T (three to five injections of casein).

The observations described above show that large quantities of amyloid protein are present in the spleen of mice soon after one or two injections of casein. This fact is in good agreement with data showing a sharp rise in the blood level of this protein in mice [10] and mink [9] 24-48 h after a single injection of E. coli endotoxin. However, the authors cited above observed amyloid formation in these experiments only on the 28th day. The appearance both of the soluble amyloid protein of the blood and of the fibrillary amyloid protein in the tissues thus anticipates active immunogenesis which begins 3-4 days after immunization [5]. The increase in the level of amyloid protein coincides in time with phagocytosis of a large volume of foreign protein. At the same time, the results of electron microscopy [13] and, in particular, of splenic tissue culture experiments [1] have demonstrated the role of macrophages in the formation of amyloid fibrils. Finally, Benson and coworkers [7] showed that the amyloid protein in the blood of mice with experimental amyloidosis has the property of depressing antibody formation in tissue culture. A comparison of all these facts suggests that specific amyloid protein is formed during phagocytosis in order to prevent overproduction of antibodies and overloading of the immune system.

Despite the high sensitivity of Coons' method, whereby protein can be detected in one synthesizing cell in a section, it was impossible to observe AFP in the cells even though very small quantities of it could be revealed extracellularly. Probably AFP acquires its antigenic properties only after leaving the cell.

The conversion of preamyloid into immature amyloid corresponds to the formation of finely granular amyloid discovered in the heart of mice with casein amyloidosis [4], which does not possess the typical staining properties. Finally, staining amyloid with thioflavin T and, in particular, with Congo red demonstrates the formation of typical amyloid fibrils, for it is the fibrillary structure of the amyloid protein which determines the binding of Congo red [8].

In sections of different organs of normal mice, incidentally, antibodies against AFP did not reveal any structures; this contradicts the observations of Linder et al. [12], who reported the discovery of synthesis of amyloid fibrils in a culture of fibroblasts from human embryonic tissues.

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