ecule are formed in SAA. In the work of Linder et al. [6] it is impossible to rule out the presence of antibodies against connective-tissue components, for these workers used whole patient's serum as the source of antigen SAA (the SAA content was judged by the precipitation test in agar with antiserum against this protein). It was most likely fluorescence of connective-tissue proteins that these workers observed in the sections through the embryonic organs.

Despite the fact that the present results were obtained in experiments on mice, they ca be perfectly and properly compared with data on Linder et al. [6], for experimental amyloidosis is an analog of secondary amyloidosis, and human and mouse SAA proteins are similar in their properties. It seems unlikely that they are synthesized by different cells.

LITERATURE CITED

1. R. Axen, J. Porath, and S. Ernback, Nature (London), 214, 1302 (1967).
2. R. F. Anders, T. E. Michaelson, J. B. Natvig, et al., Scand. J. Immunol., 4, 397 (1975)
3. E. C. Franklin, J. Exp. Med., 144, 1679 (1976).
4. G. Husby, K. Sletten, T. E. Michaelson, et al., Scand. J. Immunol., 2, 395 (1973).
5. G. Husby, J. B. Natvig, K. Nordstoga, et al., Scand. J. Immunol., 4, 811 (1975).
6. E. Linder, R. F. Anders, and J. B. Natvig, J. Exp. Med., 144, 1336 (1976).
7. R. P. Linke, J. D. Sipe, P. S. Pollok, et al., Proc. Nat. Acad. Sci. USA, 72, 1473 (1975).
8. C. Rosenthal and E. C. Franklin, J. Clin. Invest., 55, 746 (1975).

IMMUNOCYTOCHEMICAL DETECTION OF THE NONFIBRILLARY STAGE OF AMYLOID FORMATION IN THE MOUSE MYOCARDIUM

V. N. Baranov, L. N. Kapinus, and V. S. Rukosuev

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An indirect electron-microscopic immunoperoxidase method, with pure rabbit antibodies against fibrillary protein of mouse amyloid, was used to study casein amyloidosis in mice. In the early stages of development of amyloidosis deposits of finely granular material appeared in the mouse myocardium. These deposits contained an antigen similar to the fibrillary antigen of amyloid, but were without its fibrillary ultrastructure. The results of this investigation point to the existence of an early nonfibrillary stage of amyloid formation.

KEY WORDS: *amyloid; myocardium; immunocytochemistry.*

The obtaining of an antiserum against the specific antigen of amyloid fibrils — protein AA, the main component of the amyloid substance in secondary amyloidosis in man and also in experimental amyloidosis in several animals, including mice — has provided fresh opportunitie for the study of amyloid formation [1, 8, 10]. Immunologic investigations have shown that the serum of patients with secondary amyloidosis [9, 12], and also of monkeys, mink, rabbits, and mice with experimental amyloidosis [4, 11] contains a high concentration of a protein SAA corresponding antigenically to protein AA of amyloid fibrils. Protein SAA is probably a circulating precursor of fibrillary protein AA [9]. However, the possibility cannot be ruled out that both proteins are formed from a common precursor [4]. By the use of specific antiserum for the immunohistochemical study of the initial stages of experimental amyloidosis in mice it was discovered that the formation of amyloid with the typical staining properties is preceded by deposition of an antigen similar to fibrillary antigen in several organs [2, 10].

Laboratories of Electron Microscopy and General Pathological Anatomy, Institute of Human Morphology, Academy of Medical Sciences of the USSR, Moscow.

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A previous investigation [3] confirmed this fact and also showed that the appearance of structures with the antigenic properties of specific amyloid protein in the mouse myocardium coincides with the formation of interstitial deposits of an electron-dense material with a granular and not a fibrillary ultrastructure. The suggestion was made that the granular material is composed, not only of normal plasma proteins, but also of the above-mentioned antigen.

The object of this investigation was to detect an antigen similar to the antigen of amyloid fibrils in the interstitial deposits of granular material appearing in the myocardium of the mouse in the early stages of casein amyloidosis, by the use of an electron-microscopic immunoperoxidase method [6, 13].

EXPERIMENTAL METHODS

1. Preparation of Pure Antibodies. Antibodies against mouse amyloid protein were obtained from the corresponding rabbit antiserum. Noninbred male rabbits were immunized with hydrolyzed pure filbrillary protein of mouse amyloid obtained by the method of Pras et al. [14]. The collected antiserum was tested by the precipitation test and by Coon's method on sections of organs of normal and amyloid mice. Besides antibodies against amyloid protein, antibodies against plasma proteins and connective-tissue proteins also were present in the serum. After adsorption with an adequate amount of hydrolyzed mixture of homogenate of normal organs and normal plasma, the exhausted antiserum did not react with the structures of normal mouse tissues. To isolate antibodies from the adsorbed antiserum an antigen (specific amyloid protein) bound to a solid adsorbent, namely Sepharose 4B, activated by cyanogen bromide (Sigma, West Germany), by Axen's method [5], was used. Pure goat antibodies against rabbit IgG were conjugated with horseradish peroxidase (RZ = 3.0, Serva, West Germany) by a two-stage method [5].

2. Indirect Immunoperoxidase Method. Amyloidosis was induced in 10 BALB/c mice by subcutaneous injections of a 10% casein solution in 0.25% NaOH solution in doses of 1.0 ml five times a week. Preliminary testing of this method of induction showed that amyloid with the typical staining properties began to appear in the spleen of mice of this strain after two or three injections of casein. The myocardium was investigated after 12 injections of casein. Pieces of different parts of the myocardium were fixed in the cold for 1-2 h with a mixture of 2% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, with the addition of 7% sucrose. After fixation the fragments were washed for 12 h in physiological saline buffered with 0.1 M phosphate buffer in the ratio of 9:1 (BPS) and containg 7% sucrose. After rinsing the fragments in BPS, frozen sections were cut to a thickness of 25 μ. To reduce nonspecific absorption of antibodies in the tissue the sections were incubated for 15-30 min in a 0.5% solution of egg albumin in BPS. Sections of the experimental series were then incubated in a solution of pure rabbit antibodies against fibrillary antigen of mouse amyloid (0.1 mg/ml) at 4°C with constant stirring for 4-6 h. Sections of the control series were treated with a solution of normal rabbit γ-globulin (0.1 mg/ml). After washing in BPS for 10-12 h the sections of the experimental and control series were reincubated in a 0.5% solution of egg albumin, after which they were treated for 4-6 h with a conjugate of pure

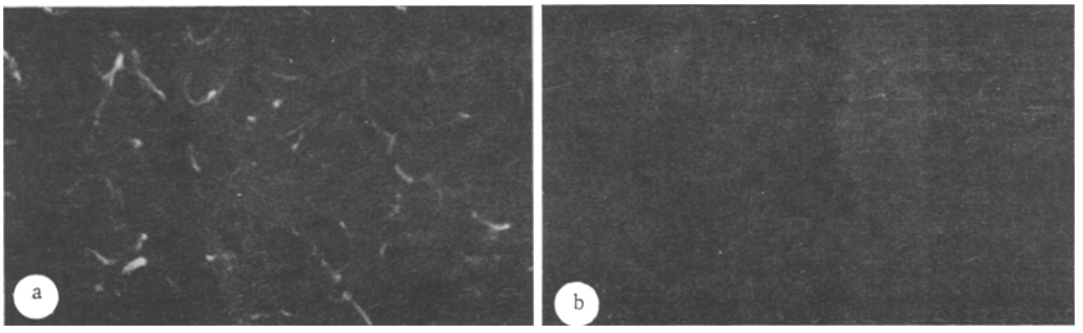

Fig. 1. Amyloidosis of myocardium of mouse after 12 injections of casein. Fluorescent microscope: 60×. a) Specific fluorescence between muscle fibers, along course of sarcolemmas. Treatment with pure rabbit antibodies against amyloid fibrils by indirect Coon's method; b) the same section, stained with thioflavin T. No amyloid visible.

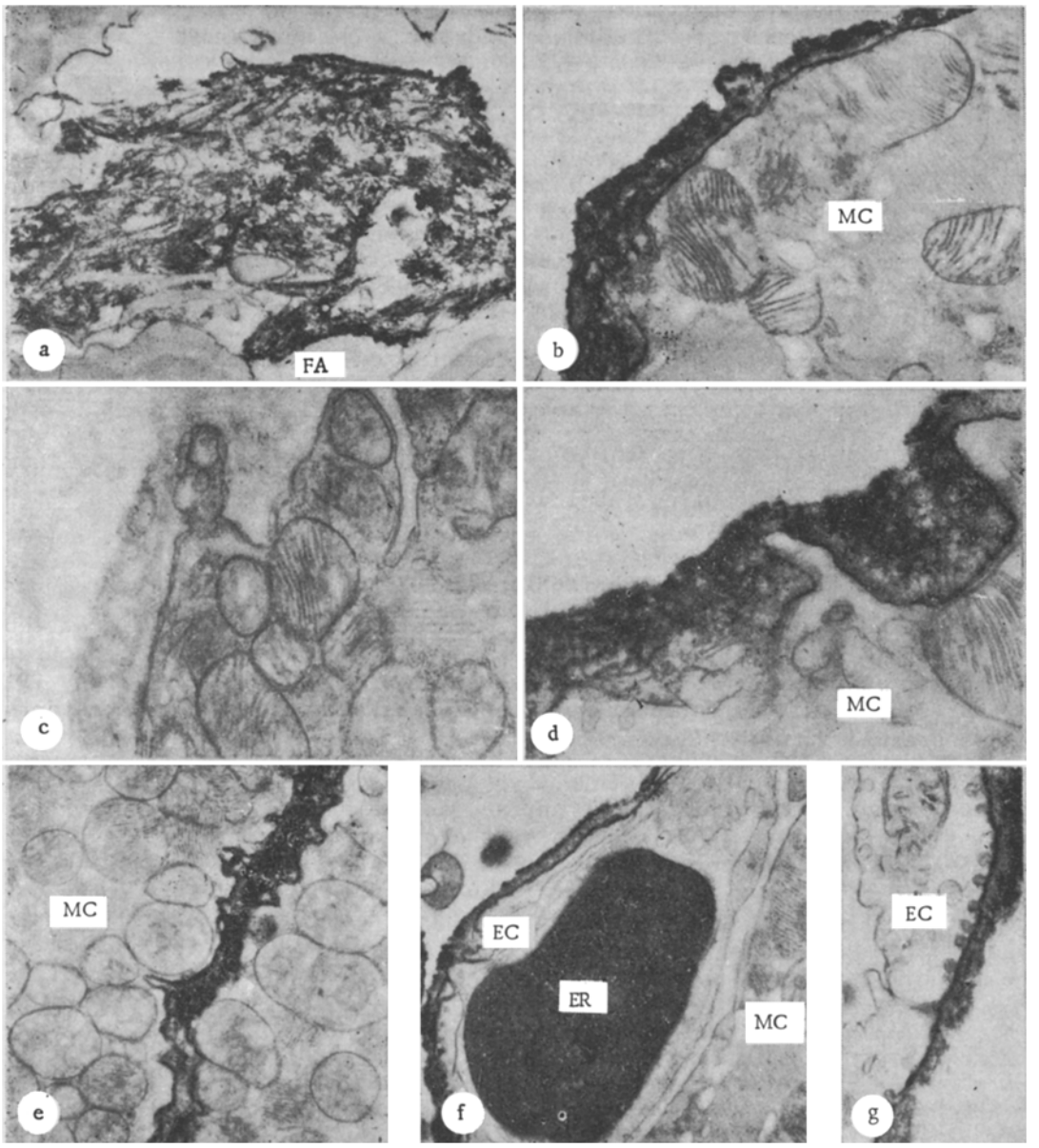

Fig. 2. Immunocytochemical demonstration of antigen of amyloid fibrils in mouse spleen and myocardium. a, b, d, e, f, g) treatment with pure rabbit antibodies against protein of mouse amyloid fibrils by indirect immunoperoxidase method; c) treatment with normal rabbit immunoglobulin by indirect immunoperoxidase method. a) Mouse spleen. Selective deposition of reaction product on fibrillary amyloid masses (FA), 12,000×; b) mouse myocardium. Selective demonstration of deposits of granular material on sarcolemma, 20,000× (here and elsewhere, MC = myocyte); c) deposits of granular material (arrows) on sarcolemma, 20,000×; d) stained granular material under high power of electron microscope, 32,000×; 3) deposits of reaction product in intercellular space of myocardium, 11,000×; f) subendothelial deposition of reaction product. ER) erythrocyte, EC) endothelial cell, 11,000×; g) deposition of reaction product beneath endothelium and in micropinocytotic vesicles, 26,000×.

goat antibodies (0.3 mg/ml) against rabbit γ-globulin with horseradish peroxidase at 4°C, with gentle stirring. The sections were washed for 14-16 h in cold BPS. After rapid washing of the sections with deionized water the reaction for peroxidase activity was carried out by a modified method of Graham and Karnovsky [7]. As a first step, the sections were incubated for 15-20 min in a solution containing 5 mg 3,3'diaminobenzidine tetrahydrochloride (DAB) in 10 ml of 0.05 M Tris-HCl buffer, pH 7.4. The secions were then incubated in the analogous DAB solution with the addition of 10 μ H_2O_2 for each 10 ml of the solution. After washing three times with deionized water the sections were fixed in a 1.33% solution of osmium tetroxide in 0.1 M phosphate buffer, pH 7.4. After rapid dehydration in acetone solutions of increasing concentration, the sections were embedded in a mixture of Epon and Araldite. Ultrathin sections were cut on the LKB Ultratome and examined without additional staining by means of the JEM-100S electron microscope.

To verify the specificity of the pure antibodies used in relation to the selective staining of fibrillary amyloid deposits, i.e., deposits known to contain the specific antigen, pieces of the spleen were taken from two mice and treated by the method described above. At the same time, material was taken from the myocardium of the mice for immunohistochemical investigation. Frozen sections were treated by the indirect Coons' method, using pure goat antibodies against rabbit γ-globulin, labeled with fluorescein isothiocyanate. Serial sections were stained with thioflavin T.

EXPERIMENTAL RESULTS

On immunohistochemical investigation of the myocardium by means of pure antibodies against amyloid fibrillary protein specific fluorescence was found along the course of the sarcolemmas, sometimes with the formation of "sheaths" around the muscle fibers (Fig. 1a), and as interrupted bands at the periphery of the vessels. No amyloid could be detected in serial sections stained with thioflavin T (Fig. 1b).

In the control immunocytochemical investigation of the spleen strict specificity of the pure antibodies against amyloid fibrillary antigen and selective staining of the fibrillary amyloid deposits in the intercellular spaces of the spleen were confirmed (Fig. 2a). Under high power of the electron microscope, deposition of the reaction product was observed exclusively on amyloid fibrils, without any staining of the interfibrillary spaces. Similar selective detection of fibrils took place during simultaneous nonspecific staining of structures possessing endogenous peroxidase activity, i.e., erythrocytes, the granules of neutrophils and eosinophils, and inclusions of macrophages.

Electron-microscopic investigation of control sections of the myocardium showed numerous deposits of finely granular material along the course of the sarcolemmas (Fig. 2c) and in the pericapillary spaces, equal in electron density to the surrounding structures. A study of sections of the myocardium of the experimental series showed selective staining of this material (Fig. 2b, f). Electron-dense granular masses of it were directly adjacent to the sarcolemma (Fig. 2d), filling the investigation, and between 0.1 and 1.2 μ in width. Small intercellular spaces were completely filled with the stained granular material (Fig. 2e). In the region of the capillaries the granular material was distributed directly under the endothelium throughout its extent. In rare cases when the capillary lay very close to the sarcolemma, subendothelial deposits of material stained only on the side of the interstitial space (Fig. 2f). The basal layer of the capillaries in places where the granular masses were deposited could not be found. Most of the micropinocytotic vesicles connected with the basal part of the plasmalemma of the endothelial cells were positively stained (Fig. 2g).

The results described above indicate that in the early stages of casein amyloidosis deposits of electron-dense material containing an antigen similar to the specific antigen of amyloid fibrils, but with a granular and not a fibrillary ultrastructure, appear in the mouse myocardium.

The problem of the origin of the protein AA and or protein SAS, corresponding to it antigenically, and of the relations between them still remains unsolved, and the antigen discovered in the present investigation cannot be identified with any one particular type of protein. However, leaving aside this problem, the results are sufficient to justify the conclusion that an early nonfibrillary stage of amyloid formation exists in the mouse myocardium and is characterized by the intercellular deposition of a protein with the antigenic properties of the specific protein of amyloid fibrils.

LITERATURE CITED

1. V. S. Rukosuev, Byull. Éksp. Biol. Med., No. 12, 99 (1973).
2. V. S. Rukosuev, Arkh. Patol., No. 5, 73 (1974).
3. V. A. Shakhlamov, V. N. Baranov, V. S. Rukosuev, et al., Arkh. Patol., No. 2, 58 (1977).
4. R. Anders et al., J. Immunol., 118, 229 (1977).
5. R. Axen et al., Nature (London), 214, 1302 (1967).
6. J. Burns, Histochemistry, 43, 291 (1975).
7. R. Graham and M. Karnovsky, J. Exp. Med., 124, 1123 (1966).
8. G. Husby and J. Natvig, Clin. Exp. Immunol., 10, 635 (1972).
9. G. Husby and J. Natvig, J. Clin. Invest., 53, 1054 (1974).
10. C. Isersky et al., J. Immunol., 107, 1690 (1971).
11. P. Keith et al., Fed. Proc., 35, 196 (1976).
12. M. Levin et al., J. Exp. Med., 138, 373 (1973).
13. P. Nakane, Acta Endocrinol. (Copenhagen), 153, Suppl., 190 (1971).
14. M. Pras et al., J. Clin. Invest., 47, 924 (1968).
15. L. Sternberger, Immunocytochemistry, Englewood Cliffs, New Jersey (1974).

CYTOSPECTROPHOTOMETRIC INVESTIGATION OF GABA-TRANSAMINASE IN THE RAT CEREBELLAR CORTEX

G. É. Galust'yan and V. A. Pryanishnikov

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577.152.261.087.4:543.42

A modification of a histochemical method for the detection of GABA-transaminase is suggested. Optimal concentrations of substrates and coenzymes were chosen on the basis of an investigation of the enzyme reaction in frozen sections through the rat cerebellar cortex by a quantitative microspectrophotometric method.

KEY WORDS: *GABA-transaminase, cytospectrophotometry; kinetics of histochemical reaction; cerebellar cortex.*

A number of investigations into the connection between GABA, the role of which in inhibition has been studied in detail [4, 10], and the regulation of behavior and the action of various psychotropic drugs have been published in recent years [3, 5, 14]. In 1965 Van Gelder suggested a histochemical method for the detection of GABA-transaminase (GABA-T; 4-aminobutyrate: 2-oxoglutarate aminotransferase, EC 2.6.1.19), the principal enzyme concerned in the metabolism of GABA, with the use of tetrazolium salts [12].

However, no methods of quantitative evaluation of this reaction in individual structures of the section have yet been worked out. The object of the present investigation was the histochemical study of the enzyme reaction for GABA-T in the presence of different concentrations of substrates and coenzymes and at different temperatures in frozen sections of the rat cerebellar cortex.

EXPERIMENTAL METHODS

Experiments were carried out on 15 male albino rats weighing 150-200 g. The animals were killed by decapitation, the brain was removed, pieces of cerebellar tissue measuring 0.5 × 0.5 cm were cut out and frozen in iso-octane cooled with liquid nitrogen. Histochemical reactions were carried out on frozen sections 10, 15, and 20 μ thick. GABA-T in sections of the cerebellar cortex was detected by Van Gelder's method [12]. The pH was adjusted to 8.4 with 1 N NaOH. Agar was replaced by dextran (mol. wt. 20,000) in a concentration of 60 mg/ml. The effect of α-ketoglutarate (α-ketoglutaric acid, disodium salt; from Boehringer

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