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ORIGINAL ARTICLE

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Ultrastructural evidence for colocalization of κ light chain- and β_2-microglobulin-derived amyloids using double labelling immunogold electron microscopy

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Abstract In systemic amyloidosis, it is essential to decide what type of amyloid protein is deposited in tissues before the therapy can be selected and the prognosis assessed in each patient. We examined tissues affected by amyloid deposition from a patient with multiple myeloma by immunohistochemistry and double labelling immunogold electron microscopy and demonstrated colocalization of κ light chain- and β_2-microglobulin-derived amyloids in the same tissue. β_2-Microglobulin-derived amyloid had ultrastructurally characteristic features that distinguished it from κ light chain-derived amyloid. This is the first report of the colocalization of two different amyloids by immunoelectron microscopy.

Key words Systemic amyloidosis · κ Light chain-derived amyloid · β_2-Microglobulin-derived amyloid · Colocalization · Immunogold electron microscopy

Introduction

Amyloidosis is a disorder of protein metabolism characterized by deposition of amyloid fibrils in various tissues and organs. To date, 15 types of amyloid proteins have been identified in humans [12, 25]. Amyloid proteins derived from serum amyloid A (AA), immunoglobulin light chains (AL: Aκ, Aλ), transthyretin (ATTR), and β_2-microglobulin (Aβ2-M) are major constituents in systemic amyloidosis. Despite the close histological resemblance of all amyloid deposits, their pathogenesis appears to differ. In general, a single type of amyloid protein is deposited in any individual with systemic amyloidosis, although the coexistence of two or more different types of amyloid proteins has been reported following immunohistochemical investigations [3, 7, 11, 15, 24].

Only one case of amyloidosis showing the coexistence of two different types of amyloid fibrils has been reported in the literature, and both immunohistochemistry and conventional electron microscopy had been used in the study [7]. Whether the coexistence of different amyloid proteins in a single case is merely a phenomenon of trapped serum proteins within amyloid deposits or whether it represents a coincidental deposition of different amyloid proteins is not clear.

In the present study we identified the colocalization of Aκ and Aβ2M amyloid fibrils in systemic amyloidosis using the double labelling technique of immunogold electron microscopy.

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Materials and methods

The patient, a 62-year-old man, presented with proteinuria at the age of 50 years in 1981, with a clinical diagnosis of hypertensive nephropathy. His renal function steadily deteriorated, and haemodialysis using cuprophan membrane dialyser was conducted in 1987. The cause of renal failure was not confirmed because no renal biopsy was performed during the disease. At that time severe anaemia continued and laboratory data revealed a monoclonal κ light chain spike in serum and urine. Bone marrow aspiration disclosed a diffuse infiltrate of plasma cells comprising 29.6% of the total cell count. Some of these plasma cells had an immature appearance and had two or three nuclei, supporting the diagnosis of multiple myeloma. In 1990, multiple nodules ranging from 3 to 20 mm in greatest dimension appeared in the shoulders and wrists, and in 1992 in the upper eyelids, ear lobes, and tongue. In 1990, a biopsy specimen from the right shoulder disclosed eosinophilic amorphous deposits in the interstitium. In preparations stained with alkaline Congo red [22], the deposits showed birefringence and dichroism under polarized light, indicating that the deposits were amyloid. On immunohistochemical examination (described in detail below), the amyloid reacted with both anti-human amyloid-κ (Aκ) and anti-human β_2-microglobulin (β2-M) antisera. VMP (vindesine, melphalan, prednisolone) chemotherapy for mul-

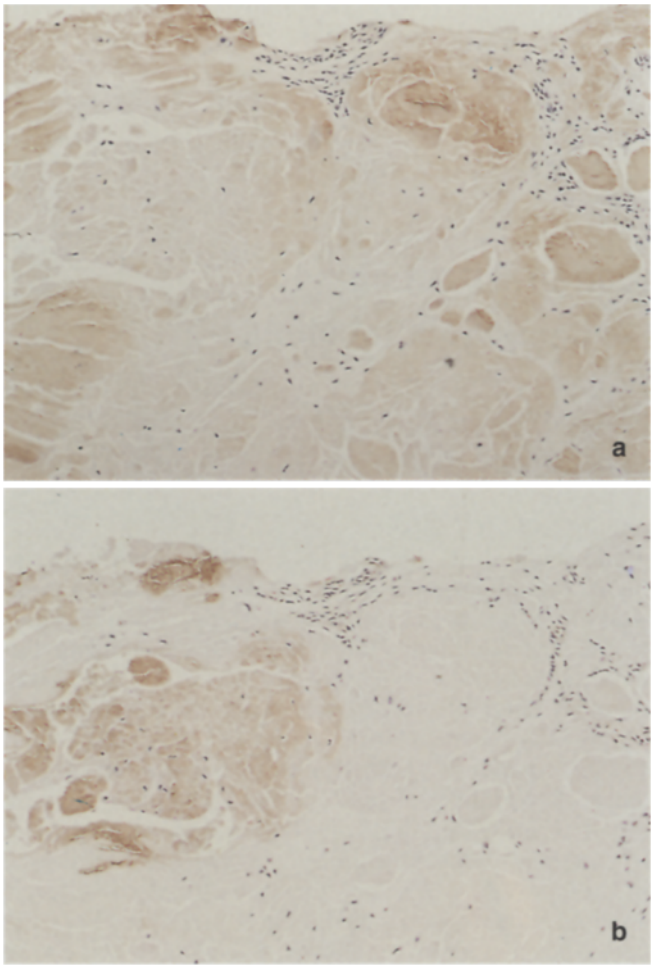

Fig. 1a, b Immunohistochemical staining of the tissue from the right shoulder. **a** Amyloid deposits reacted mainly with anti-Aκ antiserum. **b** A serial section shows focal staining with anti-β_2–microglobulin antiserum. LSAB method, haematoxylin counterstain, ×50

tiple myeloma resulted in a partial remission, but the patient died of acute cardiac failure in his home at the age of 62 years. No autopsy was performed.

Biopsy specimens from the right shoulder, right wrist, right eyelid, and rectum were used for further evaluation of the type of amyloid proteins. Paraffin sections were stained with haematoxylin and eosin and with alkaline Congo red. Formalin-fixed, paraffin-embedded sections of tissues from the right shoulder, right wrist, and right eyelid were used for immunohistochemical study. Small fragments from the right shoulder were fixed in a buffered 2.1% glutaraldehyde solution for electron microscopy.

For immunohistochemistry, formalin-fixed, paraffin-embedded sections were prepared according to the labelled streptavidin biotin (LSAB) method [23]. Primary antibodies used in the present study were: monoclonal anti-human amyloid A protein (AA) antibody (1:5000; Dakopatts, Glostrup, Denmark), polyclonal rabbit anti-human Aκ (1:4000) and anti-human Aλ (1:8000) antisera (anti-human Aκ and Aλ antisera were kindly provided by Dr. George G. Glenner, Department of Pathology, University of California, San Diego, School of Medicine, La Jolla, California; Aκ was produced from patient A68, $V_{\kappa I}$ and Aλ from patient A66, $V_{\lambda II}$ or $V_{\lambda IV}$; production and specificity of these antisera have been described elsewhere [13]), polyclonal rabbit anti-human transthyretin (TTR) antiserum (1:600; Dakopatts), and polyclonal rabbit anti-human β_2-microglobulin (β2-M) antiserum (1:2000; Nordic Immunological Laboratories, Tilburg, The Netherlands). These primary antibodies were incubated overnight at 4° C.

For immunogold electron microscopy glutaraldehyde-fixed tissues were postfixed in 1% osmium tetroxide for 1 h, dehydrated in alcohol, and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate for conventional electron microscopy.

For the immunoelectron microscopic procedure, the blocks were prepared by standard procedures for electron microscopy. Silver grey sections of selected areas were cut and mounted on nickel grids. Before immunocytochemical labelling, thin sections were pretreated for 60 min in a saturated aqueous solution of sodium metaperiodate according to the method of Bendayan and Zollinger [2]. After washing in 0.01 M phosphate-buffered saline (PBS), pH 7.4, the pretreated thin sections were incubated for 30 min in 1% bovine serum albumin in PBS to block nonspecific binding sites. The sections were then transferred onto a drop of polyclonal rabbit anti-Aκ (1:4000) or anti-β2-M (1:10000) antiserum and incubated for 4 h. After a rapid wash in PBS, the sections were incubated with colloidal gold (10-nm and 20-nm gold particles, respectively)-labelled goat anti-rabbit IgG antisera (1:10; 10-nm gold particles: Janssen Biotech, Olen, Belgium; 20-nm gold particles: EY Laboratories, San Mateo, USA) for 1 h. All incubations were performed at room temperature. After washing in PBS, rinsing in distilled water, and drying, sections were stained with uranyl acetate and lead citrate.

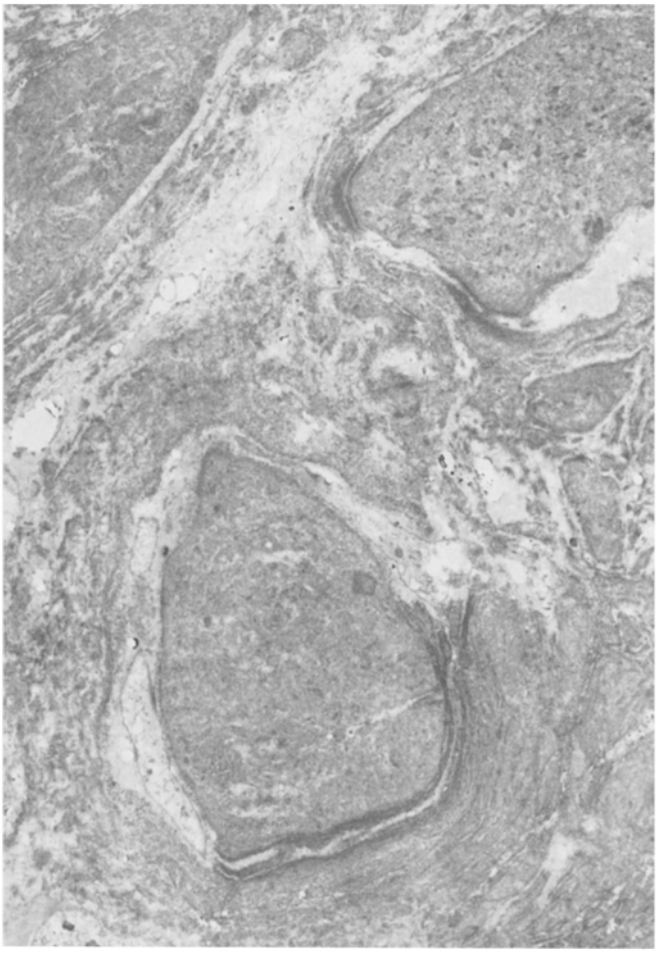

Fig. 2 At a low magnification of conventional electron microscopy, amyloid deposits show a nodular configuration with a rim of electron-dense lamellae. ×2800

The double labelling procedure of immunogold electron microscopy was done according to the method of Bendayan [1]. The tissue sections were mounted on uncoated nickel grids. In this way the tissue sections have two faces exposed. Immunolabelling of one face of the grid was performed as mentioned above with anti-human β2-M antiserum as the primary antibody and colloidal gold-labelled goat anti-rabbit IgG antiserum (20 nm). The other face of the grid was then labelled with anti-human Aκ antiserum as the primary antibody and colloidal gold-labelled goat anti-rabbit IgG antiserum (10 nm). Care was taken not to wet the opposite face during the incubations. The sections were stained on only one face with uranyl acetate and lead citrate. They were examined with Hitachi H-800 and H-7000 electron microscopes. The specificity of immunoelectron microscopic staining was confirmed by replacing the primary antibodies with anti-AA antibody, anti-Aλ antiserum or nonimmune goat serum. Sections from AA, Aκ, Aλ, or Aβ2M amyloidosis were used as positive controls.

Results

The tissue from the right shoulder, right wrist, right eyelid, and rectum revealed massive deposits of eosinophilic homogeneous materials in the interstitium or around small vessels. All stained with alkaline Congo red and showed apple green birefringence under polarized light, indicating the presence of amyloid deposition.

Immunohistochemically, amyloid deposits in the right shoulder and right wrist reacted mainly with Aκ (Fig. 1a), focally with β2-M (Fig. 1b) and weakly with Aλ, but not at all with AA or TTR antiserum. Amyloid reacted with Aκ antiserum tended to be deposited in a nodular configuration, and that reacted with β2-M antiserum appeared to locate around the Aκ amyloid nodules. The tissue from the right eyelid reacted only with Aκ antiserum.

Ultrastructurally the deposits consisted of diffusely distributed fine fibrils. Small nodular to patchy accumulations of the fibrils were occasionally observed. In the areas of the nodular accumulations electron-dense fibrils were seen at the rim of the nodules arranged in a lamellar fashion (Fig. 2). At a higher magnification the inner area of the nodules was composed of rigid, straight, nonbranching fibrils, measuring 8-11 nm in diameter and of indeterminate length, indicating ordinary amyloid fibrils reported in AL or AA amyloidosis. At the rim of the nodules there was a different kind of fibril, which measured 12–14 nm in diameter and had a "curvilinear" or comma-like appearance (Fig. 3). In the diffuse area of amyloid deposits, these two thin and thick fibrils seemed to be intermingled with collagen fibres.

In the single labelling preparations of the immunogold technique, the reaction product (10-nm gold particles) to anti-Aκ antiserum labelled specifically thin amyloid fibrils (8-11 nm diameter) at the inner area of the nodular deposits. These amyloid fibrils were haphazardly arranged without forming bundles (Fig. 4a). However, 20-nm gold particles to anti-β2-M antiserum labelled thick amyloid fibrils (12–14 nm diameter) at the rim of the nodules specifically. These tended to form short curved bundles and were arranged in a whorling fashion (Fig. 4b).

The double labelling preparation clearly demonstrated the topographic localization of the different sizes of gold particles on individual amyloid fibrils in the nodular deposits (Fig. 4c). These different sizes of gold particles did not decorate any amyloid fibril together. In the diffuse area of amyloid deposits, different-sized amyloid fibrils were intermingled and the thin amyloid fibrils were labelled with 10-nm gold particles to anti-Aκ antiserum and the thick ones with 20-nm gold particles to anti-β2-M antiserum (Fig. 5). This immunolabelling was always observed on the amyloid fibrils and no labelling was seen on nonfibrillar structures.

Immunoreaction with anti-human AA and human Aλ antibodies was not seen in amyloid fibrils using immunogold electron microscopy. In sections from positive controls (AA, Aκ, Aλ, or Aβ2M amyloidosis) gold particles with the respective antibodies labelled amyloid fibrils in each type.

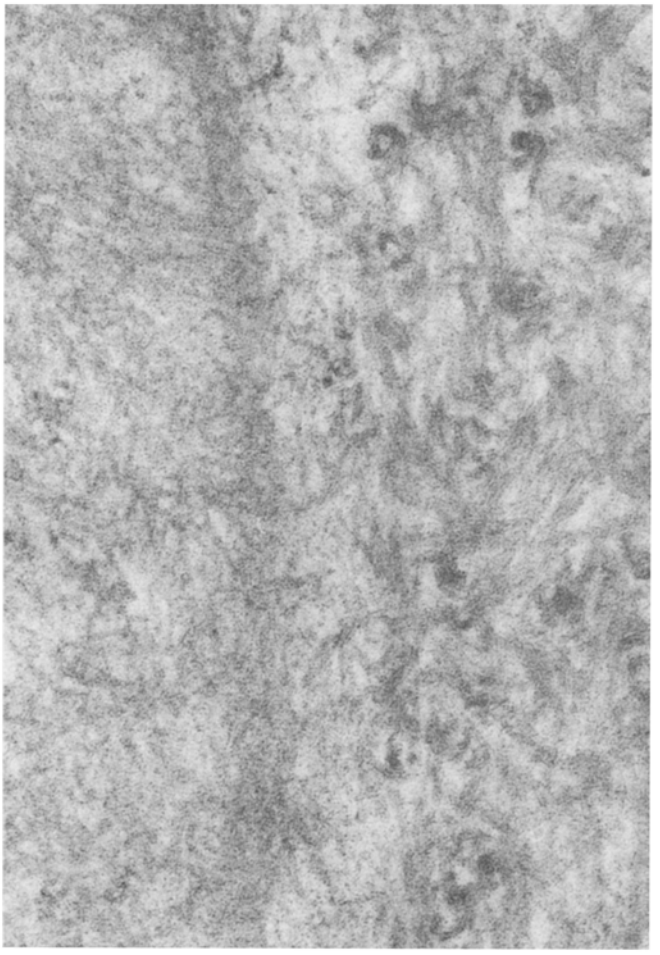

Fig. 3 A higher magnification of the nodular area of amyloid deposit shows the presence of two morphologically different fibrils. One has a thin, rigid, straight configuration and the other has a thick, curvilinear or comma-like appearance. ×110000

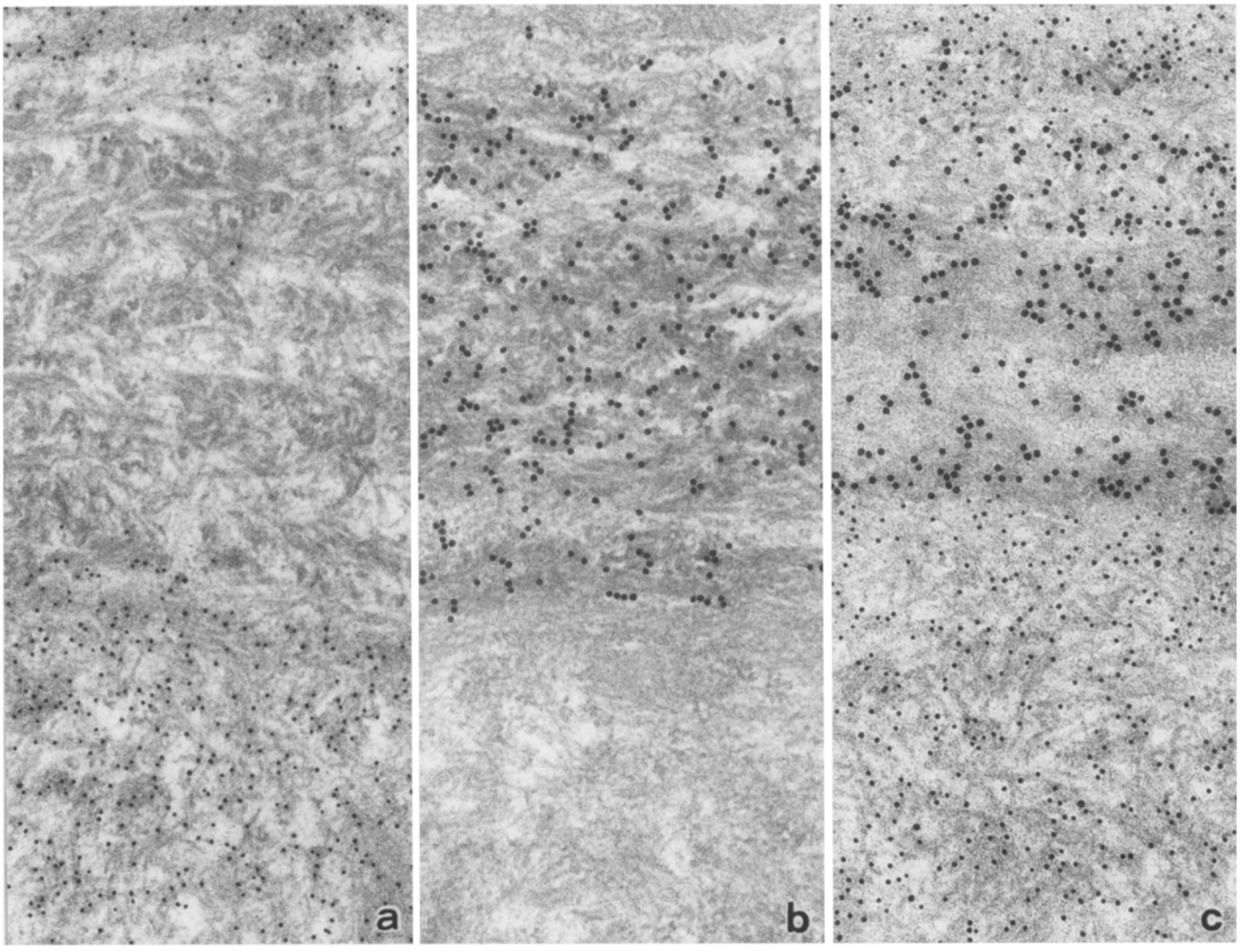

Fig. 4a–c Immunogold electron microscopy at the nodular area of amyloid deposit. **a** Immunoreaction product (10-nm gold particles) to anti-Aκ antiserum is located on thin amyloid fibrils. Single labelling procedure, ×56000. **b** Immunoreaction product (20-nm gold particles) to anti-β_2-microglobulin antiserum is seen on thick amyloid fibrils. Single labelling procedure, ×56000. **c** A double labelling preparation demonstrates different sizes of gold particles on respective amyloid fibrils. Double labelling procedure, ×56 000

Discussion

The immunogold electron microscopic technique has recently been applied for the identification of the subcellular topographic localization of antigenic sites. In amyloidosis, several investigations have been conducted using this technique for the classification and identification of different amyloids using antibodies against amyloid proteins [6, 14]. In the present study, we used a double labelling method with a two-surface reaction in which each antibody bound to antigenic sites was detected by a particular size of colloidal gold probe. Colocalization of Aκ and Aβ2M amyloid fibrils has been clearly demonstrated in the same tissue in a patient with systemic amyloidosis: different-sized gold particles decorated different amyloid fibrils (10-nm gold particles to anti-Aκ antiserum on thin amyloid fibrils and 20-nm gold particles to anti-β2-M antiserum on thick ones).

By light microscopic immunohistochemical methods, coincidental immunoreactivities for two or more amyloid proteins in a single patient with systemic amyloidosis have been reported by several investigators [3, 7, 11, 15, 24]. The coexistence of AA and Aβ2M amyloidoses was most frequently demonstrated in various tissues and organs. There has been no reported case of the coexistence of AL and Aβ2M amyloids. In most of the reported cases two different amyloids showed different distributions in different organs, or even in the same organ. To our knowledge, only three cases in which colocalization of two different amyloids (AA and Aβ2M) has been demonstrated in the same tissue have been found in the literature [3, 7, 11]. In one of them, two morphologically different amyloid fibrils – curvilinear microfibrils characteristic of Aβ2M fibrils and crossed straight microfibrils representing other types of amyloid fibrils – were also demonstrated by conventional electron microscopy [7].

In the present study the amyloid deposits reacted not only with anti-Aκ and anti-β2-M antisera but also with anti-Aλ antiserum on light microscopic immunohisto-

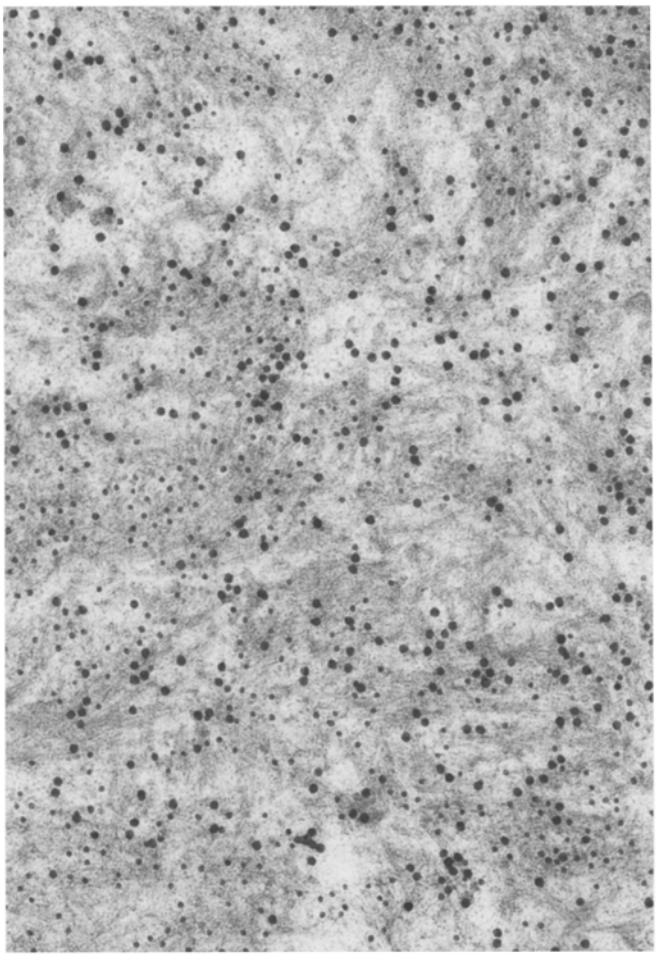

Fig. 5 Immunogold electron microscopy in a diffuse area of amyloid deposit. Two cytochemically different amyloid fibrils, thin amyloid fibrils labelled with 10-nm gold particles to anti-Aκ antiserum and thick amyloid fibrils labelled with 20-nm gold particles to anti-β_2-microglobulin antiserum, are intermingled. Double labelling procedure, ×110000

chemistry. Immunostaining with AL antibodies in AA or Aβ2M amyloidosis seems to result from nonspecific trapping of immunoglobulins within amyloid deposits [20]. However, it has been suggested that in immunogold electron microscopic preparations the relevant antigenicity for trapped plasma proteins is not preserved by the tissue processing or revealed by the etching procedure [6]. In our study, no labelling of gold particles was seen on the amyloid fibrils when anti-Aλ antiserum was applied as primary antibody. Therefore it is reasonable to assume that the immunoreactivity for Aλ in the present light microscopic immunohistochemical study was due to trapped immunoglobulin λ-light chain in amyloid deposits.

Aβ2M amyloidosis affects mainly synovial membranes in patients undergoing chronic haemodialysis and causes osteoarthropathies [8]. Recently Aβ2M amyloid deposits have been demonstrated in various organs other than the osteoarticular region [4] and Aβ2M amyloidosis is generally accepted to be a systemic disease. Controversy exists about the electron microscopic appearance of Aβ2M amyloid. Initial reports demonstrated a peculiar type of fibril with a "curvilinear" appearance [5, 17, 19], but since these several ultrastructural reports have demonstrated β2-M-derived amyloid fibrils with features common to all other chemical types of amyloid [21, 26]. These differences might be related to the fixation of affected tissues, staining of thin sections, or duration of the deposit. Using immunogold electron microscopy, we have studied several patients with Aβ2M amyloidosis who were undergoing chronic haemodialysis, and the fibrils in all patients so far examined have had a peculiar curvilinear appearance and formed short bundles (unpublished data) similar to those seen in the present study. These findings indicate that Aβ2M amyloid shows characteristic ultrastructural features in some circumstances, which distinguish it from other amyloidosis.

AL amyloidosis is one of the best known systemic forms and is often associated with multiple myeloma or related conditions. AL proteins consist of either the whole molecule or fragments of monoclonal immunoglobulin light chains. Fibrillogenesis of AL and AA amyloidosis involves the partial proteolytic cleavage of precursor proteins into fragments with a propensity for aggregating into anti-parallel β-pleated sheets [9]. However, Aβ2M amyloid is composed of the entire sequence of the amyloid proteins (β2-microglobulin), which strongly suggests the polymerization of intact β2-M in tissues [10]. Linke et al. have reported that β2-M fragmentation is common in amyloid deposits and suggested that limited proteolysis with lysine-specific protease(s) play s an important part in the pathogenesis of Aβ2M amyloidosis [16].

Since it is uncertain whether Aκ and Aβ2M amyloids in the present case are derived from whole proteins or fragments and what protease(s) effect(s) in cleavage of the precursor proteins, the underlying mechanism of fibrillogenesis of the amyloids remains unclear. However, predisposing amyloid deposits might be attributable to another amyloid deposition, because amyloid fibrils thereof are known to possess the activity of amyloid-enhancing factor [18].

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