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Widespread aluminium deposition in extracerebral organ systems of patients with dialysis-associated encephalopathy

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Abstract We have described new silver-staining methods for the demonstration of lesions in senile dementia of the Alzheimer type. The same procedure was used to visualize characteristic aluminium (Al)-containing inclusions in choroid epithelium, glia and neurons of the central nervous system in dialysis-associated encephalopathy (DAE). Here we describe the patterns and degree of Al deposition in extracerebral tissues of 12 DAE autopsy cases. Light microscopy of silver-stained paraffin sections demonstrated autonomic ganglion cells filled with numerous intracytoplasmic black-stained fine granular inclusions, which were also seen in endocrine tissues (pituitary, parathyroid and adrenal) and in Leydig cells. Heart, liver cells and the testicular tubules were involved, but decalcified bones, haematopoetic elements, hyperplastic epithelium and one case of malignant epithelium lacked inclusions. Laser microprobe mass analysis revealed prominent Al-related mass signals within the en-bloc silver-stained inclusions which were seen at low intensity in adjacent non-stained structures. Electron microscopy demonstrated accumulations of small electron-dense granules intermingling with lipopigments.

Key words Silver staining · Aluminium
Laser microprobe · Electron microscopy
Dialysis-associated encephalopathy

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Introduction

The possible dangerous role of aluminium (Al) compounds for human health in lung fibrosis or osteomalacia was first suggested in 1957 by Campbell et al. The toxic properties of Al became a serious problem with the introduction of haemodialysis (HD) for chronic renal failure. In 1964, one year after starting HD, Kerr and Ward first described "Newcastle bone disease", defined in 1968 as fracturing dialysis-osteopathy (Schorr). In 1972, Alfrey et al. reported a new encephalopathic syndrome, which was elucidated in 1976 (Alfrey et al.) as Al-induced dialysis encephalopathy. The most recently reported symptom was hypochromic microcytic anaemia (Short et al. 1980). In addition to long-term HD, undialysed patients with chronic renal failure developed Al encephalopathies (Bakir et al. 1986; Russo et al. 1992), and one antacid-induced osteomalacia (Kassem et al. 1991) has been reported. By atomic absorption spectrometry the Al content in various organs has been demonstrated to be 4–40 times higher in bones, muscle and grey matter of the brain when compared with control subjects (Alfrey et al. 1972; McDermott et al. 1978; De Broe et al. 1984; Van de Vyver et al. 1987).

Morphologically, apart from aluminon staining in undecalcified, methacrylate-embedded bones (Maloney et al. 1982) no method has been available to demonstrate and localize Al-containing deposits in paraffin sections. Recently we reported a new silver staining method for the demonstration of Alzheimer lesions in light microscopy (LM) (Reusche 1991; Rosenwald et al. 1993) and at the electron microscope (EM) level (Reusche et al. 1992). The same procedures allowed us, for the first time, to visualize Al-containing deposits in choroid epithelium, glia and neurons of the central nervous system in patients with dialysis-associated encephalopathy (DAE; Reusche and Seydel 1993).

Here we report the morphology and distribution patterns of silver-stained Al-containing inclusions in extracerebral tissues as revealed by LM and EM. Laser

microprobe mass analysis (LAMMA) demonstrated the Al content of the lesions.

Materials and methods

We selected 12 patients with a history of long-term HD showing distinct morphological changes of DAE (Reusche and Seydel 1993). Another 20 haemodialysed patients, with no changes in the CNS, were investigated. Five patients of different ages and sex and suffering from heart disease or carcinoma served as controls.

All available stored material was silver-stained on paraffin sections (Reusche 1991): a colloidal developer was prepared by mixing 1 volume of 2% gelatin, which had been dissolved in 1% formic acid with 2 volumes of 50% silver nitrate solution. Deparaffinized sections, 10 μm thick, were incubated with the developer-silver solution in a moist dark chamber, in a horizontal position and at room temperature, for 45–60 min. During incubation they were covered by a coverslip. Repeated rinsing with distilled water followed. After 2 min of incubation in a 3% sodium thiosulphate solution the slides were rinsed in tap water for 30 min. Finally the slides were dehydrated, cleared and mounted with a synthetic resin.

After evaluation of the silver-stained paraffin sections, small pieces from paraffin blocks of corresponding regions with severe morphological changes were cut out and silver-stained en bloc, as described previously (Reusche et al. 1992).

LAMMA was performed with a LAMMA 500 instrument, a transmission-type microprobe (Leybold-Heraeus, Cologne, Germany), on en-bloc silver-stained semithin sections of 1 μm thickness, which were mounted on electron microscope grids. LAMMA permits the mass spectrometric analysis of small sample volumes. A focussed NdYAG laser pulse ($\lambda = 265$ nm, irradiance 10^8 W/cm^2, spot size ≈ 1 μm) evaporates microvolumes – typically 1 μm^3 to a few μm^3 – in selected areas of the sample, for example within a single cell. The analysed area is selected by an optical microscope. The elemental and molecular ions simultaneously generated in the analysed volume are accelerated in a time-of-flight mass spectrometer (mass resolution m/Δm 650) and detected by an open electron multiplier, which is linked to a transient recorder (LeCroy TR 8818, Heidelberg, Germany) with a sampling rate of 10 ns. Further data processing (e.g. mass calibration) is done by means of a computer (HP A600+, Hewlett-Packard, Bad Homburg, Germany). All generated elemental ions are detected with a sensitivity down to ppm range. In particular for Al the overall sensitivity was determined to 0.2 ppm (Guest 1984). Only qualitative measurements were performed.

Results

Routine haematoxylin and eosin-stained specimens did not reveal any specific changes. Silver-stained paraffin sections of the 12 patients showed varying degrees of Al deposition in various organ systems (Table 1). In particular, ganglion cells of Auerbach's and Meissner's plexuses, as well as neurons in autonomic ganglia (Fig. 1), revealed conspicuous Al inclusions with numerous black-stained fine granules in the cytoplasm (Fig. 1A, B). The endocrine system was strikingly involved in Al storage. The parathyroid glands (Fig. 2A), adeno- and neurohypophysis, cortex of adrenal gland and Leydig cells (Fig. 3A) presented numerous cytoplasmic inclusions. Heart muscle fibres demonstrated deposits of black-stained granules (Fig. 4A), which can be easily distinguished from intermingled lipofuscin with its brown-coloured, larger and tightly packed granules. While striated muscle fibres contained occasional small subsarcolemmal deposits, no significant changes were evident in smooth muscle fibres. Cells of seminiferous

Table 1 Distribution patterns and variable degrees of silver-stained aluminium-containing depositions in paraffin sections of patients with dialysis-associated encephalopathy (*DAE*). None of the patients died of *DAE*. (0 No deeply black, fine-granular silver-stained inclusions; 1 few granules in single cells; 2 granules in several cells; 3 numerous granules in nearly all cells; – not available; *a* autolysis; *h* hyperplasia; *L* lipofuscin; *f* female; *m* male)

Case number	1	2	3	4	5	6	7	8	9	10	11	12
Age	45	56	52	73	65	62	65	50	38	62	55	52
Sex	f	m	m	f	f	m	f	m	m	m	m	m
Year of autopsy	92	92	91	91	89	88	88	88	87	86	84	81
Years of dialysis	6	12	13	6	9	–	8	3	3	6	4	12
Choroid epithelium	3	3	3	3	3	3	3	2	2	2	–	3
Neurons in central nervous system	1	2	3	3	2	1	1	1	1	1	–	1
Autonomic ganglia	3	3	3	–	–	–	–	3	–	–	–	3
Auerbach plexus	2	2	3	3	2	3	3	2	2	–		3
Adenohypophysis	2	3	3	3	2	2	2	1	–	–	–	–
Neurohypophysis	2	3	3	3	2	3	2	2	–	–	–	–
Parathyroid gland	3	3/0h	3	3	–	3	–	2	1/0h	–	2	–
Adrenal gland	3	3	3	a	2	2	3	2	3	3	2/a	3/a
Langerhans islets	a	a	2	3	1	2	a	1	2	3	2	3
Leydig cells	–	3	3	–	–	3	–	2	2	–	2	3
Heart muscle	2	2	3	3	2	1	3	1	1	2	1	3
Striated muscle	–	1	1	–	–	–	1	–	–	–	–	1
Seminiferous tubules	–	2	3	–	–	2	–	1	1	–	2	3
Pancreas	a	a	1	3	1	1	a	1	2	3	1	3
Thyroid gland	2	2	3	3	3	1	2	1	2	2	1	2
Liver cells	2L	2L	2L	2L	2	2	1L	1L	2	3	2	3L

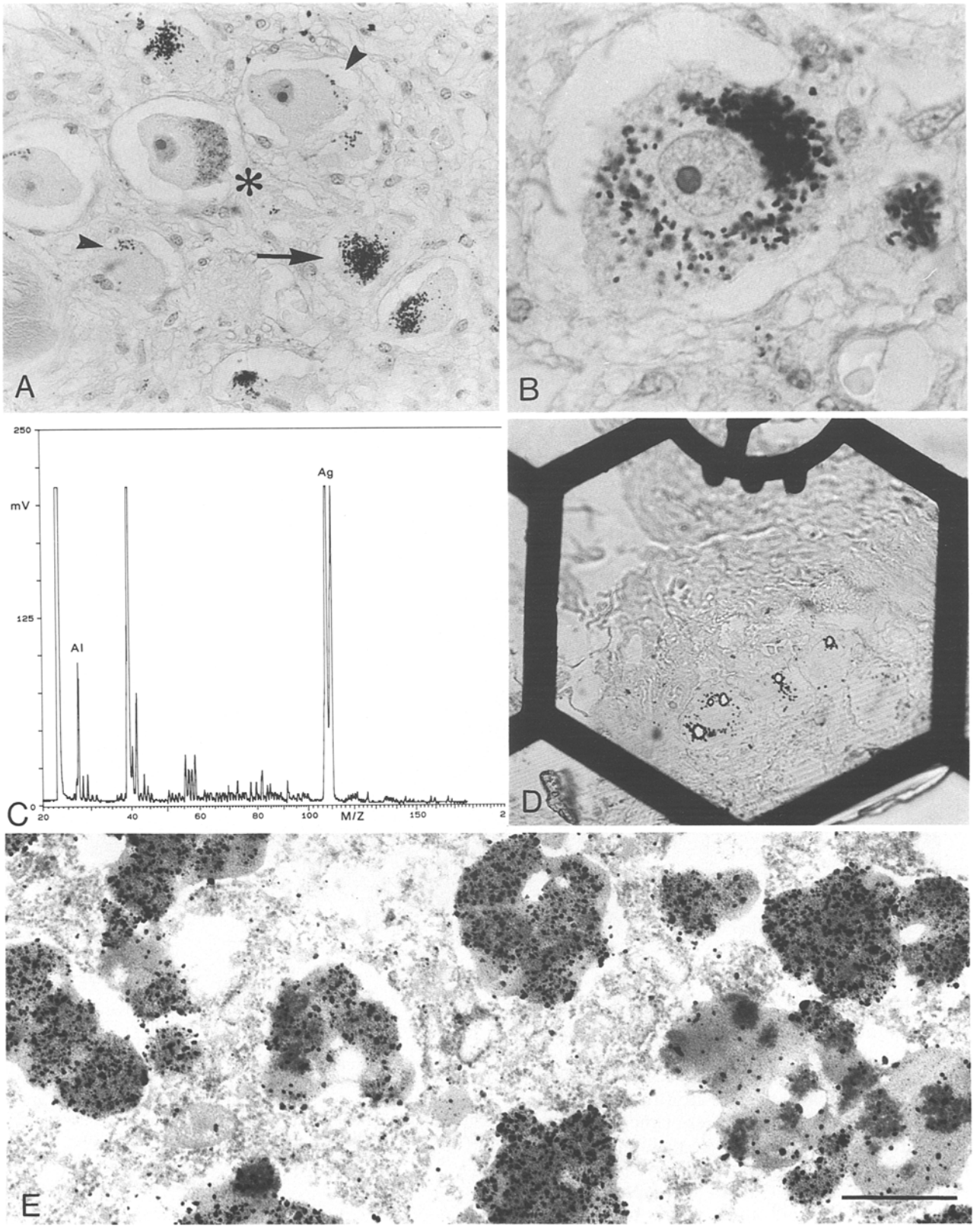

Fig. 1A–E Neurons of the coeliac plexus. **A** Ganglion cells with deposits of numerous (*arrow*) and sparse (*arrowhead*) black-stained neuronal inclusions and faintly stained lipofuscin (*asterisk*); silver-stained, paraffin, × 360. **B** Higher magnification with deeply black-stained granular cytoplasmic aluminium (Al)-containing inclusions, surrounding an intact nucleus, silver-stained, paraffin, × 1040. **C** Laser microprobe mass analysis (LAMMA) spectrum with Al-related mass signal m/z 27 and m/z 107/109 for silver. **D** LAMMA grid with several ganglion cells and laser-beam-induced perforations, directed to silver-stained structures; en-bloc silver-staining, × 240. **E** Electron micrograph from neuronal cytoplasm with non membrane-bound amounts of small electron-dense granules, intermingled with loose lipopigment, × 38 400, *bar* = 0.5 µm

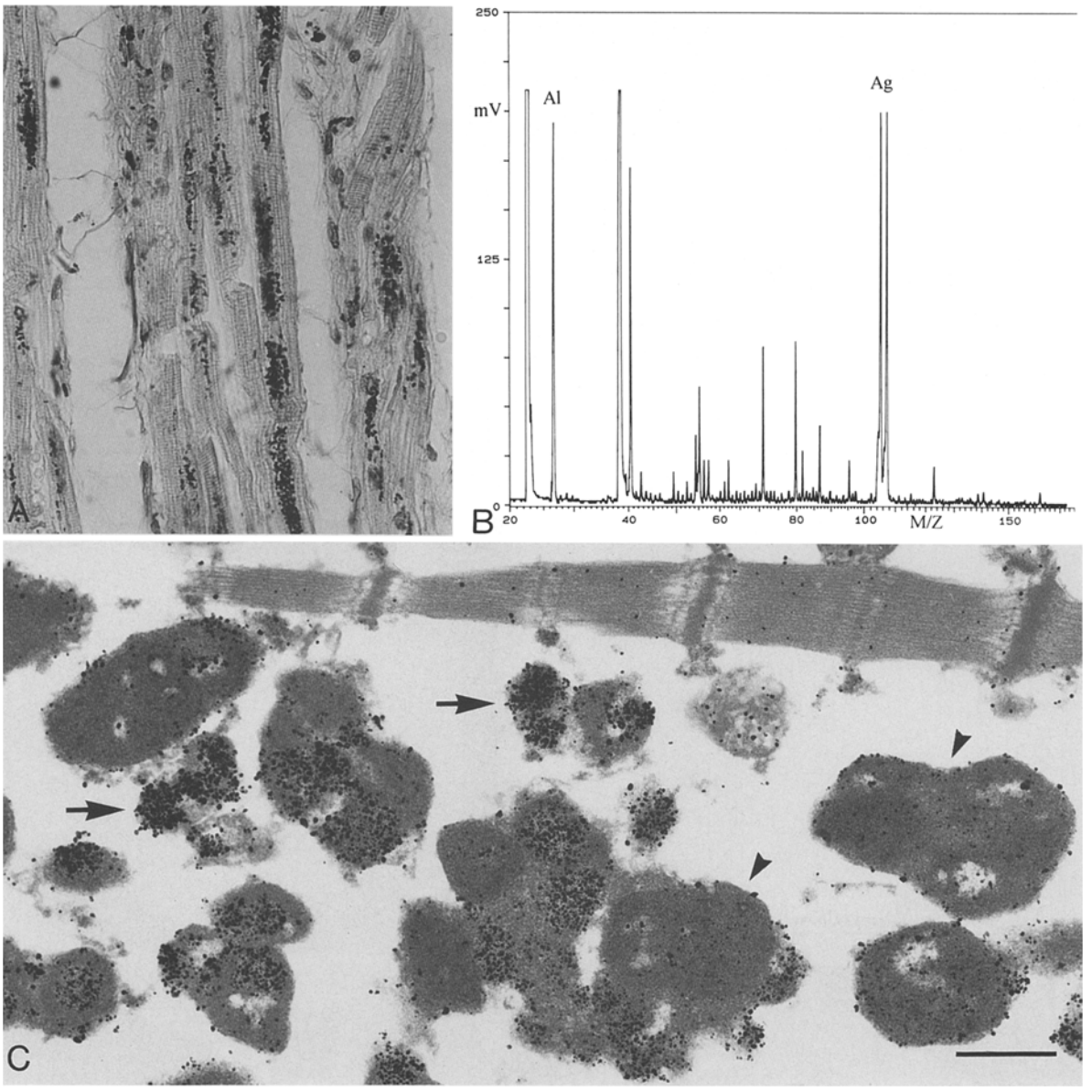

Fig. 2A–C Heart muscle. **A** Longitudinal section of muscle fibres with massive deeply black-stained aluminum (Al)-containing deposits; silver-stained paraffin section, ×220. **B** LAMMA spectrum with prominent Al-related mass signal at 27 and at m/z 107/109 for silver. **C** Muscle fibre and different intensity of non membrane-bound fine granular electron dense deposits (*arrow*), intermingling lipofuscin which is partly free of inclusions (*arrowhead*); electron micrograph × 31 900, *bar* = 0.5 μm

tubules (Fig. 3A) were distinctly involved. Liver cells presented amounts of coarse (periodic acid-Schiff-positive) lipopigment granules beside typical inclusions. Kupffer cells sometimes revealed silver-stained granules. Nonspecific precipitates were seen in thyroid gland epithelium and the cortical structures of end-stage kidneys were covered with massive silver precipitations. Postmortem autolysis of adrenal glands and pancreatic tissue in particular caused a loss of argyrophilic reactions.

Cells of the lymphoid and haematopoetic series in the spleen, bone marrow and blood vessels and hyperplastic epithelial cells (case 2, Fig. 2A) and one carcinoma (case 6, Fig. 5A) displayed a lack of silver-stained Al inclusions. Lung tissue did not contain apparent Al inclusions. Decalcified paraffinized bones did not show silver-stained granular deposition. The interface between calcified bone and osteoid was demonstrated as a faintly stained structure, similar to the intensity of staining shown in the control cases.

Five control cases and the 20 HD patients without DAE changes showed no typical silver-stained inclusions.

LAMMA revealed prominent mass signals at m/z 27 in silver-stained structures within the cytoplasm of en-bloc stained specimens, confirming the Al content of the

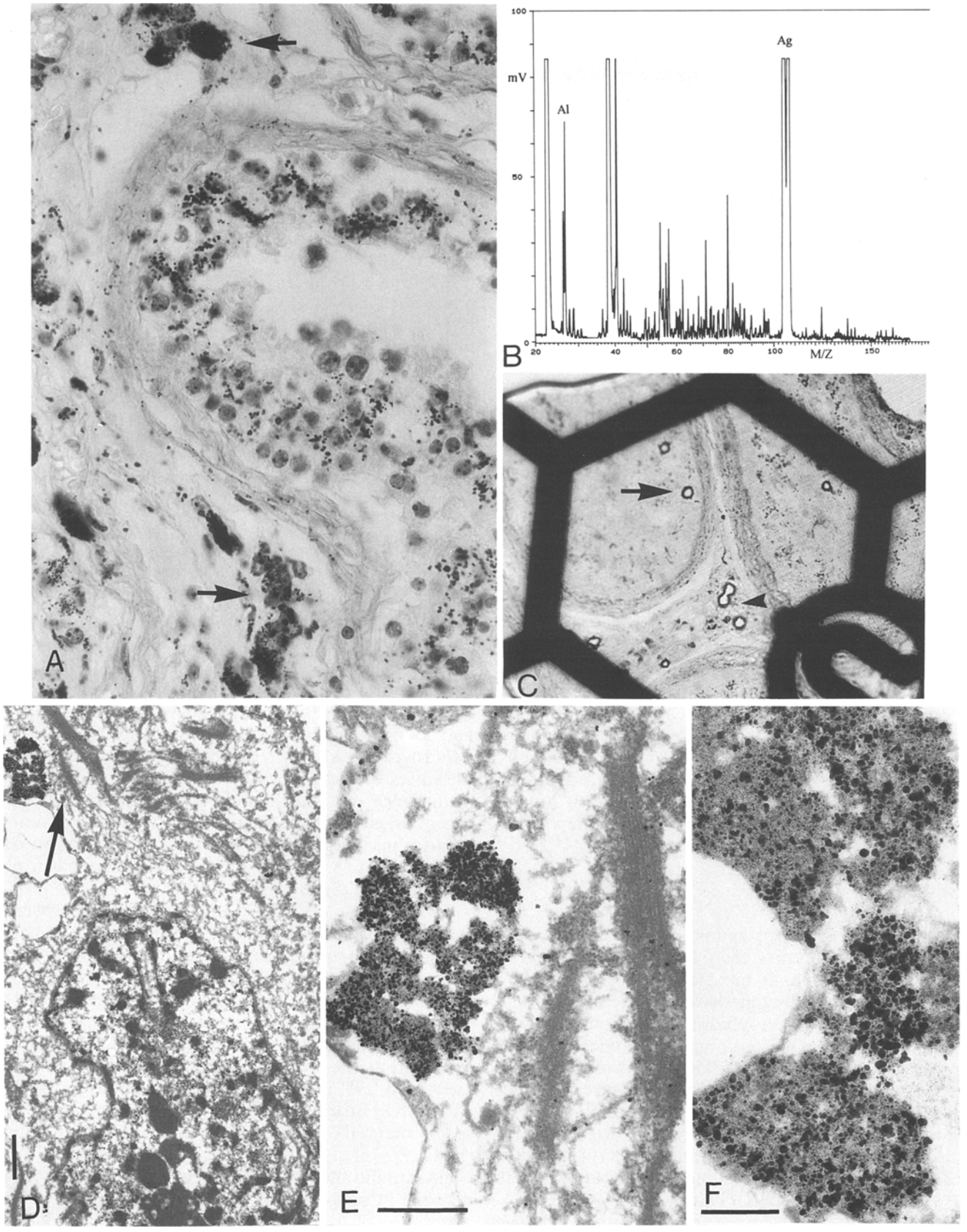

Fig. 3A–F Testis. **A** Section of seminiferous tubule with numerous black-stained inclusions in tubules and Leydig cells (*arrow*); silver-stained, paraffin, × 440. **B** LAMMA spectrum of silver-stained inclusions in tubules with prominent mass signal at 27 for Al and at 107/109 for silver. **C** LAMMA-prepared grid with laser-beam-induced perforations in tubules (*arrow*) and Leydig cells (*arrowhead*), directed to en-bloc silver-stained Al-containing structures. **D** Sertoli cell with cytoplasmic electron-dense inclusions and Charcot-Boettcher's crystalloid, (*arrow*, electron micrograph, × 8150, *bar* = 1 μm); **E** Higher magnification of **D** (*arrow*) with non membrane-bound electron dense granules, close to a Charcot-Boettcher's crystalloid (electronmicrograph, 32 400, *bar* = 0.5 μm); **F** Leydig cell cytoplasm with amounts of electron dense small granules, mingled with loose lipopigment (electronmicrograph, × 68 800, *bar* = 0.2 μm)

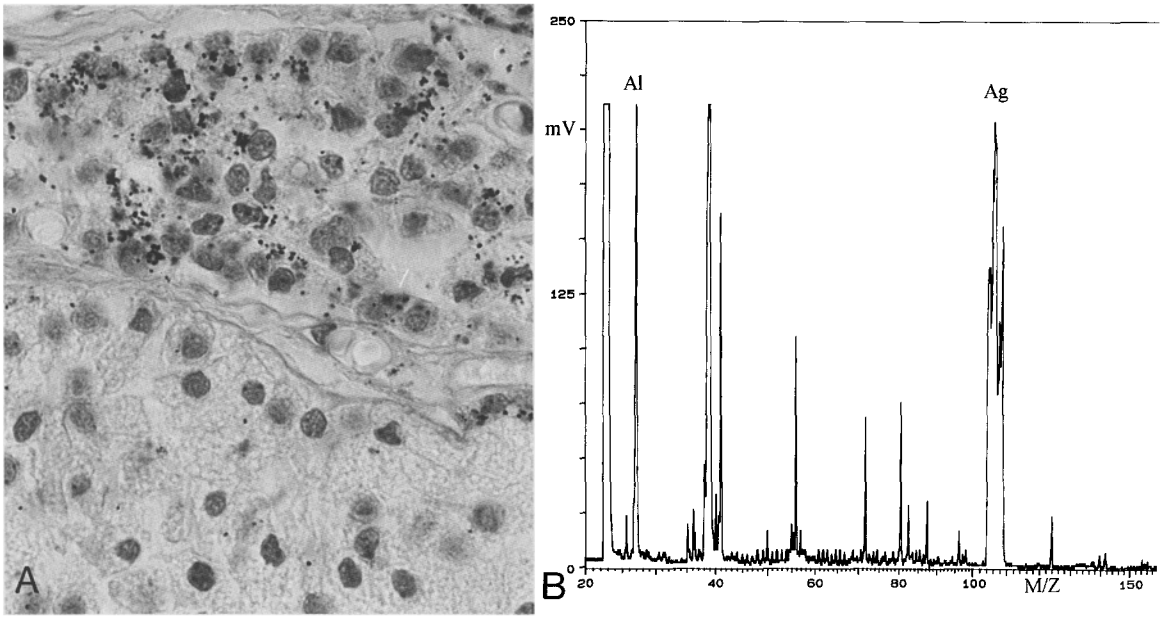

Fig. 4A, B Parathyroid gland. **A** Glandular tissue with numerous cytoplasmic Al inclusions (*upper half*) and hyperplasia with a striking lack of black-stained inclusions (*lower half*); silver-stained, paraffin, × 860. **B** LAMMA spectrum with prominent Al-related signal at m/z 27

lesions (Figs. 1C, 2B, 3B, 4B). LAMMA signals of low intensity for Al were obtained in nuclei and adjacent non-silver-stained structures. Carcinoma cells lacked Al-related signals (Fig. 5B).

EM of heart muscle fibres showed accumulations of non-membrane-bound electron-dense fine granules, intermingled with large amounts of lipopigment (Fig. 4C). Similar fine granular non-membrane-bound deposits, intermingled with loose lipopigment, were found in the cytoplasm of neurons (Fig. 1D), Leydig cells, seminiferous tubules (Fig. 3D–F), liver cells and parathyroid cells. Lysosomal structures (of the severely changed and re-embedded material) were not seen. EM investigation of one control case did not reveal comparable deposits.

Discussion

Although the Al intoxication syndrome has been known for over 20 years, there has been no method to visualize Al compounds in paraffin-embedded tissues. With our silver-staining method we have been able to demonstrate the intracytoplasmic deposition of Al-containing inclusions in various organs light microscopically. Laser microprobing confirmed the considerable Al content of the silver-stained lesions. EM revealed accumulations of electron-dense granules, intermingled with amounts of lipopigment. The latter have been described both experimentally in rats (Galle et al. 1980) and in neurons of long-term HD patients (Sabouraud et al. 1978; Reusche and Seydel 1993).

In 1978 four cases were reported of young long-term HD patients who had died after sudden cardiac events, without a history of hypertension or ischaemic heart disease (Elliott et al.). Whether there is an effect of Al deposition in autonomic ganglion cells resulting in autonomic dysfunction (Zucchelli et al. 1985) is uncertain and what effect might be caused by severe Al deposition in heart muscle is unknown. It is also uncertain what the effect of Al deposits in central neurons, pituitary gland and testes might be on the arrest of spermatogenesis (Holdsworth et al. 1977), on libido and on potency. Disturbances of these functions are said to be caused by disturbance of the hypothalamo-pituitary-testicular axis in uraemia (Campese and Liu 1990). Al-containing deposits may suppress the parathyroid gland as an Al-induced relative hypoparathyroidism described in osteodystrophia (Schorr 1968; Ellis et al. 1977).

Proliferating cells in haematopoesis, hyperplastic states and in a carcinoma lacked evidence of Al inclusions, thus supporting the assumption that time is needed to store visible quantities of Al compounds.

A well-known method of demonstrating Al in dialysis-osteopathy is the histochemical aluminon reaction on undecalcified, methacrylate-embedded bone, which shows Al at the osteoid/calcified bone interface (Maloney et al. 1982), demonstrated by previous LAMMA studies (De Broe et al. 1984) and in atomic absorption spectrometry (AAS) with 70% of the entire Al in bones (Van de Vyver et al. 1987). Our material was from decalcified paraffin blocks, so we could not demonstrate Al deposits in silver-stained paraffin sections, due to the dissolution of Al compounds by the concentrated acids of the decalcifying agents. LAMMA investigations were only qualitative providing confirmation of the Al content in en-bloc silver-stained intracytoplasmic inclusions. Comparative AAS investigations could not be done on the stored paraffin-embedded material.

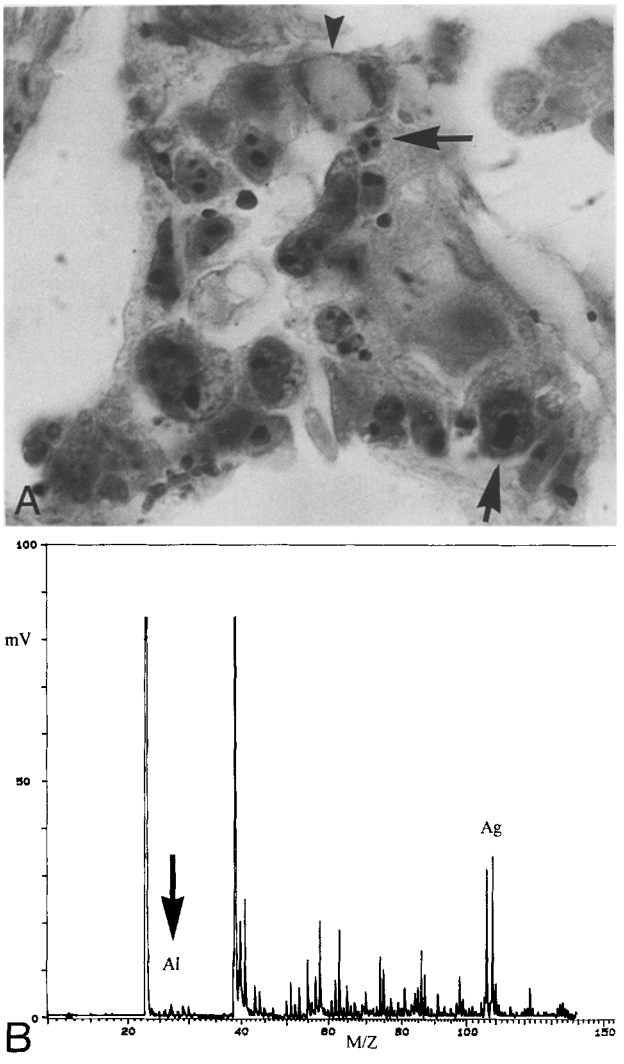

Fig. 5A, B Carcinoma. **A** Adenocarcinoma with a signet ring cell (*arrowhead*), and increased numbers of brownish-coloured, partly enlarged nucleolar organizer regions (*arrows*). Note the lack of black-stained fine granular Al inclusions; silver-stained, paraffin, ×820. **B** LAMMA spectrum with lack of Al peak at m/z 27 (*arrow*); low peaks at m/z 107 and 109 result from silver contamination

Originally, the silver-staining methods were developed for the demonstration of nucleolar organizer regions (NORs; Howell and Black 1980; Ploton et al. 1982). We applied the procedures for the demonstration of Alzheimer lesions, including neurofibrillary (tau protein) changes as well as all kinds of β/A4 amyloid protein (Reusche 1991; Reusche et al. 1992; Rosenwald et al. 1993).

Certainly, the silver-staining reactions described are not specific for Al. However, there are obviously sensitive argyrophilic reactions to Al-containing (proteinaceous?) compounds, which stain the characteristic intracytoplasmic inclusions seen exclusively in DAE cases (Reusche and Seydel 1993). Loss of argyrophilia in autolytic tissues points to staining reaction with Al/protein compounds. We could not visualize the Al of air-borne origin in lungs (Skalsky and Carchman 1983).

The effects of the duration of dialysis and/or ingestion of Al-containing phosphate binders (Russo et al. 1992) on Al deposition requires statistical evaluation. Even in younger patients there is an accelerated deposition of lipopigments, possibly as the result of failing lysosomal digestion (Ghadially 1988).

Widespread intracytoplasmic Al deposition in various organ systems, visualized for the first time, may provide additional information in understanding the manifold clinical symptoms of long-term a HD.

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