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Neurofilament profile in olfactory mucosa of patients with a clinical diagnosis of Alzheimer's disease

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Abstract In an attempt to find a reliable peripheral marker of Alzheimer's disease (AD), pieces of olfactory mucosa were removed by biopsy from 11 patients with probable AD and from eight control patients. The samples were analysed immunocytochemically using monoclonal and polyclonal antibodies. The olfactory and peripheral neurons of the olfactory mucosa in both AD and control patients typically exhibited immunoreactivity to neurofilament (NF) triplet proteins, including both phosphorylated and non-phosphorylated epitopes, as well as to synaptophysin, but lacked reactivity to other intermediate filament proteins, microtubule-associated protein 2 and tau. Our results do not support the recent findings suggesting the lack of NF proteins in olfactory neurons or the preferential phosphorylated status of NF proteins in olfactory neurons solely in AD.

Key words Alzheimer's disease · Antibodies
Neurofilament · Olfactory mucosa

Introduction

In spite of progress in clinical and pathological diagnosis of Alzheimer's disease (AD), a reliable peripheral marker of the disease is still lacking. The demonstration of neurofibrillary tangles and senile plaques in brain autopsy is currently the requirement for definite diagnosis of AD (McKhann et al. 1984). A peripheral biological or diagnostic marker would greatly facilitate the clinical diagnosis of AD and might even replace the neuropathological examination.

The human olfactory mucosa, which contains neurons of central origin, can be reached and a piece removed by biopsy relatively easily (Lovell et al. 1982). In addition, several clinical studies have demonstrated abnormalities in the ability to detect and identify odours in AD; olfactory deficit may even be one of the earliest symptoms of AD (Ferreyra-Moyano and Barragan 1989).

The nasal epithelium from AD patients has been reported to contain areas of increased reactivity to some neurofilament (NF) antibodies and abnormal neuronal structures, while the normal olfactory mucosal nerves lack NF proteins (Talamo et al. 1989; Trojanowski et al. 1991). Recently Tabaton et al. (1991) demonstrated that the olfactory mucosa, removed by biopsy from AD patients, contains tau- and ubiquitin-reactive neurites, and the neuronal changes are morphologically identical to those found in the brain.

In the present study we have attempted to confirm the earlier nasal findings in AD using immunocytochemical markers similar to those used in previous studies. To characterize possible changes in nasal mucosa further, a more extensive immunocytochemical examination with some other NF and intermediate filament antibodies was included. Our results do not support the differential expression of phosphorylated NF proteins in AD.

Materials and methods

Nasal mucosa biopsy samples were obtained from 11 patients with probable AD and eight control patients (Table 1). The demented patients were clinically evaluated and diagnosed as having probable AD according to currently accepted clinical criteria (McKhann et al. 1984; American Psychiatric Association 1987). If two or more patients with probable AD were found in the same family, the diagnosis was familial AD. All the demented patients underwent computed tomography of the brain, laboratory tests for exclusion of secondary dementias, electroencephalography, ^{99m}Tc-HM-PAO single photon emission tomography, Mini-Men-

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Table 1 Clinical characteristics of probable Alzheimer's disease (*AD*) and control patients (*MMSE* Mini-Mental State Examination)

AD patients (initials)	Sex	Age (years)	Clinical diagnosis	MMSE score
EK	Male	55	AD, moderate	19
EB	Male	72	AD, moderate	16
PO	Male	56	AD, moderate	15
AS	Female	69	AD, moderate-severe	10
LS	Male	72	AD, moderate, familial	16
KS	Male	76	AD, severe	Non-testable
SS	Female	60	AD (frontal type), mild	21
ER[a]	Male	52	AD, mild, familial	26
MP	Female	58	AD, mild, familial	18
AA	Female	54	AD, moderate	14
SV	Female	55	AD, moderate	15
Controls				
EV	Female	51	Recidivous maxillary sinusitis	
PR	Female	32	Recidivous maxillary sinusitis	
VH	Male	17	Nasal septal deviation	
FP	Female	72	Dysphagia, polymyositis	
TA	Female	40	Recidivous maxillary sinusitis	
BL	Female	78	Laryngeal carcinoma	
SP	Female	71	Laryngeal carcinoma	
OS	Male	62	Metastatic carcinoma of neck	

[a] This patient's father had a neuropathologically confirmed AD

tal State Examination (Folstein et al. 1976; see Table 1) and neuropsychological investigation. Hachinski's ischaemic score (Hachinski et al. 1975) was 2 or less in all cases. The accuracy of antemortem diagnosis has varied in our previous patient series from 82% to 89% (Sulkava et al. 1983; Erkinjuntti et al. 1988). The control patients were neurologically healthy persons undergoing operation in the nasal cavity. Informed consent was obtained from every patient or his/her relative. The study was accepted by the ethical committees of the Departments of Neurology and Otorhinolaryngology of the University of Helsinki.

Biopsy of olfactory mucosa was performed using the modified technique of Lovell et al. (1982). Briefly, under local anaesthesia (lidocaine 4%) a piece of olfactory mucosa (1–3 mm^2) was removed with a cup forceps from the upper part of septum using a rhinoscope. The biopsy sample was immediately frozen in liquid nitrogen and stored at −80° C until used. No complications, except for slight temporary haemorrhage, occurred during biopsy.

For indirect immunofluorescence the frozen samples were cut into 4, 10 or 15 μm sections. Haematoxylin and eosin staining was used to control the histological quality of the specimens. Then they were exposed to primary monoclonal (mAb) or polyclonal antibodies followed by fluorescein isothiocyanate coupled second antibodies (Cappel Laboratories, West Chester, Pa., USA). The mAb used were: mAb 13AA8, specific for all NF 200 kDa epitopes (Virtanen et al. 1985); 1A3, specific for the phosphorylated 200 kDa NF protein; 14BA8, reacting with the 68 kDa and 200 kDa NF proteins; a mAb against the 68 kDa NF protein (Dakopatts, Glostrup, Denmark); 65EE3, reacting with vimentin (Virtanen et al. 1986); 37EE3, reacting with desmin (Virtanen et al. 1986); 2A4, reacting with cytokeratins 8, 18 and 19 (Virtanen et al. 1985); and SY 38, reacting with synaptophysin (Wiedenmann and Franke 1985). mAb against the microtubule-associated protein 2 (MAP2) and two independently raised mAb against tau, clone tau-2 and clone tau, were obtained from Sigma (St. Louis, Mo., USA) and Chemiscon (Temecula, Calif., USA). The polyclonal antisera used were rabbit antibodies to the 68 kDa, 150 kDa and 200 kDa NF proteins (Dahl 1983), and those recognizing a phosphorylated epitope in the 200 kDa (Con 59) and in the 68 kDa (Con 57) NF protein, or recognizing both the phosphorylated and the non-phosphorylated 200 kDa (Con 62) NF protein (Mencarelli et al. 1987; kindly provided by Prof. V. Pallini, Department of Biology, University of Siena, Siena, Italy).

All the specimens were also examined by the sensitive immunohistochemical bridge method of alkaline phosphatase anti-alkaline phosphatase (Dako). This method gave identical results, but for documentation immunofluorescence was preferred.

Results

In routine histology the specimens were well preserved and mostly showed an intact epithelium, mainly as described by Nakashima et al. (1984). The immunohistochemical results are summarized in Table 2. In olfactory tissue of the controls the olfactory nerves, close to the

Table 2 Results from immunostaining of olfactory epithelium (*OE*) cells (*MAP* microtubule-associated protein; *NF* neurofilament; *p* phosphorylated epitope; *PNS* peripheral nervous system; *SYN* synaptophysin; + present; − absent)

Peptide	Perikaryon of OE neuron	Olfactory nerve	PNS nerve
NF 68	−	+	+
NF 150	−	+	+
NF 200	−	+	+
NF 150(p)	−	+	+
NF 200(p)	−	+	+
MAP2	−	−	−
SYN	−	+	+
Tau	−	−	−
Vimentin	−	−	−

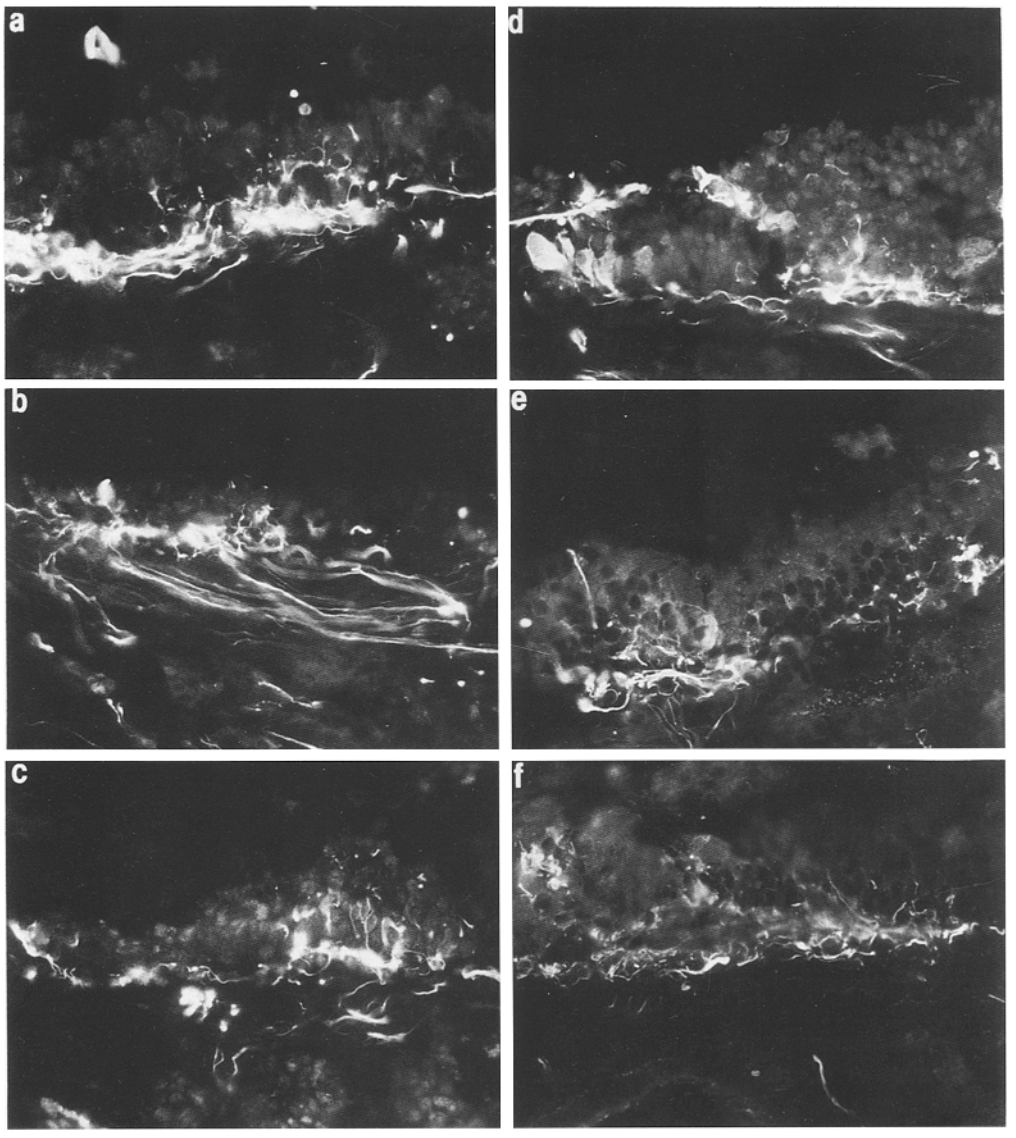

Fig. 1 Immunohistochemistry of specimens from olfactory mucosa of control (**a–c**) and Alzheimer's disease (AD) patients (**d–f**) reacted with the con 57 antiserum (**a, d**), the monoclonal antibody (mAb) 13AA8 (**b, e**) and mAb 1A3 (**c, f**). Note that dense arrays of subepithelial immunoreactive nerve fibres are seen in all cases together with fibres amidst the epithelial cells. (× 200)

olfactory epithelium (OE), showed bright immunoreactivity to phosphorylated NF 68 as revealed with the Con 57 polyclonal antiserum (Fig. 1a) and with other antibodies to NF 68 used. Similar immunoreactivity was seen with the mAb 13AA8 reacting with all NF 200 epitopes (Fig. 1 b) and with the mAb 1A3 reacting with a phosphorylated variant of NF 200 (Fig. 1c).

Identical reactions were obtained with the same antibodies in olfactory nerves taken from AD patients (Fig. 1d–f). In the specimens from both controls and AD patients also the peripheral nerves, deeper in the tissue, showed an identical immunoprofile for NFs as shown with the mAb 13AA8 (Fig. 2a–d). Double staining of control (not shown) and AD specimens demonstrated that the NF-reactive olfactory nerves, as revealed with polyclonal anti-200 NF serum (Fig. 2e), did not show immunoreactivity to vimentin (mAb 65EE3; Fig. 2f).

Immunoreactivity to synaptophysin was similarly seen in fibres close to the OE (Fig. 3a) and in peripheral nerves (Fig. 3 b) in both the control (not shown) and the AD specimens. When tested with antibodies for MAP2 and tau, all the specimens were negative. However, in control experiments both mAb against tau as well as the mAb against MAP2 reacted strongly with neuronal structures and neurofibrillary tangles found in frozen brain sections from patients suffering from AD.

Discussion

The results of the present study suggest that both central and peripheral neurons in freshly frozen specimens of human olfactory mucosa exhibit NF immunoreactivity, and so do both their phosphorylated and non-phosphorylated epitopes. The distribution of NF-positive neurons coincided with that of synaptophysin, a ubiquitous neuronal marker. These results partially differ from recent studies (Talamo et al. 1989; Trojanowski et al. 1991) reporting lack of NFs in normal olfactory mucosa but not in axonal profiles or "dystrophic neurites" in

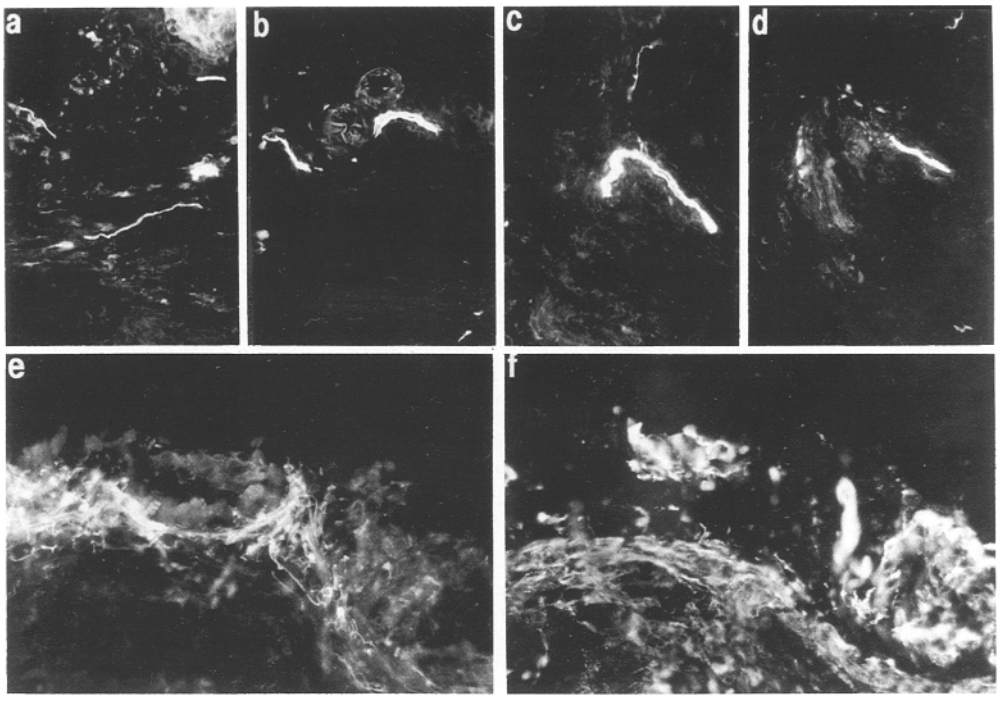

Fig. 2 Both in specimens from control (**a, c**) and AD patients (**b, d**) the mAb 13AA8 (**a, b**) and the mAb 1A3 (**c, d**) reveal immunoreactivity in peripheral nerves. Neurofilament 200 immunoreactive subepithelial nerve fibres (**e**) do not contain vimentin immunoreactivity in AD specimens (**f**). (× 200)

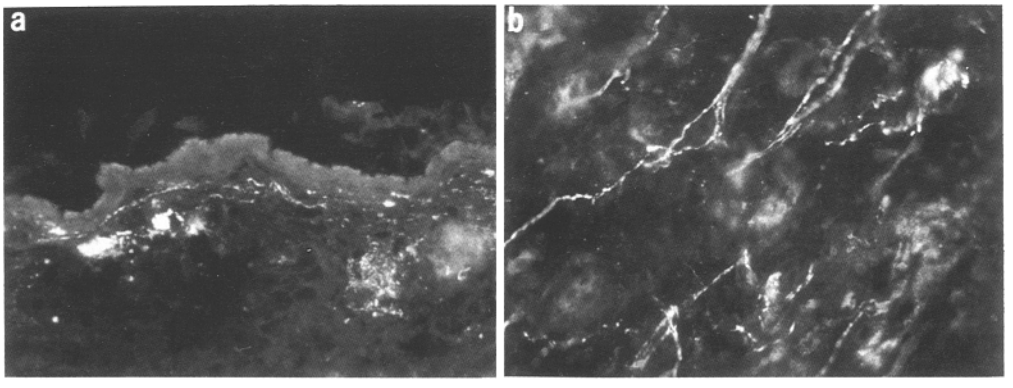

Fig. 3 The mAb SY38, specific for synaptophysin, reveals subepithelial (**a**) and deeper located nerve fibres (**b**) in specimens from AD patients. (× 200)

patients with AD and in the majority of old control patients. Furthermore, we could not confirm the previous findings of the presence of MAP2 and tau in olfactory mucosa of AD patients, although we tested the specimens with two independent mAb. The reason for the ubiquitous presence of synaptophysin in the specimens is most likely due to the fact that it belongs to the pore forming proteins present in neurosecretory granules of axonal profiles, also in the peripheral nervous system (Wiedenmann and Franke 1985).

We have demonstrated previously that the accuracy of clinical diagnosis of AD, verified post mortem, is about 82% (Sulkava et al. 1983). The similarity of our immunohistochemical findings between patients and controls and the difference from those above is therefore unlikely due to misdiagnosis, although we did not carry out brain biopsies. Nor was a postmortem examination performed since all the AD patients are still alive. The differences between our findings and those of Talamo et al. (1989) and Trojanowski et al. (1991) are more likely due to the fact that we studied fresh specimens while they had only postmortem paraffin specimens. We also think that the stage of AD is an insufficient explanation for the differences between our results and those mentioned above, because our material also included severe cases.

Trojanowski et al. (1991) also report in their study on postmortem specimens that olfactory nerves exhibited vimentin in line with the results of Schwob et al. (1986) on adult rats. However, the result differs from our double immunofluorescence findings which showed lack of vimentin in olfactory nerves as in mature nerves elsewhere, as reported in several previous studies (for a review, see Lazarides 1982). Interestingly enough, studies on rat tissues have resulted in contrasting findings on the presence of NF proteins in olfactory neurons: Gorham et al. (1991) reported their absence whereas Bruch and Carr (1991) reported their presence in rat olfactory mucosa. Thus, while our earlier results from inner ear (Anniko et al. 1987) and those of many others

on several other neurons (Lazarides 1982) have shown that neurons may lack NFs, this seems not to be the case with human olfactory neurons.

The results of Trojanowski et al. (1991) also imply that the differential phosphorylation of NF proteins in olfactory neurons of AD patients would be significant for the pathogenesis of the disease. However, numerous studies have implied that the phosphorylation of several neuronal proteins (Hemmings et al. 1989) and NF proteins in particular (Sternberger and Sternberger 1983; Matus 1988) is ubiquitous in neurons and important for the function and organization of NFs. The present immunohistochemical studies on human olfactory neurons support this view.

Our experience thus suggests that biopsies of human nasal olfactory mucosa can be used for immunohistochemical studies to avoid problems related to autolysis of postmortem specimens and masking of antigens in paraffin-embedded and decalcified tissues. However, the NF profile studied did not reveal any specific changes for AD.

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