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Characterization of an amorphous deposit in the lamina propria in oral snuff users in the Sudan as collagen

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Histological and ultrastructural features of 25 oral snuff dipper's lesions with distinctive subepithelial hyaline deposits were investigated. Periodic acid-Schiff reaction with and without diastase digestion demonstrated the presence of glycogen and other carbohydrates, but histochemical stains for normal collagen, elastin and fibrin showed a weak variable reactivity of the deposit. Although in 7/25 cases the deposit was in close proximity to labial salivary glands and on occasions ducts were found within the deposit, the presence of mucin was not a consistent feature. Congo red staining and immunohistochemical investigation with an anti-amyloid antibody did not support the previous contention that such deposits were amyloid in nature. Immunohistochemically, collagen antibodies also provided negative results, but ultrastructural features of three biopsies studied suggest that the bulk of this deposit is made up of collagen, as typical cross-striated fibrils were found. The pathogenesis of this deposit could therefore be interpreted as over-production and/or reduced turnover of collagen by resident fibroblasts, which is further altered by the ingredients of toombak. The deposit does not appear to be a secretory product.

Key words: amorphous deposit; amyloid; collagen; oral mucosa; smokeless tobacco; toombak; ultrastructure

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Smokeless tobacco use is widespread among rural populations in the Sudan, and recent epidemiologic studies have recorded the habit to be prevalent among 42% of adult males (1). The product used, a mixture of tobacco and sodium bicarbonate, referred to as toombak, is high in carcinogenic tobacco-specific N-nitrosamines (TSNA) (2). Habitués commonly place the toombak-quid in their lower labial sulcus for prolonged periods and some are also known to retain it during sleep. We recently described the histopathology of toombak-induced oral lesions in a case-series of 156 chronic users from Northern Sudan (3). While in a large majority of these oral biopsies, all taken from toombak users, several metaplastic changes related to keratinization (96%) and alterations in thickness (66%) were observed, the prevalence of epithelial dysplasia was low (7%). In this initial report we commented on the occurrence of an amorphous deposit in the lamina propria in 25/156 (16%) biopsies. Such mucosal amorphous deposits were first described in snuff dipper's lesions from Denmark (4). Subsequently, Hirsch *et al.* (5) identified this type of deposit in 2% of non-inflamed lesions in 50 Swedish habitual snuff dippers. However, there is controversy as to the nature of these deposits. While Lyon *et al.* (6) consider them to be amyloid, in a later report Archard & Tarpley (7) could not identify amyloid. Axell *et al.* (8) have speculated that these deposits could be altered collagen. We report here the detailed light and electron microscopic features of amorphous deposits in a series of oral biopsies from the Sudan based on extensive histochemical, morphologic and ultrastructural investigations.

Material and methods

The material for this study was obtained during an epidemiological survey of toombak-associated oral mucosal lesions in the Sudan carried out in 1994 (3). All subjects were interviewed on their smoking and toombak usage and received a comprehensive oral mucosal examination. The toombak-associated oral lesions were visually graded 1–4 by criteria described earlier (3). Twenty-five oral biopsies from this series were used for further studies; all were from lower labial mucosa/sulcus that, on assessment of haematoxylin and eosin-stained sections, demonstrated a band of homoge-

neous eosinophilic amorphous deposit in the connective tissue within the lamina propria and the submucosa. The amount of deposit present was graded on a semiquantitative basis using a micrometer eye-piece graticule and scored as +, ++ or +++. The clinical characteristics and toombak habits of these patients are listed in Table 1.

To characterize the nature of this amorphous deposit, 4 μm serial sections were prepared. These were separately stained with periodic acid-Schiff (PAS) with and without a diastase digestion stage for demonstration of glycogen and other carbohydrates, by the van Gieson technique for dense connective tissues (collagen), by Verhoeff's method for elastic fibers, by Highman's Congo red method for amyloid, by the Martius scarlet blue (MSB) technique for fibrin, and with Alcian blue (pH 2.5) for glycoprotein. These histochemical techniques were adapted from BANCROFT & STEVENS (9). Congo red-stained sections were viewed by semi-polarized light to note any dichromic birefringence.

Ultrastructural examinations were carried out utilizing three representative formalin-fixed, paraffin-embedded blocks each from examples of mild, moderate and severe accumulation of the deposit. The area of the amorphous deposit was microdissected and 10 μm thick sections were mounted on glass slides and deparaffinized in xylene. These were then rehydrated in a descending acetone series, post-fixed in osmium tetraoxide and dehydrated in an ascending acetone series. One or two drops of a 1:1 mixture of acetone and epoxy resin was placed over each section. After 20 min incubation the mixture was replaced by pure epoxy resin and polymerized at 60°C. Ultrathin sections were cut with a glass knife, stained with lead citrate and uranyl acetate and examined in an AEI 6B transmission electron microscope.

Immunohistochemistry

Immunohistochemical methods were performed to examine sections for amyloid, collagen and cytokeratin, using a murine monoclonal antibody that recognizes human amyloid fibril protein AA (mol wt 7000–9000) (DAKO: mc 1), polyclonal donkey/rabbit anti-human collagen types I, III, IV, V, (Novacastra & Sigma) and antihuman pan-cytokeratin (Dako: MNF116). Antibodies against collagen types used here react against conformational determinants on human and bovine collagen, and an individual antibody exhibits<10% cross-reactivity with other collagen types. Immunolocalization was performed using a streptavidin-biotin immunoperoxidase method, as previously described from our laboratory (10). Endogenous peroxidase activity was blocked by a 20-min treatment with 0.5% hydrogen peroxide in 70% methanol. Sections stained for amyloid and cytokeratin were pretreated with trypsin for 10 and 15 min, respectively. Sections used for demonstration of collagen were subjected to antigen retrieval by heating in 10 mM citrate buffer (pH 6.0) using an 800 W microwave oven (setting 5) twice for 5 min, replacing the buffer at the end of the first 5 min. The slides were bench cooled for 15 min. Following a blocking step with normal sheep or goat serum (1:30), tissues were incubated with primary antibodies for either anti-amyloid (dil 1:50), anti-collagen (dil 1:500 for collagen IV; 1:40 for other types) or with anti-cytokeratin (dil 1:50) for 1 h. Biotinylated anti-mouse, anti-rabbit (1:100) or anti-donkey (1:150) IgG was applied for 50 min followed by streptavidin peroxidase at a dilution of 1:200 for an additional 50 min. PBS buffer (pH 7.4) was used as the dilutant and for rinsing/washing between staining steps. Slides were developed using 0.05% 3,3′-diamine benzine tetra hydrochloride (Sigma) and 0.1% H_2O_2.

Results

A description of the subjects with an amorphous deposit by personal charac-

Table 1. Personal characteristics and the histological features of the subepithelial hyaline deposit with various special stains

Case	Age (yr)	Duration (yr)	Degree	H&E	PAS	DPAS	HVG	EVG	C. red	MSB	Alcian blue	Cytokeratin
142	55	28	0	+++	+++	+++	+	±	−	−	−	−
146	60	46	2	+++	+++	+++	±	±	+	++	++	±
168	40	12	2	+++	+++	+++	+	−	±	+	−	−
174	−	−	−	+++	+++	+++	±	±	−	−	−	−
176	55	30	2	+++	+++	+++	−	−	±	+	−	+
181	28	6	3	+	+	+	−	−	−	−	−	−
185	24	5	2	+	+	+	−	−	−	−	−	±
186	25	5	1	+	+	+	−	−	−	−	−	−
189	35	10	3	+++	+++	+++	++	±	−	−	−	±
192	−	−	−	++	++	++	±	±	−	−	−	−
194	28	−	3	+++	+++	+++	±	±	−	−	−	±
195	45	25	3	++	++	++	−	−	−	−	−	−
196	40	15	2	++	++	++	−	−	−	−	−	−
208	34	6	3	+++	+++	+++	+	+	−	±	−	−
209	38	30	−	+++	+++	+++	−	−	−	−	±	−
211	25	18	1	++	++	++	+	−	−	−	−	−
214	58	−	−	+	+	+	−	−	−	−	−	−
232	27	14	3	+	+	+	−	−	−	−	−	−
233	50	34	3	++	++	++	−	−	−	−	−	−
239	50	−	−	+++	+++	+++	+	++	±	++	−	−
249	40	−	−	+++	+++	+++	±	±	−	±	−	−
264	31	12	−	+++	+++	+++	+	−	±	±	−	−
268	−	−	−	+	+	+	−	−	−	−	−	−
271	55	36	1	+	+	+	−	−	−	−	−	−
274	55	20	1	+++	+++	+++	−	−	−	−	−	±

Duration: duration of toombak use; degree: clinical degree on scale 0–4 (see ref. 3); HVG: van Gieson; EVG: Verhoeff's; C. red: Highman's Congo red; MSB: Martius scarlet blue.

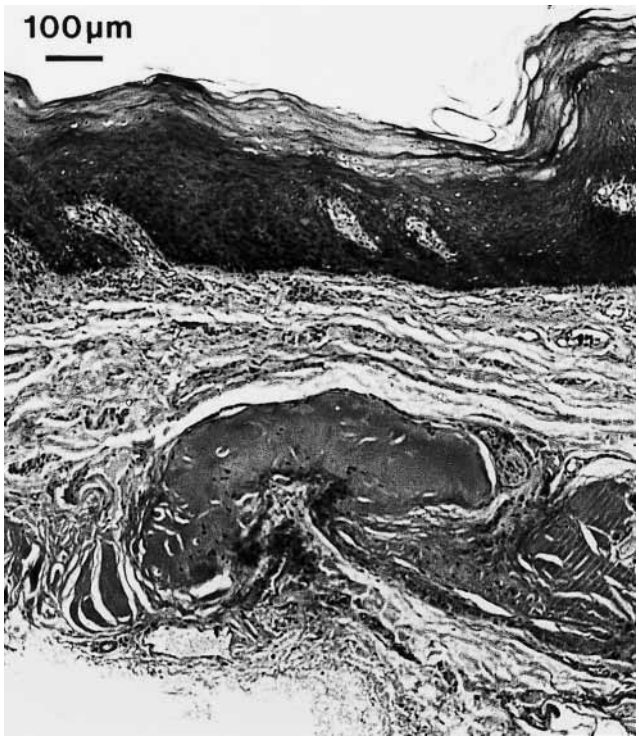

Fig. 1. Grade III hyaline deposit in lower labial mucosa. The photomicrograph shows slightly acanthotic, parakeratinised squamous cell epithelium, a light inflammatory infiltrate in the lamina propria and a broad band of hyalinised eosinophilic material below this (H&E, ×75).

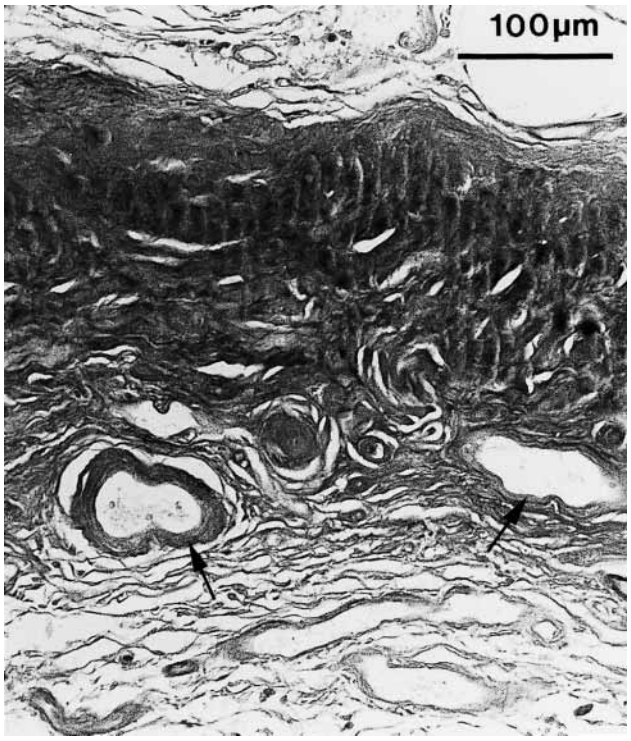

Fig. 2. Higher powered view of a hyalinised submucosal deposit showing some fibriller texture, particularly at the periphery of the main mass and surrounding ducts (arrowed) of minor salivary glands (H&E, ×203).

teristics and their tobacco habits is shown (Table 1). On light microscopy an amorphous eosinophilic deposit of varying size and staining intensity was observed as a continuous wavy band (without any lamination) located in the lamina propria and submucosa, often just above and adjacent to the minor salivary glands of the lip (Fig. 1). The intensity of the deposits in relation to clinical characteristics is shown in Table 1. In some specimens (7/25) ducts of the labial glands were seen to be incorporated within the deposit (Fig. 2). In none of the specimens was any associated inflammatory infiltrate or host reaction to the deposit observed. The staining characteristics of the deposit are detailed in Table 1. The periodic acid-Schiff reaction with and without diastase demonstrated a uniformly stained band in all 25 cases. With other stains, however, the deposit showed varied reactions. Verhoeff's method showed irregular linear brown-black staining in 10 sections, the intensity was strong in two and weak in eight; the remaining 15 sections were negative. By the van Gieson method minute red areas were seen within the deposit in 12 of the sections. The intensity of the stain was strong in one, moderate in six sections and weak in five sections; the remaining 13 sections were negative. In no instance was the whole deposit uniformly stained with these reaction products. Five sections stained weakly red with Congo red, but none showed red/green birefringence when viewed with polarized light. Seven sections stained red with MSB; the staining intensity was strong in two sections, moderate in two sections and weak in three sections, and the remaining 18 sections were negative. Two of the deposits stained blue with Alcian blue; one was moderately intense. Selected examples with moderate/strong staining patterns to elastin, collagen, fibrin and mucin are illustrated in Figs. 3–5. Immunostaining with anti-human pan-cytokeratin (Dako:MNF116) showed positive reactions for cytokeratin in seven sections; one was moderately, and six were weakly, positive. Immunostaining of 10 cases with polyclonal donkey-antihuman and bovine collagen types, I, III, and V, and rabbit anti-human collagen type IV, showed multiple foci of positive reactions within the deposit and surrounding lamina propria in three sections only. The bulk of the collagen visualized consisted of type IV. One section was strongly positive with collagen type IV and the remaining two were weakly positive. With collagen type I, all three showed a weak positive reaction. One section was moderately positive with collagen type III and the remaining two were negative. Two were positive with collagen type V and one was negative. Weak staining with collagen type IV was seen in the basement membrane zone and around minor salivary glands and blood vessels in the connective tissues of the lamina propria and submucosa in all ten sections.

The ultrastructural features of the deposit in the three preparations showed amorphous lakes with bundles of electron-dense fibrils of variable size arranged in whorls or layers (Figs. 6, 7). The fibrils measured 20–120 nm in diameter and demonstrated a marked axial periodicity. The length of each period was estimated to be approximately 52 nm. The fibrils were densely distributed in two cases where the deposit was intense and sparsely in the third specimen. No calcifications were noted among the fibrils. In some areas (Fig. 8) the collagen fibrils appeared hollow – with the periphery of the fibril

stained – suggesting this was tubular collagen. This was most likely a staining artefact. In other areas (Fig. 8) the fibrils comprising the fibres appeared very electron-lucent, giving the appearance that they had completely failed to stain. This is consistent with the feature referred to as 'negatively stained collagen'. Based on these EM appearances, particularly the banded fibrillar pattern, collagen was seen to be a major component of the deposit in all three

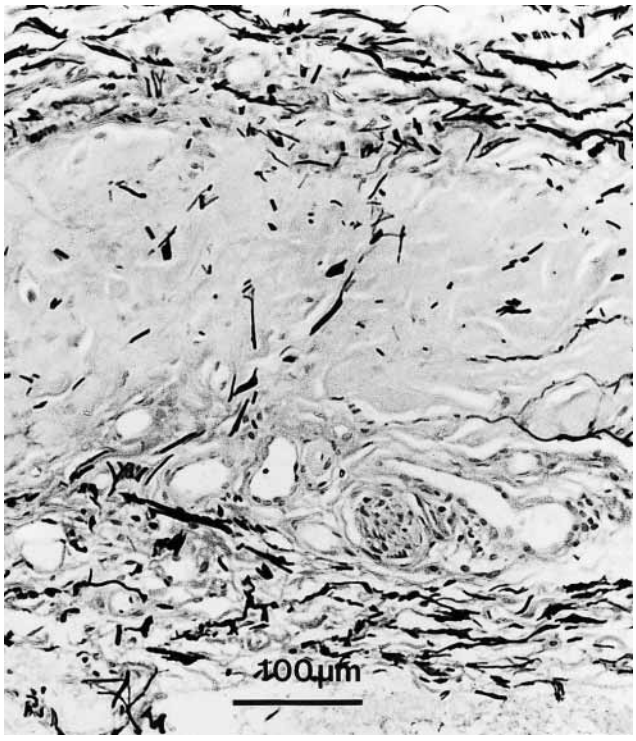

Fig. 3. A hyaline submucosal deposit showing elastin fibres peripheral to the main mass and occasionally embedded within it (elastin–van Gieson, ×178).

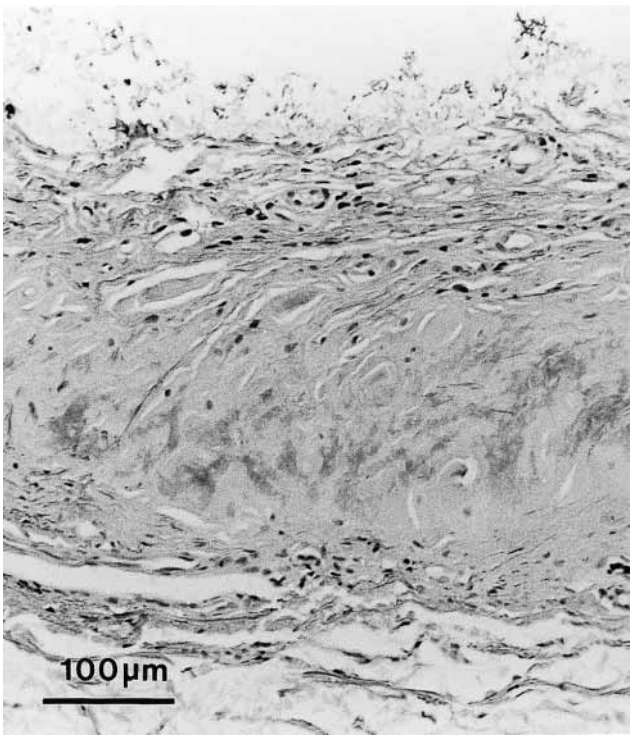

Fig. 4. A hyaline deposit stained by Martius scarlet blue (MSB)/haematoxylin in an attempt to identify fibrin. The mass is almost entirely acellular and shows patchy MSB staining of central areas (×178).

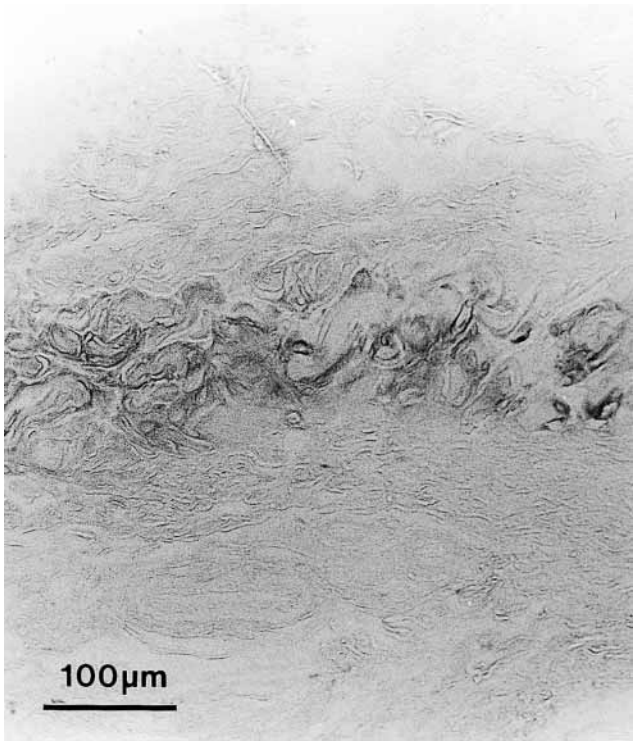

Fig. 5. Alcian blue staining at pH 2.5 reveals slight positivity, indicative of glycoproteins, in the centre of a hyaline deposit (×178).

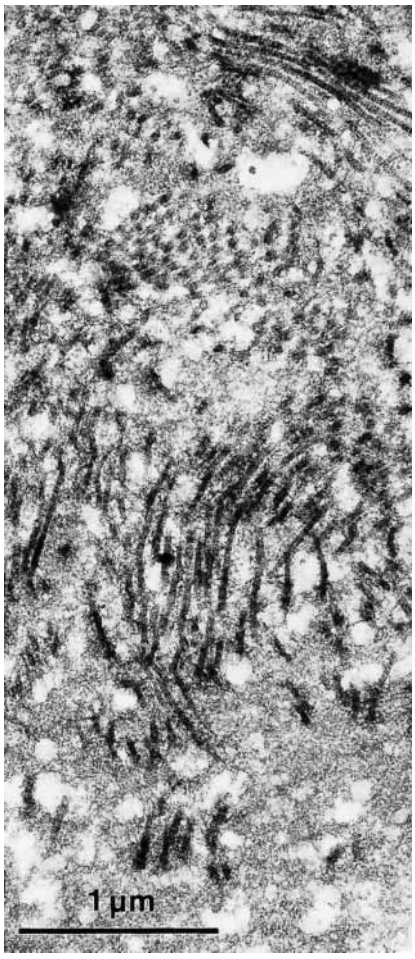

Fig. 6. Collagen fibers are seen in both long- and cross-section within a diffusely granular matrix (×27000). Ultrathin section of hyalinised deposit microdissected from paraffin block and embedded in resin, post-fixed in osmium tetraoxide and stained with lead citrate and uranyl acetate.

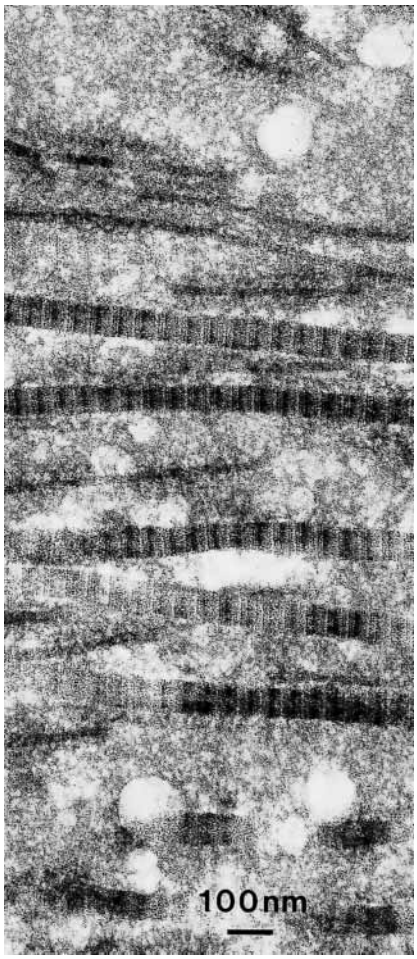

Fig. 7. Types I and III collagen fibres with characteristic collagen banding are revealed at 52 nm (×61000). Ultrathin section of hyalinised deposit microdissected from paraffin block and embedded in resin, post-fixed in osmium tetraoxide and stained with lead citrate and uranyl acetate.

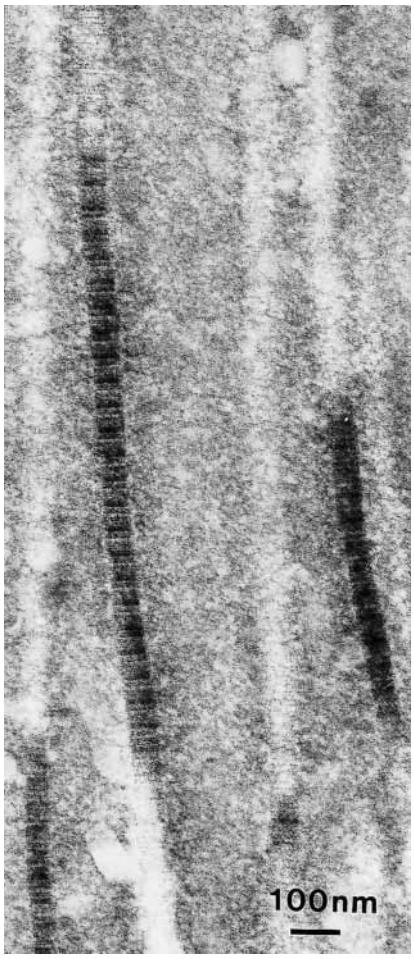

Fig. 8. Individual collagen fibrils are seen in longitudinal section, exhibiting both positive and negative staining (×67,000). Ultrathin section of hyalinised deposit microdissected from paraffin block and embedded in resin, post-fixed in osmium tetraoxide and stained with lead citrate and uranyl acetate.

biopsies examined ultrastructurally. The extracellular matrix around the cross-striated fibrils was difficult to interpret. This could consist of collagen type VI (not studied here) or non-collagenous glycoproteins but, due to poor tissue preservation a specific matrix could not be differentiated.

Discussion

The amorphous deposit in all of the 25 cases was, by light microscopy, morphologically similar to those previously reported from Scandinavia among snuff dippers (4, 6, 7). Pindborg & Poulsen (4) associated these deposits with a particular brand of snuff called Copenhagen snuff, while Axell *et al*. (8) reported that the deposits were specific to two brands of Swedish snuff, called Ettan and Roda Lacket, but not to other brands used by Swedes. The results of the present study suggest that such amorphous deposits in the connective tissues may be more globally widespread than earlier thought, as the biopsy samples described here originated from toombak users in the Sudan. The nature of the deposit we describe is somewhat heterogeneous. Characterisation of the deposits as PAS-positive, diastase-resistant carbohydrates is consistent with the earlier reports. The most significant finding of our study is the detection of collagen, as demonstrated by the van Gieson-positive stains, sometimes by immunohistochemistry, and by the ultrastructural finding of characteristically banded fibrils. While the presence of collagen has not been confirmed in any of the earlier studies, Axell *et al.* (8) have proposed that the amorphous deposit could be an 'altered collagen'. Detecting collagen by immunohistochemistry in formalin-fixed tissues is quite difficult (11), and many studies have failed to detect the typical staining pattern for oral mucosa. Our report is the first study to examine the ultrastructural features of an amorphous deposit in snuff users. The presence of 52 nm banded fibrils is consistent with the range of 52–62 nm for collagen in ultrathin sections (12) and corresponds to a periodicity of 64–70 nm in the wet preparations, as measured by x-ray diffraction. The artefactually produced, presumably negatively stained, collagen fibers are a well-recognised ultrastructural observation in connective tissues (13). Whether this is pathologically altered collagen is not known. Tissues for this study were initially fixed in buffered formal saline and the sizes of biopsies were not optimal for electron microscopy. Al-

terations due to poor tissue preservation may account for negative staining.

It has been previously contended that the homogeneous deposit in snuff dipper's lesions is predominantly composed of amyloid. Our findings do not support the presence of an amyloid component. Although a few of the tissue sections showed weakly positive red foci with Congo red staining, red/green birefringence was absent and immunohistochemistry gave consistently negative results. These observations are consistent with the reported lack of amyloid in Copenhagen snuff dippers by ARCHARD & TARPLEY (7). It has been reported that the Congo red birefringence test is highly selective and sensitive (14). Recently described anti-amyloid antibodies are known to be specific for immunolocalization of amyloid in many sites (15). Electron microscopic findings also did not support the presence of amyloid. It has been affirmed that electron microscopy often confirms a doubtful result of the Congo red test by demonstrating the characteristic unbranched, usually straight, thread-like filaments and fibrils of amyloid that are virtually impossible to confuse with other fibrillar structures (13, 16).

There was a varied localized positive reaction with stains for elastin, mucin, and fibrin. The seven positive results with MSB staining suggest the presence of fibrin in parts of the deposit but ultrastructural studies did not support this. The few sections that showed positive staining with Alcian blue may confirm the close location of the deposit with minor salivary glands, suggesting that mucin may occasionally leak into the adjacent amorphous deposits. Our findings do not support the amorphous deposit to be a secretory product.

While hyaline collagen deposits in the lamina propria are well described in areca nut chewers (17, 18), snuff dipping appears to result in a more heterogeneous band composed principally of collagen. The pathogenesis of both appears to be related to upregulation of the synthesis of collagen in fibroblasts following chronic irritation of the mucosa among habitués, perhaps together with downregulation of the collagenolytic arm of normal collagen turnover. There is clearly also masking or alteration of epitopes on the surfaces of collagen fibres, which disturbs the specificity of immunochemical reactions. The molecular and cytokine pathways associated with these alterations need detailed examination.

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