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A comparative immunohistochemical study of cytokeratin and vimentin expression in middle ear mucosa and cholesteatoma, and in epidermis

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Summary. Cytokeratin expression was studied in human middle ear cholesteatoma lesions, using a variety of immunohistological techniques and a wide range of polyclonal antisera and monoclonal antibodies against cytokeratin (CK) subgroups or individual CK polypeptides. The expression of the other cytoskeletal proteins, vimentin and desmin, was also investigated. Middle ear mucosa and epidermal tissues were used as reference tissues. Our investigations also included epithelial structures present in the cholesteatoma perimatrix and in dermal tissues.

The results indicate that, compared with epidermal tissues, the expression profile of CKs in cholesteatoma matrix is representative of a hyperproliferative disease. Evaluating the presence of a marker of terminal keratinization – the 56.5 kD acidic CK n° 10 – we found supportive evidence of a pronounced retardation of its expression, which did not parallel histological differentiation. In epidermal tissues, the first prickle cell layers are CK10 positive whereas in many cholesteatomas this finding was observed near the stratum granulosum only. Probing the early stages of keratinization – the 58 kD basic CK n° 5 and the 50 kD acidic CK n° 14 – we regularly observed an extended staining area in the cholesteatoma matrix. In epidermal reference tissues, only the basal and nearest suprabasal layers were convincingly labeled.

As a rule, non-epidermal CKs did not belong to the cholesteatoma CK set. However, exceptions to that rule were noticed as a focal or more extended expression of one or more non-epidermal CKs in about half of the cases. Together with the extended CK5 topography, this is further evidence that CK expression is seriously affected by the diseased state. CK expression in the perimatrix is limited to mucous glands, either normal, atrophic or hyperplastic. CKs n° 4, 5, 7, 14, 18 and 19, also displayed by middle ear mucosa, were consistently observed. Where ductal arrangements were present, CK10 was also detected, in analogy with the CK10 registration in ductal portions of mucous glands in the external ear canal skin. The absence of CK8 in mucous glands of the perimatrix, however, strongly differentiates these structures from the mucous gland acini and ducti in the external ear canal, where CK8 is systematically expressed. Vimentin staining was restricted to dendritic cells of the matrix (Langerhans cells) and to perimatrix fibroblasts, blood cells and vascular endothelium. Coexpression of CK and vimentin was not observed.

Key words: Cytokeratins – Middle ear mucosa – Aural cholesteatoma – Immunohistochemistry

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Introduction

Aural cholesteatoma is a relatively common, locally invasive and insidiously destructive lesion, affecting the mucosa of the middle ear and the air cell system of the temporal bone. The basic feature of this disorder is the presence in the middle ear cleft of an expanding stratified squamous epithelium, accumulating squamous debris. It causes a serious disturbance of the physiology of hearing and

a vigorous attack on the underlying connective and bony tissues. Basic data on the aetiology, pathology, histology and clinical aspects of cholesteatoma are described in the Proceedings of the Second International Conference on Cholesteatoma (Sadé 1982). The particular differentiation pattern of cholesteatoma epithelium has been largely unexplored, while a better knowledge of this phenomenon may provide helpful insights in the pathogenesis of the complex lesion as a whole.

Two markers of cholesteatoma keratinization have already been the subject of a quantitative histochemical study, namely, the covalent strengthening of structural polypeptides by $-S-S-$-cross-links during terminal stages of keratinization and the progressive differentiation and ultimate fate of epithelial nuclei (Broekaert et al. 1982a, 1988).

Here, we report on an in-depth study of the cytokeratin (CK) expression in this pathological type of keratinization. It is now well established that all epithelia express a limited and generally well-defined number of CK polypeptides. Human CKs make up a large family of about 19 polypeptides, subdivided according to their molecular mass and isoelectric point into two discrete subfamilies (Moll et al. 1982; Sun et al. 1984). The rules governing the differential expression of CKs, including the tissue distribution and the obligatory coexpression of different members of each subfamily have been outlined in a unifying model by Sun et al. (1984).

These CKs generate tonofilaments belonging to the class of intermediate-sized filaments (IF). The nature of structural polypeptides constituting IFs in various types of mature eukaryotic cells have been shown to correlate with the embryonic origin of the cells. Five types of IFs, each composed of different structural polypeptides, are distinguished biochemically and immunochemically (Weber and Osborn 1982), and antibodies to the different types of IF polypeptides have become useful tools for cell typing, tissue recognition and tumour diagnosis (e.g. Ramaekers et al. 1983a).

Using a variety of immunohistochemical techniques and a wide range of polyclonal antisera and monoclonal antibodies against either CK subgroups or individual CK polypeptides, we tried to unravel the CK expression pattern of the cholesteatoma matrix and its stratum corneum, as well as of organized epithelial structures in the perimatrix. The vimentin and desmin expression of the cholesteatoma matrix and perimatrix was also investigated. Middle ear mucosa and epidermal tissues were used as a reference in our study, because certain aetiological theories consider the direct involvement of the external ear canal epidermis or tympanic membrane epithelium in the genesis of middle ear cholesteatomas (immigration, invagination and papillary proliferation theory: Sadé 1982). We discuss, among other things, our findings in relation to the established principles of the IF typing and the differential CK expression (Sun et al. 1984).

Materials and methods

Epidermal CKs were obtained from bovine snout – available in unlimited amounts – and human sole, as previously described (Gillis et al. 1980). 5 mg CK samples were dissolved by briefly boiling in SDS buffer and further purified by preparative SDS polyacrylamide gel electrophoresis in slab gels ($200 \times 200 \times 3$ mm, 14 h at 30 mA, 5% stacking gel – 10% separating gel) (Laemmli 1970). Protein bands were revealed in 0.5 cm lateral gel strips by staining with 0.05% Coomassie Brilliant Blue R-250 (Serva, Heidelberg) and the corresponding CK bands were cut out from the unstained gel, as a complete set or divided into subgroups. After homogenization of the excised gel zones, polypeptides were eluted electrophoretically (24 h, 10 mA). Protein subunit composition and approximate molecular weights were determined in analytical gels ($200 \times 200 \times 1$ mm, 25 µg proteins per lane) in the presence of a calibration kit (LMW kit Pharmacia, Uppsala) (Laemmli 1970).

Rabbits were immunized subcutaneously with approximately 3 mg CKs, dissolved in 200 µl 1% SDS, 0.2% 2-mercaptoethanol, enriched with 200 µl Freund's complete adjuvant (0638-60, Difco, Detroit), injected at multiple sites in the back (Vaitukaitis et al. 1971). Before immunization, preimmune sera were collected. From the third week onwards, rabbits were bled and boosted repeatedly (up to six times with 3-week intervals) with 100 µl CK solution (1.5 mg) and 45 µl incomplete Freund's adjuvant (0639-60, Difco).

Antibody titer determination was performed by spontaneous immunodiffusion on modified Ouchterlony agarose plates (Yen et al. 1976). A serial dilution of antisera in Coons buffer pH 7.2 was tested against an epidermal CK solution (6 mg/ml in 0.86% NaCl, 2% SDS and 0.5% Triton X-100) in the central well.

The specificity of antisera was verified by immunoblotting, involving spontaneous capillary transfer of total epidermal CKs or subgroups in comparable concentration, from polyacrylamide gels onto nitrocellulose sheets (Millipore Hahy-type, 0,45 µm pore size, 20 h at 20° C) in the following blotting buffer: 25 mM Tris, 150 mM glycine pH 8.6, 20% methanol, 0.1% SDS. The nitrocellulose membranes were saturated with 3% BSA or 3% skimmed milk powder in PBS (2 h, 30° C), prior to the application of the primary antiserum (diluted 1/20–1/50 in PBS containing 1% BSA or milk powder, 15 h at 30° C). Antigenic CK bands were visualized by the direct immunofluorescence technique, using FITC-conjugated goat anti-rabbit IgG as a second antibody (Nordic, Tilburg, 1/10–1/20 in PBS containing 1% BSA or milk powder, 3 h at 30° C).

The following antisera were selected for immunohistochemical investigations. The K109, K112 and K113 antisera, directed against the total bovine epidermal CK set, and the K150 and K151 antisera, directed against bovine epidermal CK subgroups, were all broadly crossreacting when tested by immunoblotting against human epidermal CKs (Broekaert 1984).

Table 1. Characteristics of the monoclonal antibodies

Antibody	Antigen(s)[a]	Panel	References
6B10	CK4	I	Van Muijen et al. 1986
RCK102	CK5, 8	I	Broers et al. 1986
RCK105	CK7	I	Ramaekers et al. 1987a, b
LE41	CK8	II	Lane 1982
RKSE60	CK10	I	Ramaekers et al. 1983a
LPH1	CK10	II	unpubl.
LH3	CK10	II	unpubl.
1C7	CK13	I	Van Muijen et al. 1986
2D7	CK13	I	Van Muijen et al. 1986
LH8	CK14	II	unpubl.
EKH4	CK14	II	Eto et al. 1985
M9	CK18	I	Sanbio BV, Uden (NL)
RCK106	CK18	I	Ramaekers et al. 1987a, b
RGE53	CK18	II	Ramaekers et al. 1983c
CK18-2	CK18	I	Broers et al. 1986
LE61	CK18	II	Lane 1982
LP2K	CK19	I, II	RPN. 1165, Amersham (Bucks.)
clone 80	CKs[b]	I	Van Muijen et al. 1984
RCK103	CK5[c]	I	Ramaekers et al. 1987b
Vim 9	vimentin	I	Van Muijen et al. 1985
RV202	vimentin	I	Ramaekers et al. 1987b
RD301	desmin	I	Ramaekers et al. 1987a

[a] CKs numbered according to Moll et al. 1982
[b] broad spectrum CKs
[c] plus some unidentified CKs and neurofilament polypeptides

The crossreactivity of the K148 antiserum was restricted to CK bands in the 56–67 kD range, and did not involve CK14 (50 kD) and CK16 (48 kD). Both these CKs were detected by the K149 and K192 antiserum (Broekaert 1984). A broadly crossreacting affinity purified polyclonal antiserum to human callus CKs (pKer (K40): Ramaekers et al. 1983a) was also included in our panel.

Two panels of monoclonal antibodies were used in our study (panel I and II: Table 1). Ten cholesteatoma cases were investigated using panel I, while 11 other cases were screened with panel II. Tissue culture medium from non-producing hybridomas was included as a control.

Thirty five samples of cholesteatoma, representing a wide variety of clinical appearances, were incorporated in this study. These specimens were frozen in liquid nitrogen immediately after surgery and processed at the appropriate time for one of the following slide preparation procedures: Paraplast Plus embedding (Oxford Laboratories International Corporation) after fixation in 3.7% formaldehyde neutralized with 0.03 M phosphate buffer, pH 6.8 (18 h, 20° C) (Broekaert et al. 1982b), when polyclonal antisera, except pKer (K40), were used. Cryosectioning (5 µm, Kryostat type 1720, Leitz, Wetzlar) after mounting in Jung embedding medium (nr 08926) when monoclonal antibodies and pKer (K40) were tested. Serial sections were stretched on gelatin (1%)-glycerin (1/1, v/v) coated glass slides, air-dried (2 h, 20° C), fixed in acetone (15 s or 10 min, 20° C) and rehydrated in PBS for 5 min.

Small biopsies of retroauricular and external auditory canal skin and of the healthy part of middle ear mucosa were regularly taken from the same patients. Other human epidermal biopsies (sole, thumb, abdomen, breast) were also included in this comparative study as reference tissues.

Immunostaining of formaldehyde-fixed tissues was carried out by the peroxidase-antiperoxidase (PAP) and the indirect fluorescence technique (Broekaert 1984). To enhance exposure of antigens, tissue slices were regularly pretreated with type II trypsin from porcine pancreas (Pinkus et al. 1985). Preimmune sera, or sera repeatedly absorbed with a purified total epidermal CK preparation, were systematically included as negative controls.

Cryosections were processed for the indirect immunoperoxidase technique. Rehydrated sections were incubated with the monoclonal antibodies (1:5–1:25 in PBS, 30 min at 20° C in a moist chamber), followed by extensive washing in PBS (3 washes, 10 min each; second wash containing 0.1% Tween-20). Rabbit anti-mouse Ig conjugated to peroxidase (1:40 in 10% normal human serum in PBS) was used as a second antibody (30 min, 20° C), followed by extensive washing. Visualization of antigen sites was achieved using either 3,3′-diaminobenzidine tetrahydrochloride or 3-amino-9-ethylcarbazole as the electron donor. Mayer's haematoxylin (30s) was applied as a counterstain.

Coexpression of vimentin (Vim 9) and CKs (pKer(K40)) was verified using the double label indirect immunofluorescence technique (Ramaekers et al. 1983b) with fluorescein conjugated goat anti-rabbit IgG and rhodamine-conjugated goat anti-mouse IgG. All immunological reagents were from Nordic (Tilburg, The Netherlands). Slides were viewed with a Dialux 20 (Leitz) microscope, equipped with a Ploemopak epi-illuminator (type 2.4) and the appropriate filter blocks for FITC (I2) and TRITC (N2).

Results

Initially, immunocytochemical investigations were performed on routine paraffin sections using CK antisera directed against the epidermal CK set (K109, K112, K113). Generally, an homogeneous immunostaining was achieved throughout the cholesteatoma matrix, similar to the labeling of epidermal tissues. However, certain antisera (e.g. K109) generated a low degree of differential staining, the details of which in this discussion of differential CK expression, are irrelevant. At the cellular level the immunostaining was homogeneous to slightly granular in the cytoplasm, with some accumulation of label near the nucleus and the cell membrane. Cholesteatoma corneocytes and the remaining keratinous debris were heterogeneously labeled, whereas in epidermal reference tissues, the immunostaining abruptly diminished in the transition area, leaving only a minor antigen detection in the stratum corneum (Fig. 1A, B). In a small number of epidermal corneocytes, the immunostaining was as intense as in keratinocytes.

The K150 and K151 antisera revealed significant differences in epidermal tissues at various sites of the body and discriminated between cholesteatoma tissues. E.g. K151 exhibited either a non-discriminative staining pattern (breast and sole) or a preferential labeling of all suprabasal stages (abdominal, meatal and retroauricular epidermis). Concerning cholesteatoma, while some specimens

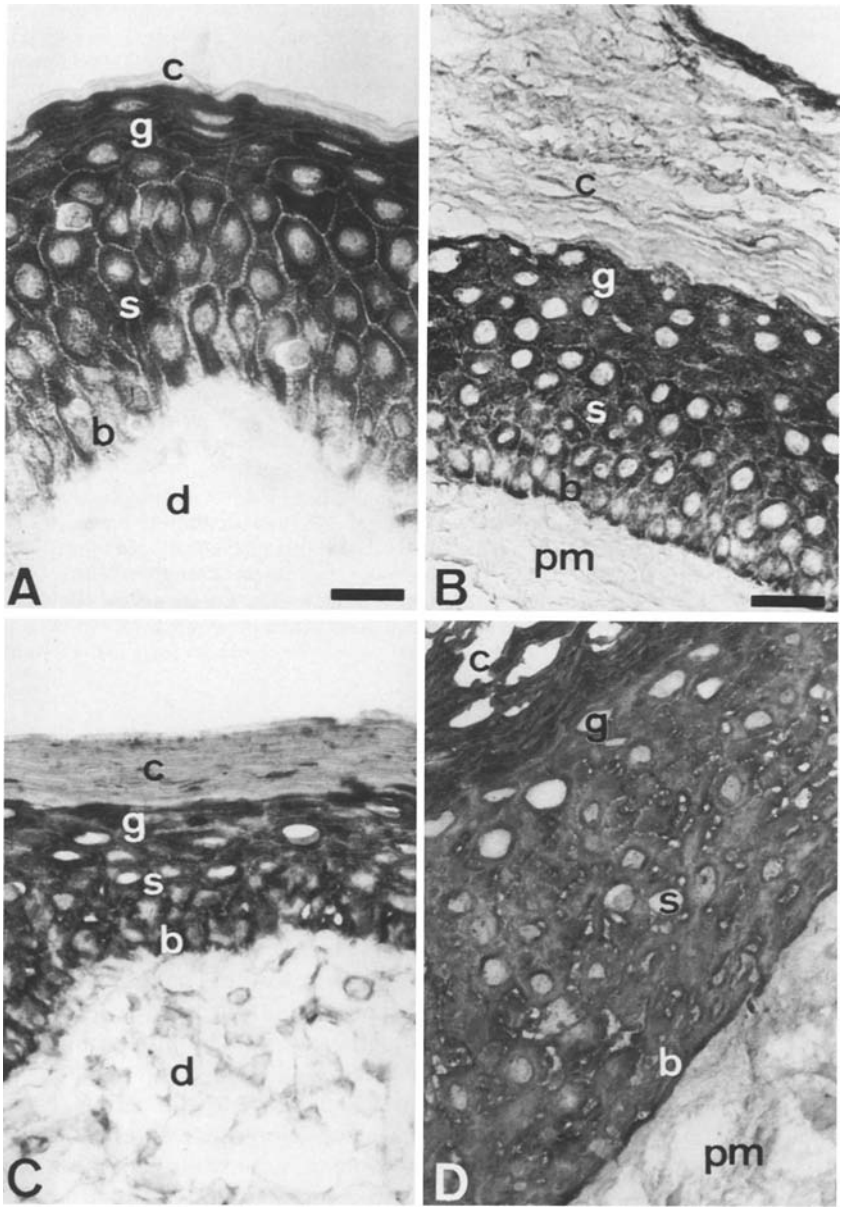

Fig. 1 A–D. Immunohistochemical staining profiles obtained with broadly crossreacting CK antibodies in epidermal and cholesteatoma tissues. An homogeneous immunostaining reaction of basal, spinous and granular keratinocytes was achieved with the polyclonal antiserum K113 in the retroauricular epidermis, while only a minor antigen detectability was noticed in corneocytes (**A**: ×330; paraffin section). Generally, cholesteatomas gave similar staining patterns, except in corneocytes and keratinous debris, which were heterogeneously labeled (**B**: ×200; paraffin section). Using cryoslices and a monoclonal antibody with a broad CK spectrum (pKer (K40)), similar staining profiles were revealed, now completed with a prominent marking of the stratum corneum (**C–D**: ×330, respectively retroauricular epidermis and cholesteatoma tissues). Tissue slices were routinely counterstained with Mayer haematoxylin. The horizontal bar in **A, C** and **D** represents 30 μm, in **B** 50 μm. Abbreviations: *b*: stratum basale; *c*: stratum corneum; *d*: dermis; *g*: stratum granulosum; *m*: matrix; *pm*: perimatrix; *s*: stratum spinosum.

(6/11) revealed no corresponding sites at all or reacted (very) weakly, others showed a strong but uniform expression of the corresponding determinants in basal and Malpighian layers (5/11).

Finally, pKer (K40) tests on cryosections of both epidermal and cholesteatomatous tissues revealed a similar staining pattern, consisting of a prominent staining of basal and suprabasal keratinocytes, a persistent or slightly decreased staining of prickle cell layers and granular keratinocytes, and a significant staining of the stratum corneum (Fig. 1C, D).

The availability of antisera specifically directed against certain epidermal CK subsets has enabled a first survey of the differential CK expression, interpretable at the molecular level. The staining profiles of human epidermal tissues were highly reproducible and well-differentiated. The K148 antiserum, detecting CKs in the 56.5–67 kD range, elicited a specific staining of spinous and terminal keratinocytes, which persisted in the stratum corneum (Fig. 2A). The complementary staining of basal and nearest suprabasal keratinocytes was convincingly seen with the K149 and K192 antisera directed against two prominent low MW CKs (n° 14 and 16) (Fig. 2B). The corresponding determinants were only weakly recognized in more advanced keratinization stages of thick epidermal tis-

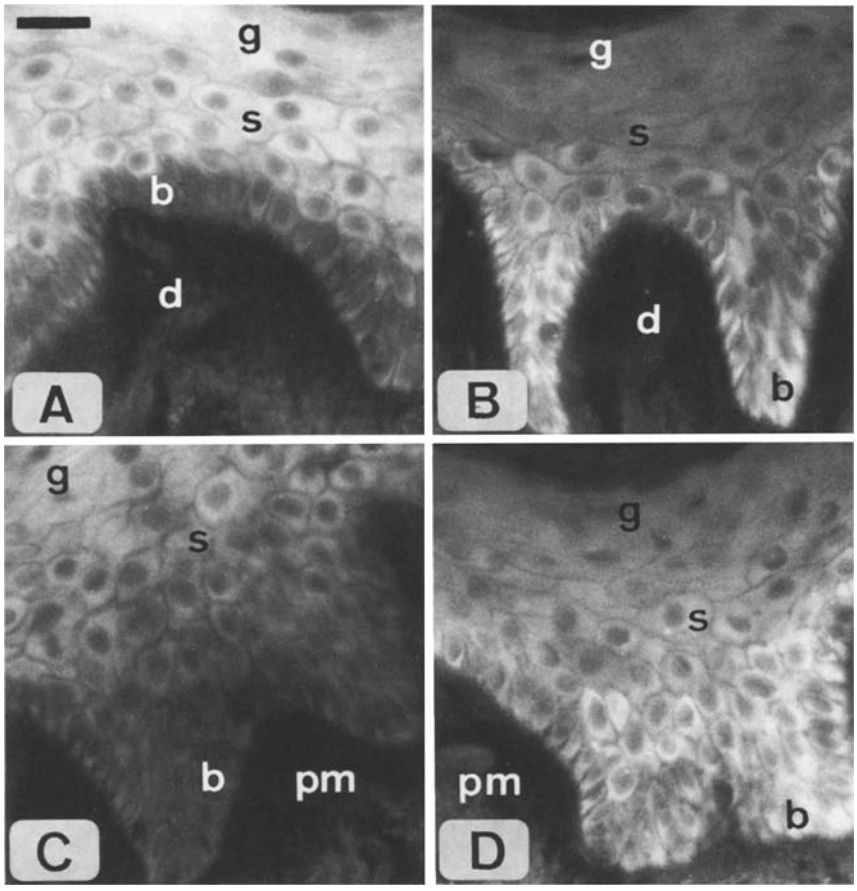

Fig. 2A–D. Immunofluorescent staining patterns of epidermal and cholesteatoma tissues, obtained with polyclonal antisera to specific subsets of CKs. The K148 antiserum, reactive in the 56.5–67 kD range is a fairly selective indicator of spinous and terminal keratinocytes, leaving the stratum basale unlabeled (**A**: sole epidermis, paraffin section; ×330). Complementary fluorescent staining of basal and nearest suprabasal keratinocytes was noticed with the K149 antiserum, directed against CK14 and 16 (**B**: ×330). Typical immunofluorescent profiles of cholesteatoma tissues show on the one hand a delay of the K148 reactivity (**C**: ×330), and on the other, an extended area of K149 reactivity (**D**: ×330). The horizontal bar in **A–D** represents 30 μm. Abbreviations: see legend of Fig. 1.

sues. In thin epidermal tissues, CK14/16 staining persisted at, and beyond the spinous level, and included the stratum corneum.

Data on cholesteatoma tissues strongly suggest major deviations from the epidermal situation described above (see also: Broekaert 1984). The expression of high MW CKs (K148 antiserum) was more or less retarded in 6 out of 11 cholesteatoma lesions (Fig. 2C). It accompanied terminal keratinization in another three cases (3/11). On rare occasions, K148 staining focally anticipated terminal keratinization. Low MW CKs, probed by the K149 and K192 antisera, were only locally selective markers of basal and nearest suprabasal keratinocytes. Mostly, a decreasing staining intensity was noticed from the germinative towards the cornified layers (8/11) (Fig. 2D). Non-discriminating labelling was observed in other cholesteatoma tissues (3/11).

An in-depth study of the intermediate filament expression was performed on 21 cholesteatoma lesions. Staining profiles of highly and weakly keratinized epidermal tissues (of the thumb and meatus respectively) were simultaneously investigated as references. The results obtained have unequivocally confirmed that the CK expression in cholesteatoma matrix and stratum corneum corresponds qualitatively to the epidermal CK composition. However, there are important quantitative and topographic differences in the CK distribution.

CK staining in epidermal tissues was achieved with the following antibodies. The antibody of clone 80 (broad spectrum CKs) mostly elicited an increasing staining intensity in maturing keratinocytes and a persistent maximal staining of corneocytes. The RCK102 monoclonal antibody (CK5, 8) produced a maximal labeling in basal keratinocytes, with emphasis on the proximal cytoplasm and the serrated zone in the shallow rete ridges (Fig. 3A). Staining decreased throughout the Malpighian layers to a minimum level in terminal keratinocytes and corneocytes. RCK103 (recognizing among others CK5) produced a similar staining of living cells, but, quite unexpectedly, an intensive staining in cornified layers also. CK10 (antibodies: RKSE60, LPH1 and LH3) was totally absent in basal keratinocytes but became a prominent marker of the immediate suprabasal differentiation

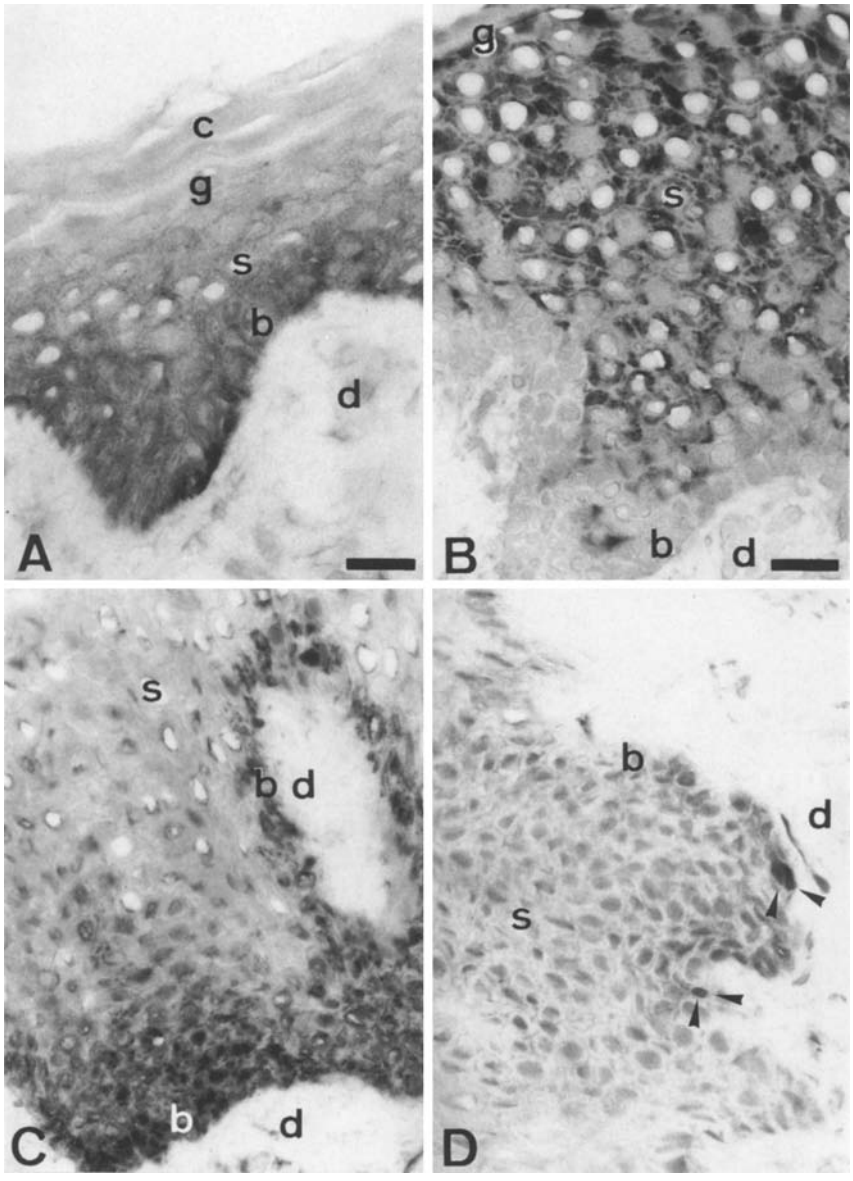

Fig. 3A–D. Immunohistochemical staining profiles of epidermal tissues, obtained with monoclonal CK antibodies. A maximal labeling with RCK102 (CK5, 8) of basal keratinocytes is reached, locally emphasising the proximal cytoplasm but decreasing throughout the stratum spinosum and granulosum (**A**: ×485). CK10, detected with the RKSE60 probe, is absent in basal keratinocytes, but a major marker of immediate suprabasal and terminal keratinization stages (**B**: ×385). CK14, screened with the LH8 probe, is noticed in the lower cell rows. Corresponding determinants are gradually less prominent in spinous keratinocytes (**C**: ×385). Merkel cells (◀), typically arranged among basal keratinocytes display CK7 (RCK105 probe; **D**: ×385). Tissue slices were routinely counterstained with Mayer haematoxylin. The horizontal bar in **A** represents 20 μm, in **B–D** 25 μm. Abbreviations: see legend of Fig. 1.

stage and of all terminal keratinization stages (Fig. 3B). CK14 staining (antibodies LH8 and EKH4) was patchy and heterogeneous in the lower part of the epidermis. The corresponding determinants were gradually less prominent in the upper layers, almost absent in terminal keratinocytes and never observed in cornified layers (Fig. 3C). Finally, Merkel cells, spatially arranged among basal keratinocytes in deep rete ridges displayed CKs n° 7 (Fig. 3D), CK n° 8, 18 and 19.

The following conclusions can be drawn concerning the cholesteatoma CK reactivity. The antibody from clone 80 recurrently provided a maximal labeling of cornified layers. In two samples, the initial labeling of living cells was postponed to prickle cell layers or even to the stratum granulosum. In 6 out of 10 cases, decoration of matrix keratinocytes remained minimal. Antibody RCK102, specific for CK5 and CK8, provided an "epidermal"-type staining pattern in 4 out of 10 cases. However, in 6 cases, determinants were maximally located in the stratum granulosum. Absence of staining or a minimal staining of cornified stages was observed in 6 out of 10 cases, while in 4 cases a remarkable labeling of the cornified debris was noticed. Clone RCK103 elicited a substantial but locally heterogeneous or "spotty" staining of matrix cells, slightly accentuating basal cells near the basement membrane and spinous cells near the cell periphery. Stratum corneum

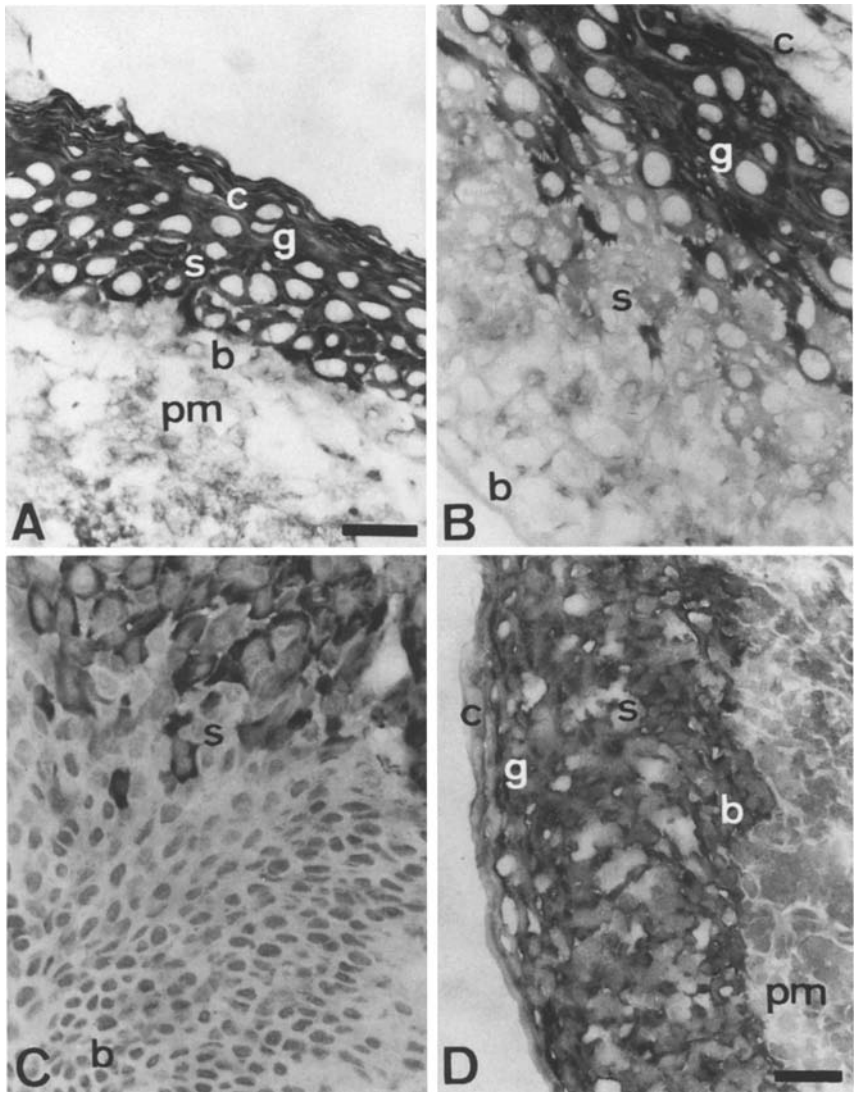

Fig. 4A–D. Expression of CK10 (clone RKSE60) and CK14 (clone EKH4) in middle ear cholesteatoma. The expression of CK10 generally starts in the lower prickle cell layers (**A**: ×330), or is postponed to more advanced stages of the matrix differentiation, leaving numerous keratinocytes in the stratum spinosum unlabeled (**B**: mosaic-pattern; ×330). The delay of the CK10 labeling is also noteworthy and may be irregular, especially when extensive hyperplasia of the stratum basale is evident (**C**: ×330). CK14 is noticed in the entire matrix and persists in the superficial horny layers. Note severe spongiosis and infiltration from the underlying granulation tissue (**D**: ×210). Tissue slices were routinely counterstained with Mayer haematoxylin. The horizontal bar in **A–C** represents 30 μm, and 50 μm in **D**. Abbreviations: see legend of Fig. 1.

staining was generally more intense than matrix staining.

When compared with the epidermal keratinization, terminal keratinization deduced from CK10 staining patterns, was clearly postponed to more advanced stages of the histological matrix differentiation in about half of the lesions studied. In 9 lesions, this delay was less evident or only focal, but was always observed. In 2 lesions only, the CK10 expression generally started in the lower prickle layers (Fig. 4A–C).

CK14 – judged from the corresponding LH8 staining – was a preferential marker of the lower half of the matrix. Cholesteatoma terminal keratinization was associated with a drastic loss of the CK14 staining ability, which was totally absent in cornified layers in 9 out of 11 cases. In 2 biopsies, heterogeneous labeling of cornified layers was evident. The EKH4 staining pattern corresponded to the LH8 profile cited above, but was generally more intense (Fig. 4D). Perhaps due to a lower detection limit in EKH4 screening, CK14 was generally observed in cholesteatomatous cornified layers.

Overall, non-epidermal CKs (CK pair 4/13 and CK n° 7, 8, 18 and 19) do not seem to belong to the cholesteatoma CK set. However, in 12 out of 21 lesions, exceptions to that rule were noticed either as a moderate or strong, but strictly focal expression, or as a weak and more extended expression of one or more non-epidermal CKs (n° 4, 7, 8, 13, 18 and 19) (Fig. 5A–C). CK7 and 8 were involved in the disturbed CK expression in only one case.

The CK expression in reference dermal tissues was restricted to sweat glands (thumb) and mucous

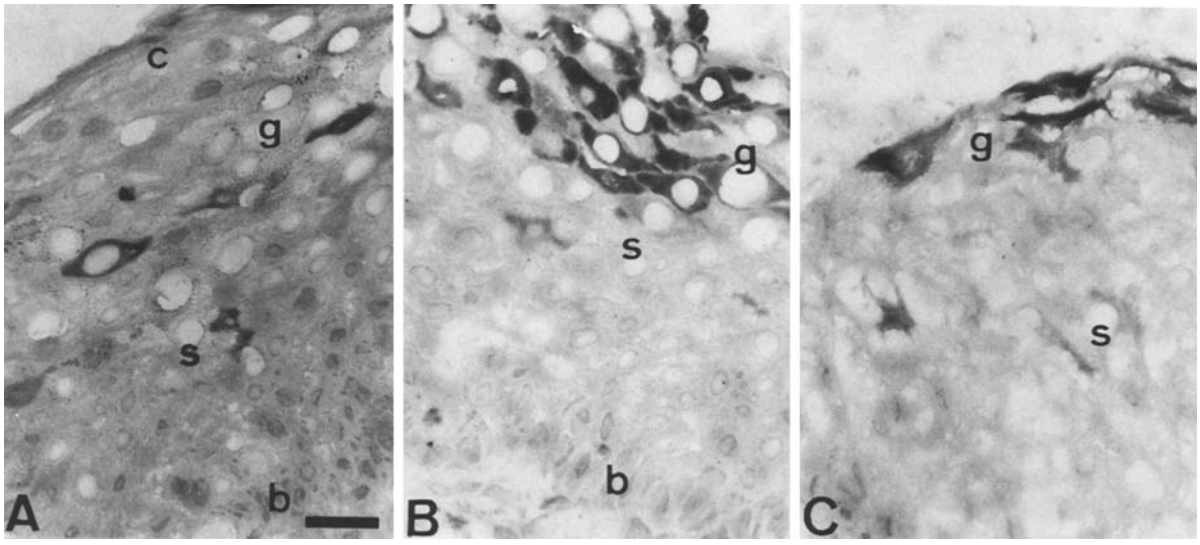

Fig. 5A–C. Expression of non-epidermal CKs in middle ear cholesteatoma. A strictly focal expression of CK4 (clone 6B10) in the stratum granulosum is shown in **A**. A more extended expression of CK4 in the upper prickle and granular cell layers is represented in **B**. Focal expression of CK19 (clone LP2K) during terminal stages of keratinization is evident in **C**. Tissue slices were routinely counterstained with Mayer haematoxylin. (**A–C**: ×330). The horizontal bar in **A–C** represents 30 μm. Abbreviations: see legend of Fig. 1.

glands (external ear canal). Sweat gland acini expressed CK n° 7, 8, 18 and 19, while ductal epithelia displayed CK n° 5, 10, 14 and 19 albeit in minimal amounts. However, the expression of CK10 in sweat gland ducti (RKSE60) was not confirmed by LPH1 assays. The absence of CK10 in acini (RKSE60) was contradicted by LPH1 screening which revealed weakly positive signals. Finally, the absence of CK14 in acinar myoepithelial cells (LH8) was clearly contradicted when EKH4 was applied. However, in the course of other studies, LH8 was found to label myoepithelial cells (unpublished results). Mucous gland acini were CK4, 7, 8, 14, 18 and 19 positive, while ductal epithelia expressed CK8, 10, 14, 18 and 19. The presence of CK4, 7 and 13 in ductal cells has not been investigated.

The CK staining in the perimatrix was limited to (sero) mucous glands, either normal, atrophic or hyperplastic. A complex CK picture was consistently revealed, fully comparable to healthy or pathological middle ear mucosa (CK4, 5, 7, 14, 18 and 19) (Fig. 6A–D). In one pathological case, CK8 was also noticed and in 3 cases, the presence of CK14 (EKH4) was not confirmed by LH8 assays. Rarely, ductal epithelia in the perimatrix were in addition CK10 positive. Furthermore, we observed a differential staining within most of the glandular structures, when individual CK polypeptides were assayed. Non-differentiated cells (so-called reserve cells) showed only a faint staining or were negative. Yet, reserve cells were rarely negative in polyclonal assays.

A concise survey of immunohistochemical staining date, obtained on cholesteatoma lesions, pathological and healthy mucosa and meatal epidermis and mucous glands is given in Table II.

Application of monoclonal antibodies, specific for other types of intermediate filaments supported the following statements. Vimentin staining was present in epidermal melanocytes and in dendritic epidermal and matrix cells (Langerhans cells) (Fig. 7). As expected, the vimentin reactivity was more pronounced in non-epithelial dermal and perimatrix tissues (fibroblasts, blood cells, vascular endothelia and macrophages). Co-expression of CK and vimentin was never observed. Desmin staining was restricted to some vascular smooth muscle cells in the dermis. Neurofilament polypeptides were only recognized in nerve fibers and fine nerve endings in the dermal layer and perimatrix.

Discussion

The results presented here are a detailed extension of the first immunohistochemical observations of Sadé et al. (1983) on the keratin expression in the cholesteatoma matrix and some perimatrix structures. Our results have unequivocally confirmed that the CK set expressed during keratinization of aural cholesteatoma corresponds to the normal epidermal CK set. Quantitative and topographic

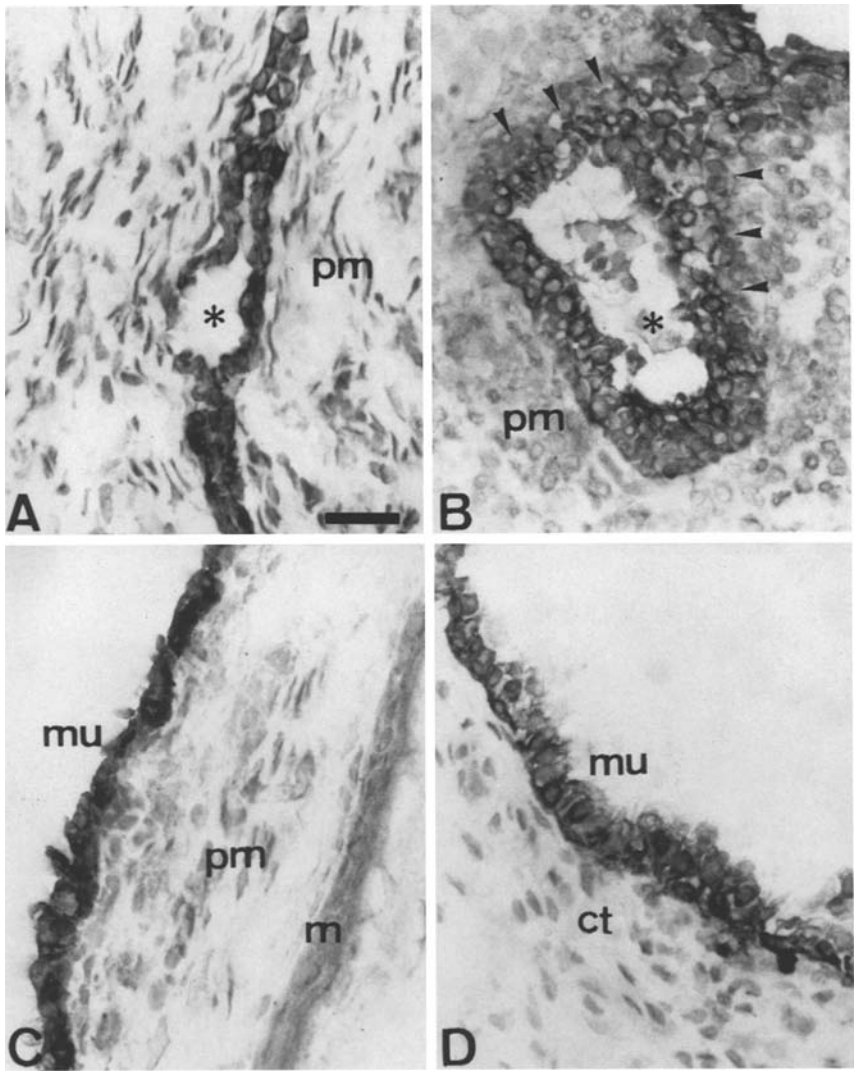

Fig. 6. Expression of CKs in the perimatrix and in middle ear mucosa. An example of a mucous glandular structure (*) with normal appearance, displaying CK5 (clone RCK102) is given in **A**. The differential expression of CK18 (clone M9) in a hypertrophic mucous gland (*) is documented in **B**. Reserve cells in the periphery of the gland (◀) are weakly stained. A thin CK18-negative cholesteatoma matrix is present under uniformly labeled, CK18-positive middle ear mucosa (**C**: clone M9). **D** illustrates the overall expression of CK5 (clone RCK102) in healthy middle ear mucosa. Tissue slices were routinely counterstained with Mayer haematoxylin. (**A–D**: ×330). The horizontal bar in **A–D** represents 30 μm. Abbreviations: see legend of Fig. 1; *ct*: (subepithelial) connective tissue; *mu*: mucosa

differences, however, were noticed in almost every case, together with a supplementary but minor expression of certain non-epidermal CKs.

The well-known concept of differential CK expression (Sun et al. 1984), was initially documented from the application of polyclonal antisera. The concept, among other things, implies that the 50/58 kD CK pair is synthesized early on during epidermal differentiation, while high M. W. CKs (i.e. the 65–67 kD members of the basic subfamily) are expressed in suprabasal, terminally differentiated cells. Our group specific K148 and K149 (K192) antisera indeed provided roughly complementary labeling. Two major acidic CKs (CK14 and CK16) have been found in basal cells, and at a reduced level later on, while high M.W. basic CKs were detected in suprabasal cells. These staining profiles are in accordance with the complementary monoclonal AE1 staining of basal cells and AE2 staining of suprabasal layers (Woodcock-Mitchell et al. 1982) and correspond to the polyclonal differential staining results of Banks-Schlegel et al. (1981), Staquet et al. (1981) and Löning et al. (1982).

Comparative investigations of middle ear cholesteatomas confirmed that the CK determinant topography was drastically different from the one described for epidermal tissues and had a strictly individual appearance. The delayed K148 determinant expression was interpreted somewhat cautiously as a retardation of the high M.W. CK expression, or in more general terms as a delay of terminal keratinization. This delay could often be correlated with an hyperplasia of the underlying stratum basale, but was also observed in areas with an apparently normal sequence of prickle cell dif-

Table 2. Survey of immunohistochemical staining data obtained with monoclonal antibodies on tissues related to the middle ear cholesteatoma disease

	Cholesteatoma			Healthy middle ear mucosa	External ear canal		
	Matrix	Perimatrix mucous glands	Residual mucosa		Epidermis	Mucous gland	
						Acini	Ducti
CK4[f]	−[a]	+	+	+	−	+	ND
CK13[f]	−[a]	−	−	−	−	−	ND
CK5[f]	+	+	+	+	+	ND	ND
CK14[f]	+	+[b]	+	+[e]	+	+[e]	+
CK7	−[a]	+	+	+	−	+	ND
CK8[f]	−[a]	−[c]	−	−	−	+	+
CK18[f]	−[a]	+	+	+	−	+	+
CK10	+	−[d]	−	−	+	−	+
CK19	−[a]	+	+	+	−	+	+

[a] exceptional expression, strictly focal or in a more extended area;
[b] 3 cases: EKH4 +, but LH8 −;
[c] one case: CK8 +;
[d] rare ductal arrangements are CK10 +;
[e] EKH4: +, but LH8 −;
[f] pairs: CK4/13, CK5/14, CK8/18

ND: not determined

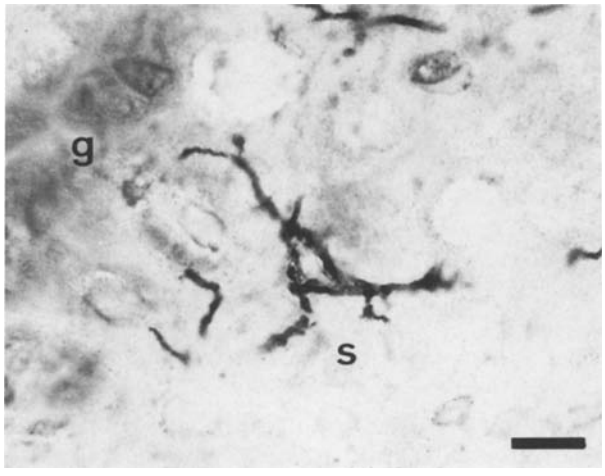

Fig. 7. Illustration of a Langerhans cell in the upper part of a cholesteatoma matrix, after staining with a monoclonal anti-vimentin probe (clone Vim9) (×830). Tissue slices were routinely counterstained with Mayer haematoxylin. The horizontal bar represents 10 μm. Abbreviations: see legend of Fig. 1.

ferentiation. In a complementary fashion, the K149 positive area tended to extend into Malpighian layers. Overall, in the cholesteatoma matrix the balance of early versus terminal keratinization markers is tipped in favour of the early ones. Terminal differentiation and cell replication are by definition two mutually exclusive events, which maintain a delicate balance in healthy keratinizing tissues (Weiss et al. 1984). In pathological conditions however, there is an approximately reciprocal alteration in the relative expression of proliferation and terminal keratinization markers, as observed here.

Our monoclonal antibody studies fully confirmed this conclusion. On the one hand, studies with three probes for CK10 – a reliable indicator of the epidermal terminal keratinization (Ramaekers et al. 1983a) – suggested a local or more general delay of the onset of terminal keratinization in cholesteatomas, and on the other hand, two probes for CK14 – an obvious marker of the basal and nearest suprabasal layers in the epidermis (Eto et al. 1985) – were located in a similar, though locally extended area in most of the cholesteatoma tissues. A minor number of cholesteatomas even conserved the CK14 determinant expression in cornified layers. The more generally observed suprabasal loss of CK14 specific staining in epidermal as well as in cholesteatoma tissues, is similar to the loss of the AE1 CK14 staining in epidermal tissues (Woodcock-Mitchell et al. 1982). However, biochemical analysis showed that CK14 persisted in suprabasal layers. Presumably, this discrepancy is due to masking of the epitope in the presence of suprabasal CKs, although a proteolytic elimination of the epitope could not be excluded (Woodcock-Mitchell et al. 1982). A hyperproliferative state of the epidermis is associated with an incomplete proteolytic removal of CK14 (Woodcock-Mitchell et al. 1982) and thus a more extended

CK14 labeling may be expected, just as we noticed in cholesteatoma tissues. Furthermore, hyperproliferative keratinocytes present in many epidermal disorders accumulate the 56/48 kD CK-pair (n° 6 and 16). These are considered to be true molecular markers of hyperproliferative diseases (Weiss et al. 1984). Unfortunately, the lack of selective monoclonal antibodies for these CKs excludes further verification of the postulated hyperproliferative state of cholesteatoma.

A positive RCK102 (CK5, 8) staining profile combined with a negative LE41 (CK8) result may indirectly but not necessarily indicate expression of CK5, since the corresponding CK8 determinant may be selectively masked. Under normal circumstances, CK8 expression is restricted to certain simple epithelia (Lane and Klymkowsky 1982; Lane 1982). CK5 is the obligate partner of CK14 in the 50/58 kD CK pair (Sun et al. 1984). Representative RCK102 staining systematically decreased from the germinative layer to the transition area, leaving only a minimal staining in epidermal squamous layers. Apparently, the corresponding determinant is unstable, although CK5 is retained during the epidermal differentiation (Woodcock-Mitchell et al. 1982). In cholesteatoma, however, we regularly noticed the persistence or accumulation of RCK102 staining in upper Malpighian layers and in the stratum corneum. This constitutes another aspect of the disturbed CK profile.

The expression of CK4 and CK13 is normally restricted to non-cornifying squamous and transitional epithelia (Van Muijen et al. 1986). The expression of CK7, CK8, CK18 and CK19 occurs preferentially in simple epithelia (see furtheron). A focal or more extensive expression of one or more of these CKs in about half of the cholesteatoma samples is a supplementary manifestation of a disturbed differentiation, interpreted as a non-epidermal and non-squamous type of differentiation. Actually, the significance of the variable composition of the cholesteatoma CK set, compared with the fixed pattern of normal epidermal CK expression, is unclear and open to speculation. Similar complexities in CK patterns are frequently seen in epidermal disorders. For a schematic representation of the spectrum of CK expression in epidermal disorders, we refer to Weiss et al. (1984). The question of whether such changes might be disease-specific has not yet been fully settled, and it would certainly appear from our analysis that a limited expression of specific new CK polypeptides occurs during the final stages of keratinization. An evaluation of the cholesteatoma CK spectrum in relation to the different hypotheses for the genesis of the lesion (epidermal versus metaplastic in origin in middle ear mucosa) favours an epidermal origin. A squamous metaplastic lesion would be expected to express a complex CK set, composed of major CKs from the recipient tissue, together with CKs representative of a stratified squamous epithelium (e.g. Gigi-Leitner et al. 1986). The composition of the cholesteatoma CK set certainly does not support this notion.

We were unable to detect Merkel cells in the cholesteatoma matrix. In addition to the "glandular" CKs n° 8, 18 and 19 (e.g. Moll et al. 1984) epidermal Merkel cells express another "glandular" CK, namely CK7. So far, this has only been observed in trace amounts in neuroendocrine tumors (Moll and Franke 1985).

Other CK-positive sites in pathological middle ears were the mucous glands in the perimatrix, with either a normal appearance or with a hyperproliferative or seriously atrophic character. These simple, pseudostratified or stratified epithelia display a complex CK pattern (CK4, 5 and/or CK8, CK7, 14, 18 and 19), comparable to that of cholesteatoma associated middle ear mucosa or mucosa from the unaffected part of the middle ear. Additionally, CK8 was observed in one case and CK10 was noticed in a few ductal arrangements of mucous glands in another case. This CK composition strongly resembles the CK pattern of mucous gland acini in the external ear canal (CK7, 8, 14, 18 and 19). Here again, CK10-specific determinants were found in ductal epithelia. This was unexpected, since CK10 is considered to be a prominent and selective marker of epidermal terminal keratinization and of certain squamous metaplasia (see above). Glandular epithelia are in general complex tissues, expressing a mixture of both typical "simple" and "stratified" types of CK (Cooper et al. 1985). Data on mucous gland CKs are scarce in literature and the only positive CK recognition was restricted to CK18 in mucous glands of other loci (e.g. Ramaekers et al. 1983c).

Dermal sweat gland acini also express a great variety of CKs (CK7, 8, 14, 18 and 19), while ducti express CK5 (and/or CK8), CK10, 14 and 19 (see also: Moll et al. 1982; Ramaekers et al. 1983c; Eto et al. 1985). The expression of CK14 by acini is strictly confined to myoepithelial cells as noted before (Eto et al. 1985). Contrary to the previous statement by Van Muijen et al. (1986) on locally CK4 positive single cells or small cell clusters in sweat gland ducts, these structures did not reveal CK4 positive cells in the present study.

Concerning the presence of other specialized cells in matrix tissues, our observations corrobo-

rate and extend earlier conclusions. The presence of Langerhans cells in cholesteatoma matrix tissues, known from morphological studies (e.g. Lim et al. 1977), was recently confirmed by immunohistochemical methods (Van Dyk et al. 1986). Because of the presence of vimentin in epidermal Langerhans cells (De Waal et al. 1984), we could demonstrate the presence of these dendritic cells in matrix tissues using vimentin monoclonal antibodies. Melanocytes, another type of intra-epithelial vimentin expression cell (e.g. Ramaekers et al. 1983a), are scarce or even absent in the cholesteatoma matrix (Lim et al. 1977). This was further confirmed by our vimentin studies.

In conclusion, our results show that the diverse types of epithelia, present in the middle ear cholesteatoma matrix and perimatrix, have specific CK expression patterns. The CK topography in the matrix provides further evidence for the notion that the CK expression can be affected by a state of disease. Broadening of the histological compartment, corresponding to early stages of keratinization, and the subsequent delay of terminal keratinization suggest that cholesteatoma involves a hyperproliferative state, also present in a number of benign hyperproliferative epidermal diseases (Weiss et al. 1983). The study of two other markers of hyperproliferative keratinization (CK6 and 16: Weiss et al. 1984) may help to elucidate the true nature of the aural cholesteatoma lesion.

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