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TECHNICAL NOTE

CRIMINALISTICS; PATHOLOGY AND BIOLOGY

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A New Approach to the Investigation of Sexual Offenses—Cytoskeleton Analysis Reveals the Origin of Cells Found on Forensic Swabs*

ABSTRACT: There are forensic inquiries in which an identification of epithelial cell types would provide important probative evidence. In cancer diagnosis, this information is yielded by histological examination of cytokeratin (Ck). Therefore, we tested 19 antibodies against different Cks (Ck1, Ck2e, Ck4, Ck5-6, Ck7, Ck8, Ck9, CK10, Ck13, Ck14, Ck15, Ck16, Ck17, Ck18, Ck19, Ck20, Ck903, PanCkAE1_3, and CAM5-2) on histological sections of epidermis, buccal mucosa, vaginal mucosa, penis, urogenital tract, and rectum and could identify two antigens unique to buccal-cell and vaginal-cell (Ck4) and skin epithelial-cell (Ck10) cytokeratin. Subsequently, we developed an immunocytological technique for distinguishing swabbed skin and mucosal cells up to at least 1 year after sampling. By the detection of the Ck4 and Ck10 mRNAs in biopsy and laser capture microdissection collected samples via quantitative real-time polymerase chain reaction, we were able to confirm our immunological findings. Hence, this study offers techniques to discriminate between skin and mucosal cells (buccal and vaginal) in forensic casework.

KEYWORDS: forensic science, immunocytology, cytokeratins, epithelial cells, sexual offense, DNA, mRNA

Advances in forensic science during recent years have made it possible to assign very small DNA amounts (1) and even single cells (2) to a specific person. A broad variety of source materials has also opened up. Technical progress has now made it possible to type degraded DNA in small reaction volumes (3) with sensitive, human-specific DNA quantitation (4) beforehand. On the other hand, it currently remains impossible to relate forensic samples to epithelial cells from different areas of the body at the same time. To say not only from whom but also from where is an investigative question whose importance is not to be underestimated and which represents one of the most important challenges in modern forensic investigation. In cases of sexual offenses or child abuse, for example, it very often would be crucial to distinguish mucosal cells and skin cells.

In the course of inquiries into sexual abuses of women and children, swabs are collected from the suspects' hands or penis to establish proof of contact with the victim by DNA analysis. When confronted with the results, however, the accused person often offers a harmless explanation: e.g., the DNA results from a handshake. Oral or vaginal contact is usually disclaimed vehemently and cannot be clearly verified by actual forensic procedures (5).

Previous studies have shown that Lugol's Iodine (6–8) and protein or ion detection (9) are not suitable for the identification of vaginal epithelial cells, for example, in penis swabs, where epidermal cells and cells of the male urogenital tract can also occur. Although it has proven possible to identify body fluids by mRNA expression (10–12), stains are required for analysis, whereas cells found on swabbed forensic samples have proven inaccessible so far.

Modern pathologic diagnosis inspired us to try an approach via the cytoskeleton, more precisely the intermediate filaments. The cytoskeleton is a filamentous network of F-actin (5–9 nm in diameter), microtubules (25 nm in diameter), and intermediate filaments (about 10 nm in diameter). Within intermediate filaments, four subtypes (vimentin filaments, lamina, neurofilaments, cytokeratins) can be distinguished. In particular, whereas vimentin filaments, lamina, and neurofilaments can be found in muscle, nucleus, and neural tissue (13), respectively, cytokeratins are restricted to the epithelial layers (14). Immunohistochemistry (IHC) is a diagnostic technique routinely used in pathology, and the DAKO Envision+™ detection system (15) makes use of cytokeratins in modern cancer diagnosis (16–19). Therefore, we tested different cytokeratins on sections of epithelial layers taken at areas of forensic interest.

Because immunohistochemical sections show only a small portion of the respective sample, which must always be embedded in a paraffin cellblock (5) with this technique, we wished to develop an immunocytological technique (ICC) in which the entire

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sample can be placed on a few slides. As the next step, we wanted to offer the possibility of selectively removing all cells of interest by laser capture microdissection (LCM) for further investigations, such as DNA analysis, an approach that has been successfully used in the separation of sperm (20–22) or male and female cells (23–25).

We also investigated mRNA to verify our immunological results. That is, the respective mRNA encoding for Ck4 and Ck10 was analyzed via quantitative real-time polymerase chain reaction (q-RT-PCR) in biopsy material, freshly taken swabs, and LCM cell samples.

Materials and Methods

Sample Collection

All tissue samples were collected from female and male cadaver donations according to guidelines approved by the University of Tuebingen Ethics Committee (reference #: 399/2005).

Histological materials were provided by the funds of the Institutes of Legal Medicine and Pathology.

All swabs (buccal dry swab, vaginal, anal, arm, and penis wet swab) were taken from voluntary subjects, then dried and stored in cardboard boxes for biological evidence samples at room temperature (RT) in the same manner as casework samples.

Fixation

Tissues were fixed in buffered formaldehyde (3%, ROTH, Karlsruhe, Germany) for at least 1 week. They were subsequently placed in aqua dest. for 5–10 min. After being passed through an increasing ethanol series, the samples were placed in xylol (ROTH) for at least 3 h. Finally, the tissues were embedded in paraffin wax.

Cells on cotton swabs (Euroturbo®; Deltalab, Rubi, Spain) were extracted and fixed with CytoRich Red® collection fluid (Thermo Electron Corporation Waltham, MA). For this, the swabs were placed in 1.5-mL reaction vessels (Biozym Scientific GmbH, Oldendorf, Germany) with 1 mL collection fluid for 5 min. To remove the cells, the swabs were rotated before being squeezed and removed. To support a suspension of possible cell clusters, the reaction vessels were subsequently placed in a shaker (Uniprep-Gyrator; Invitek GmbH, Berlin, Germany) for 10 min. The samples were then centrifuged for 10 min at $600 \times g$ to spin down the cells. The supernatant was removed, and the cells were suspended in 50–250 μL new collection fluid, depending on the pellet's size. About 25 μL of the suspended cells were applied on adhesion microscopic slides (Histo-Bond®, Marienfeld, Germany), dried and given to the staining process.

Staining

All samples in this study were stained using the DAKO Envision+™ detection system (15). Within this two-step biotin-free staining technique, the primary antibody is applied followed by a polymeric conjugate in sequential steps. The polymeric conjugate consists of a dextran backbone holding up to 100 peroxidase molecules and about 20 secondary antibody molecules. The DAKO Envision+™ detection system is easy to use and avoids the possibility of disturbing endogenous biotin.

Histological Sections IHC

All slides were examined with a light optical microscope (Leitz Aristoplan). Images were taken using a digital camera (JVC KY F75) combined with a DISKUS® software (Carl Hilgers, Koenigswinter, Germany, rel. 4.50.1075). Initially, we tested 19 antibodies specific to different Cks (Ck1, Ck2e, Ck4, Ck5-6, Ck7, Ck8, Ck9, CK10, Ck13, Ck14, Ck15, Ck16, Ck17, Ck18, Ck19, Ck20, Ck903, PanCkAE1_3, and CAM5-2) and an estrogen receptor-α (ERα) on histological sections ($n = 10$ of each type of sample) of epidermis (scalp, palm = EP), penis (glans, shaft = PE), urogenital tract (bladder [bottom], urethra [proximal part], ureter [middle section] = UR), buccal mucosa (BU), vaginal mucosa (ectocervix = VA), rectum (RE), and the respective positive control tissues specified on the antibody's data sheets. Two micrometer-thick sections were cut and placed on adhesion microslides. The sections were deparaffinized in xylol for 30 min, then rehydrated with distilled water in a decreasing ethanol series (8 min at 100%, 96%, 70% and with aqua dest.). The sections were stained according to the provider's recommended protocol and counterstained with 0.1% Mayer's hematoxylin (ROTH). Monoclonal Mouse IgG1 (DAKO, code no. X 0931) was substituted for the primary antibody in the internal negative controls.

Cytological Samples ICC

Anti-Ck4 (clone 6B 10, Novocastra, code no. NCL-CK4,) and anti-Ck10 (clone DE-K10, DAKO, code no. M 7002) were tested on cells obtained from cotton tip swabs. Because of the special premises of forensic analysis, a dye protocol for processing forensic material had to be developed by taking buccal, vaginal, epidermal, rectal, and penis swabs from different ($n = 10$) persons, then drying and storing them for at least 5 days. Different fixations (ethanol/diethyl ether, air drying, and CytoRich Red®), permeabilization steps (via digitonin, trypsin, and saponin), incubation times, and temperatures were tested and optimized (data not shown), which led to the procedure described later.

The resulting respective Ck4 and Ck10 staining method was tested on both postcoital and older samples. Vaginal ($n = 13$) and penis ($n = 38$) swabs were taken 2, 6, 12, and 24 h postcoitally and stored for at least 2 weeks before examination. Older samples (vaginal, buccal, arm, penis, and postcoital swabs, $n = 42$), stored for 6 months to 1 year, were also taken for analysis. Vaginal swabs from three different women were taken daily for 28–35 days to observe a possible change in reactivity during the menstrual cycle.

Cells were extracted and fixed with CytoRich Red® collection fluid as described earlier and applied to slides with a pipette. After rehydration of the dried solution to distilled water in a decreasing ethanol series (8 min at 100%, 96%, 70%, and with aqua dest.), the slides were transferred to 1 mM EDTA (ROTH) buffer (pH 8.0) and heated for 15 min in a food steamer (Ck4 typing) for heat-induced epitope retrieval (HIER). To block unspecific peroxidase activity, the object plates were then incubated in 3% H_2O_2 (ROTH) for 10 min. After being washed two times with tris-buffered saline (0.05 M TBS), the cells were treated for 2 min with 0.05% trypsin (ROTH) solution for permeabilization (Ck4 typing). A HIER and permeabilization step was unnecessary with the Ck10 antibody. To prevent background staining, normal goat serum (DAKO), diluted (1:10) in 3% BSA (ROTH)/TBS (0.05 M) solution, was poured over the samples and drained after 30 min at RT. The cells were then incubated with the respective primary antibody at 37°C for 1 h. In the internal negative controls (made of every specimen analyzed), the primary antibody was replaced with mouse IgG1. Primary antibodies were detected using the DAKO Envision+™ detection system. Subsequently, the object holders were washed (2×5 min in TBS) and incubated for 30 min with labeled polymer at RT. After another two washing steps (5 min in TBS), positive

cells were identified by DAB+ labeling. Finally, the samples were counterstained with 0.1% hematoxylin. Positive cells appeared now dark brown to black while negative cells were colored blue (Figs. 4 and 5).

LCM

LCM was carried out with a PALM® MicroBeam System using 1- mm PALM® membrane slides. To improve the adherence of the cells on the membrane surface, the slides were irradiated with UV light (390 nm) for 20 min and incubated with 0.1% poly-L-lysine solution (Sigma–Aldrich Chemie GmbH, Munich, Germany) in a moistened chamber for 1 h at 37°C. Finally, at least 50 cells were removed by LCM and taken for further analysis.

q-RT-PCR

To verify the immunological results, the respective Ck4 (KRT4) and Ck10 (KRT10) mRNAs were analyzed, respectively, on skin biopsies (n = 5) and on buccal (n = 7) and vaginal swabs (n = 6). Additionally, swabbed cells were placed on membrane slides and stained with 0.1% hematoxylin under RNase-free conditions. Subsequently, 50, 100, 200, 500, and over 1000 cells were removed via LCM and taken for RNA analysis.

The total RNA of the sampled cells of buccal and vaginal swabs was isolated using RNAprotect Saliva reagent (Qiagen) and the RNeasy Micro Kit (Qiagen). The skin biopsies were homogenized using Ultrathorax® (IKA, Staufen, Germany) prior to total RNA isolation with RNeasy Micro Kit (Qiagen).

The RNA quality was checked with a Lab-on-Chip-System Bioanalyser 2100 (Agilent), and the concentration was determined using a BioPhotometer (Eppendorf).

According to manufacturer's protocol, cDNA synthesis was performed with 2–50 ng of total RNA using the QuantiTect Reverse Transcription Kit (Qiagen), which includes a DNase digest to remove potential RNA contaminating DNA. For the detection of a possible genomic DNA contamination, we included a RT-negative control.

The q-RT-PCR was performed with the QuantiTect SYBR Green PCR Kit (Qiagen) in the LightCycler 480 System (Roche). Transcript-specific exon spanning QuantiTect Primer (Qiagen) were used for amplification of KRT4 (order no.: QT00052500) and KRT10 (order no.: QT00017045). The q-RT-PCR conditions were 95°C for 15 min followed by 45 cycles of 95°C for 15 sec, 55°C for 30 sec, and 72°C for 30 sec. Acquisition was performed after the 72°C step of each cycle. Melting curves were generated at the end of the run to verify the amplicon specificity. Standard curves of each amplified gene were created to obtain the PCR efficiency. The cp values of the target genes were detected by the LightCycler 480 Software applying the Second Derivative Maximum method.

Results

IHC and ICC Staining

Initially, the cytokeratin expression was investigated on sections of epithelial layers taken from areas of forensic interest to identify one or more cytokeratins that could be useful in sexual-assault investigations. In histological sections, a positive result meant that the respective tissue showed a significant dye reaction in comparison with the internal negative control (Figs. 1*A,a* and 2*B,b*).

Examination of the histological sections made it possible to identify two cytokeratins (Ck4 and Ck10) that are appropriate for discrimination between epidermal and mucosal tissues (Figs. 1, 2, and 3*A* + *B*). Another cytokeratin (Ck8) was found exclusively in the epithelial layers of the urogenital tract and rectum (data not shown). All other cytokeratins tested (Ck1, Ck2e, Ck5-6, Ck7, Ck9, Ck13, Ck14, Ck15, Ck16, Ck17, Ck18, Ck19, Ck20, Ck903, Pan-CkAE1_3, and CAM5-2) were only minimally or not at all useful for the sexual-assault questions under investigation. Reliable differentiation between vaginal and buccal cells was not possible on the basis of Ck and ERα expression.

In the next step, an immunocytological (ICC) technique was developed that offers the possibility of typing the selected cytokeratins on cells found on forensic swabs. To evaluate the efficiency of the staining procedure, an average success or dye rate within the cytological samples was determined. In this, the percentage of cells per slide showing a distinct positive reaction was quantitated in four different representative fields of view if the internal negative controls showed no dye reaction.

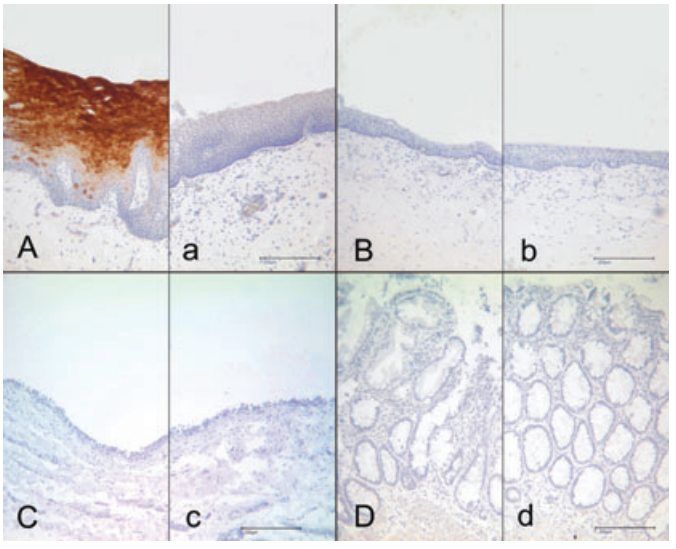

FIG. 1—*Ck4 findings in histological sections of vaginal mucosa (A,a), epidermis (B,b), bladder (C,c), and rectum (D,d). The small letters mark the respective internal negative controls.*

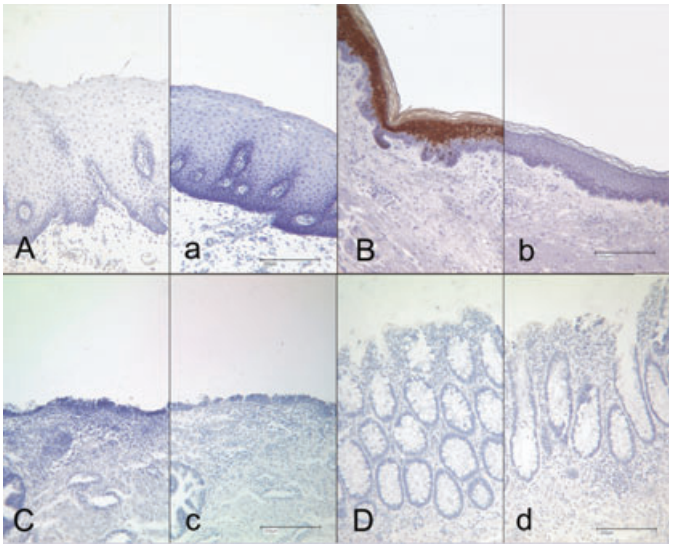

FIG. 2—*Ck10 findings in histological sections of vaginal mucosa (A,a), epidermis (B,b), bladder (C,c), and rectum (D,d). The small letters mark the respective internal negative controls.*

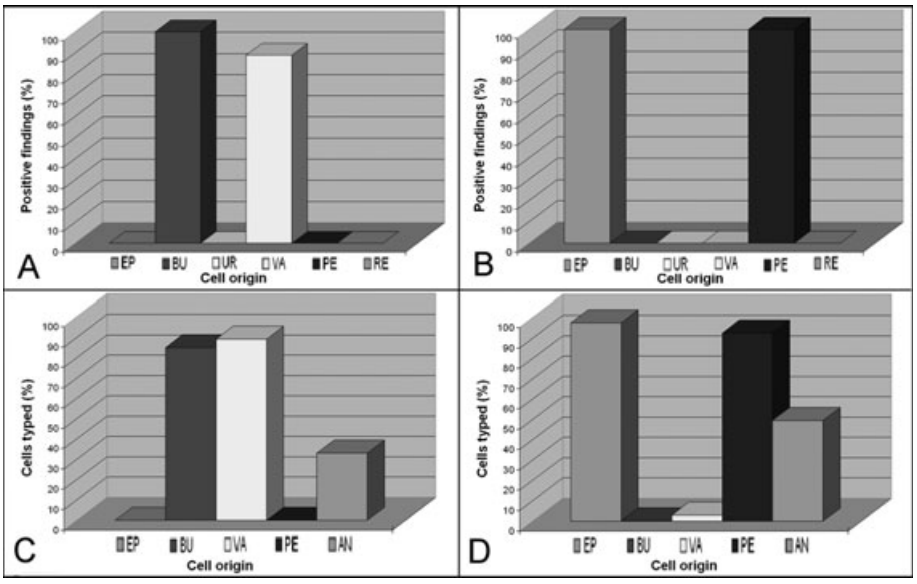

FIG. 3—*(A,B) Positive Ck4 (A) and Ck10 (B) findings (percentage of samples showing positive reaction) in histological sections (EP = epidermis, BU = buccal mucosa, UR = urogenital, VA = vaginal mucosa, PE = penis epithelium, RE = rectum). (C,D) Percentage of cells swabbed from different areas (EP = epidermis, BU = buccal, VA = vagina, PE = penis, AN = anal) showing positive Ck4 (C) and Ck10 (D) detection.*

The dye technique described here made it possible to classify the mucosal or epidermal origin of cytological cell samples from the buccal mucosa, the epidermis, and the vaginal mucosa with success rates of 85–95% (Fig. 3*C* + *D*, Figs. 4 and 5), while a small number (5–15%) of the cells or cell fragments showed no distinct dye reaction. We found no epidermal cells reacting to Ck4 typing (Fig. 4*B* + *D*) or buccal cells showing Ck10 dye reaction (Fig. 5*A*). In some vaginal swabs, a small number of cells (<10%, Fig. 3*D*) displayed a slight positive Ck10 reaction (Fig. 6*A*). In the manufacturer's product information for the anti-Ck10 antibody, a study (19) is mentioned showing that a variable amount of cells in some noncornifying stratified epithelia, including vagina, are labeled. However, because the observed Ck10 expression was comparatively low, the vaginal cells were still easily distinguished from cells of epidermal origin (Fig. 6*B*).

Subsequently, the developed Ck4 and Ck10 staining methods were tested on postcoital and older samples. Furthermore, a possible change in reactivity during the menstrual cycle was investigated. For a reliable classification of cells, it is imperative that several control samples also be run (26). Beside the internal negative controls (made of every specimen analyzed), which would reveal any unspecific dye reaction, fresh and older samples of known origin were checked (positive and negative controls) to monitor the actual quality of the staining process.

By this, detection of foreign mucosal or penis cells in the postcoital samples (Fig. 7) was possible in all cases. Hereby, we found less epidermal cells in vaginal swabs than vaginal cells in penis swabs (data not shown), which is most likely because of a different reciprocal transfer rate of cells. In some postcoital spot samples, we removed cells of one origin by LCM and we subsequently were able to type the correct alleles of the respective donor by DNA analysis (data not shown).

All samples stored for up to 1 year were typed successfully (Fig. 7) in the same manner. Within the older samples of a known single origin, we observed no significant loss of reactivity compared to fresh samples.

For detection of a possible effect of the menstrual cycle, the cells showing a positive dye reaction within the vaginal samples of the three volunteers were counted in the same manner as for the other cytological studies. The observed dye rates during the menstrual cycle revealed no significant differences, which means that we detected less than ± 5% difference in cell count (data not shown). Hence, we could not observe any influence on Ck4 and Ck10 expression.

LCM and q-RT-PCR

To verify the immunological results and to suggest another technique for the investigation of cytokeratin expression, the respective cytokeratin mRNAs were analyzed on skin biopsy material, on swabbed mucosal cells and on hematoxylin stained cells that were subsequently removed via LCM.

The PALM® MicroBeam System provided very accurate laser cutting. Isolation and catapulting of single cells or cell clusters could be performed without any difficulty.

The q-RT-PCR proved to be very sensitive, and results could be obtained from even 50 LCM-sampled mucosal cells. The low amount of RNA material in the samples and, additionally, the difficulty of analyzing a housekeeping gene in all of the samples with the same expression level led us to an analysis of the relative expression of Ck4 mRNA (KRT4) with reference to Ck10 mRNA (KRT10). The efficiency of KRT4 (1.997) and KRT10 (2.202) was included in the calculation of the relative expression of KRT4.

The analyses revealed significant differences between the amounts of KRT4 and KRT10 in buccal, vaginal (swabbed and LCM-removed samples yielded the same results), and epidermal samples (skin biopsies). We can exclude genomic DNA contamination because we did not obtain amplification curves in the RT-negative control (data not shown). The mean relative expression of KRT4 in vaginal epithelia (Fig. 8) is 1629 in relation to KRT10. In buccal samples, the relative expression of KRT4 with reference to KRT10 was, with a value of 17,546, even larger. However, the situation was reversed in the epidermal probes: we found KRT4 with a relative expression of 0.0037 compared to KRT10.

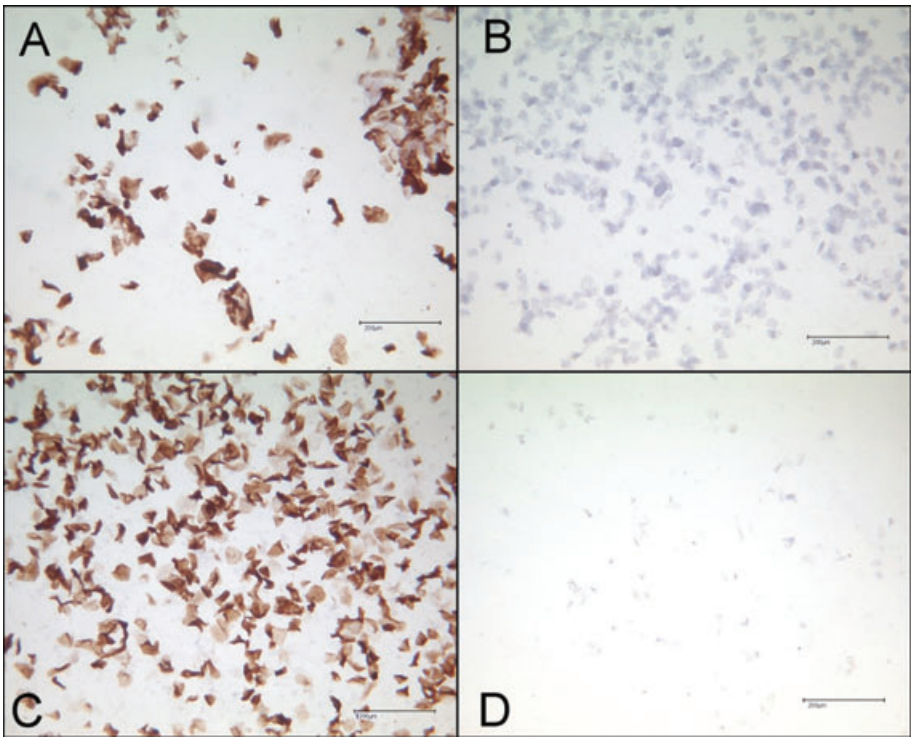

FIG. 4—*Ck4 findings in cytological samples (A = buccal swab, B = epidermal swab, C = vaginal swab, D = penis swab).*

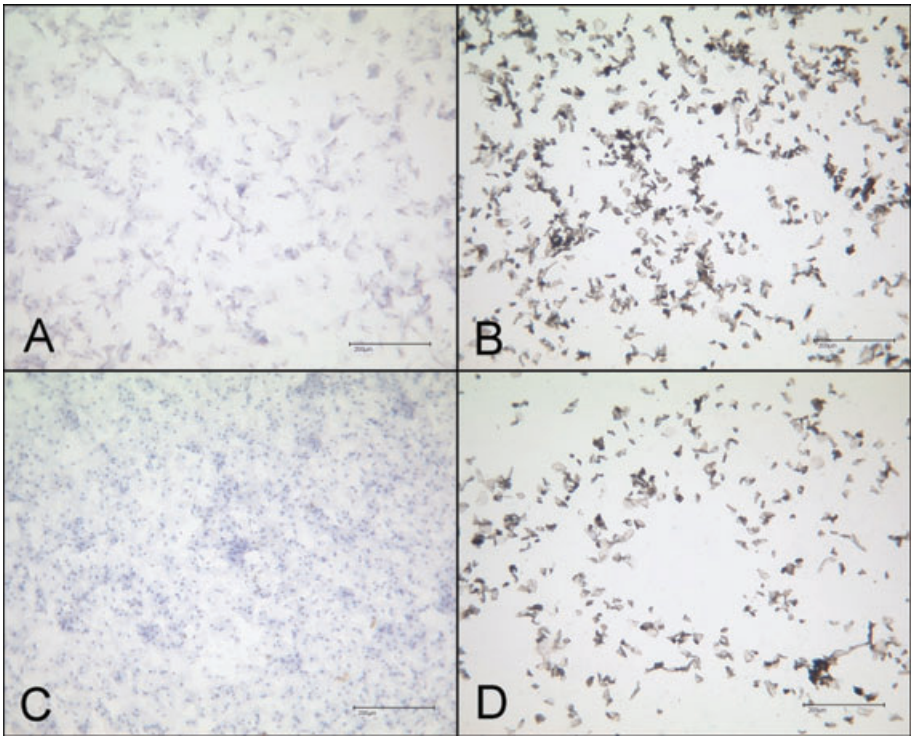

FIG. 5—*Ck10 findings in cytological samples (A = buccal swab, B = epidermal swab, C = vaginal swab, D = penis swab).*

Discussion

IHC and ICC Staining

The results obtained with the DAKO Envision+™ detection system in histological samples conform with former studies (14–16) when the same or similar tissues were examined.

The cytological dye technique described here offers the possibility of assigning cells found in forensic samples to mucosal or epidermal origins by typing them with Ck4 and Ck10 antibodies. Because it is naturally of fundamental importance to meet usual quality standards (26) when handling forensic samples, the staining process must be checked, for example, with both negative controls

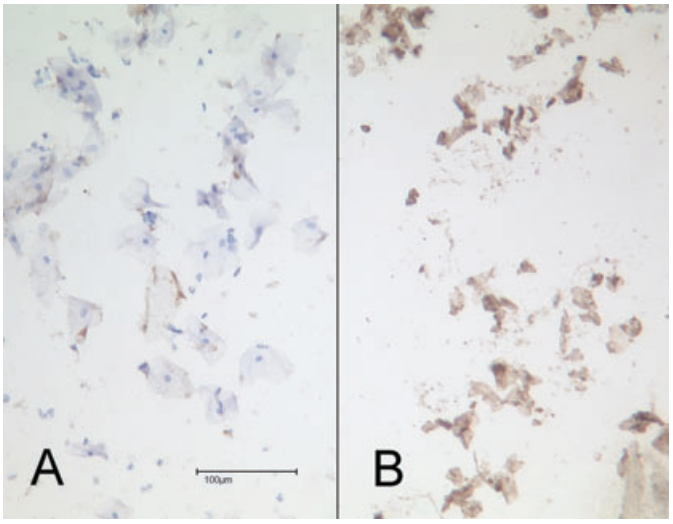

FIG. 6—*Ck10 findings in cytological samples (A = vaginal swab, B = epidermal swab) with a partial coloration of some vaginal cells and fully stained epidermal cells.*

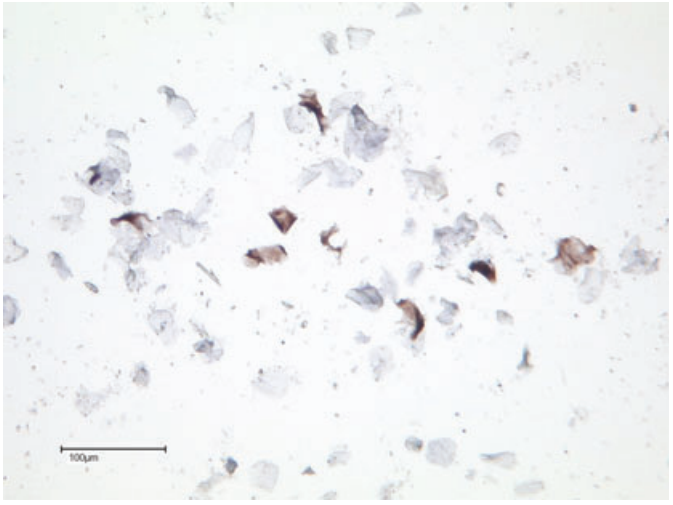

FIG. 7—*Cells extracted from a penis swab sampled 12 h postcoitally, stored for 8 months at RT and typed with Ck4 (vaginal = positive, epidermis = negative).*

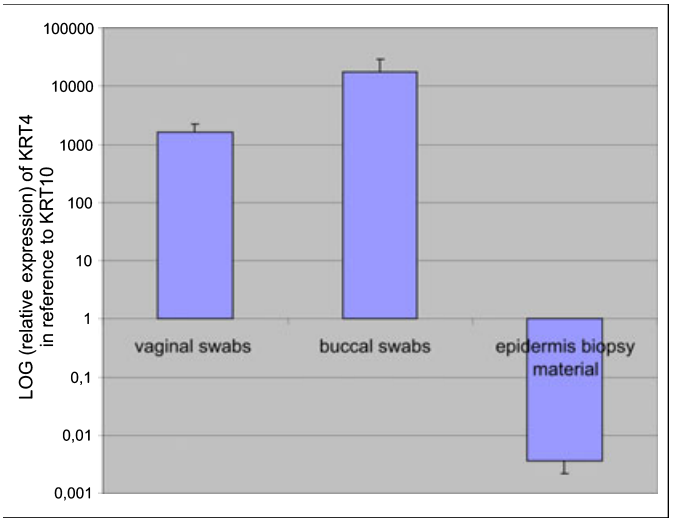

FIG. 8—*LOG (relative expression) of Ck4 mRNA (KRT4) with reference to Ck10 (KRT10) in vaginal swabs, buccal swabs, vaginal and buccal LCM cell samples and epidermis biopsy material.*

of every sample and control samples of known cell material. Only if these controls show accurate results is the typing of unknown samples reliable.

There were a small number of cells within all cytological samples that could not be typed (Ck4 *c.* 5–15% and Ck10 *c.* 5–10%). A possible reason for this might be that, in these cases, only cell fragments were left and the major part of the cytoskeleton was already lost. When dealing with postcoital samples, one cannot know if some "false-negative" cells (cells of mucosal origin showing no Ck4 and epidermal cells showing no Ck10 labeling) or cell fragments are present, but the typed control samples proved that at least the positive findings were 100% reliable. Altogether, the phenomenon of these "false-negative cells" was never a serious problem because there were always enough dyed cells left for analysis. For example, it was never a problem for us to find a large number of mucosal cells on postcoital penis swabs, even 24 h after sexual intercourse. Moreover, in our opinion, a certain number of Ck4 positive cells are necessary to prove a direct mucosal contact and to exclude a secondary transfer.

In some cells from the vaginal samples, we detected a small amount of Ck10 additionally to Ck4 (Fig. 6). This indicates that there may be a general difference between buccal and vaginal mucosa concerning their cytokeratin expression. This hypothesis is supported by the respective mRNA typing results where we found a larger difference between KRT4 and KRT10 expression in the buccal samples. However, we observed too little positive Ck10 staining reaction to clearly distinguish between the mucosal tissues immunocytologically.

Nevertheless, the technique described here clearly distinguished vaginal cells from skin cells and thus sufficed to answer most of the investigative questions common in sexual-assault inquiries. Although in our opinion discrimination between buccal and vaginal cells is not possible by means of Ck4 or Ck10 typing, it is also unnecessary in most cases, because the accused persons usually deny both vaginal and oral contact. On swabs taken from suspects' hands for example, it would be necessary to show that the DNA of victims is not the result of a handshake.

LCM and q-RT-PCR

As the PALM® MicroBeam system has already proven its suitability for forensic purposes (22), it again offered the possibility of comfortably and precisely separating the cell samples.

We found that q-RT-PCR exactly and sensitively confirmed the results of our immunological stains. Moreover, by analyzing the relative expression of KRT4 and KRT10, even the possibility of differentiating between buccal and vaginal mucosal cells is indicated. Examination of mRNA has proven to be a possible tool in identifying the origin of forensic material (10–12). However, to achieve results in these studies, a comparatively large amount of starting material was necessary. Because we have analyzed very small samples, our results look promising and it should be possible to type the intermediate filaments in forensic material via mRNA analysis. However, when evaluating the mRNA results, one should keep in mind that we tested rather fresh material that was not dyed at all (swabs and biopsy material) or hematoxylin stained (cells removed by LCM) and not labeled for cytokeratin beforehand. On

the other hand, Ck staining would be required for directed LCM in forensic case material. Thus, it still remains to be clarified whether this technique can be used to fully substitute staining results in forensic cytological samples. However, there is nothing to be said against a parallel analysis of both protein and mRNA expression if enough starting material is available.

Conclusion

The techniques presented in this study now make it feasible to distinguish between mucosal and epidermal cells found in forensic samples. The immunocytological method described here is compatible with sampling and storage conventions in the forensic community and requires no special treatment. The detection of the respective mRNAs is suggested to be another possible option for the investigation of forensic material.

These results are very promising for forensic investigation. The use of other intermediate filaments might offer the possibility of relating cells to a broad variety of tissues, after which selective DNA typing should be established. This offers the possibility of answering very specific investigative questions.

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