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SHORT REPORTS

Cryosectioning of Hair Follicles

An Improved Method using Liquid Nitrogen Conduction Freezing

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Horizontal sectioning of scalp biopsies is especially useful in hair diseases characterized by a reduction of follicles in size or number. Horizontal sectioning using standard cryo-microtomes is hampered by the differences in cutting properties between hair follicles and fatty tissue, resulting in loss of topography. We report a simple, inexpensive method to temporarily cool the specimen to an appropriate temperature by means of a conduction system using liquid nitrogen. Immunohistological methods such as immunofluorescence and immunoperoxidase techniques can be applied without restrictions. ***Key words: Horizontal cryosectioning; Fatty tissue; Hair diseases; Alopecia areata; Immunostaining.***

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For routine histopathology and immunohistological analysis vertical skin sections are used. For hair diseases, a better approach would be the use of horizontal sections. The practical value of this approach in frozen sections is limited due to differences in the optimal cutting temperature for hair follicles and connective or fatty tissue. The optimal temperature for cutting fatty tissue is below −30°C and thus beyond the range of standard laboratory cryomicrotomes.

We report a simple, inexpensive method for cooling the biopsy on the tissue holder to an appropriate temperature by means of a conduction system using liquid nitrogen. By using this technique, sections similar in quality to paraffin sections can be obtained. In order to show that the expression of common antigens is not altered, the sections were processed with standard techniques for immunofluorescence and immunoperoxidase staining. In normal hair follicles, the expression of cytokeratin 13/16 wasanalysed. In order to evaluate this approach in hair diseases, we chose alopecia areata as a disorder with a prominent T cell infiltrate (1, 2) for staining with an antibody with anti-CD2 reactivity.

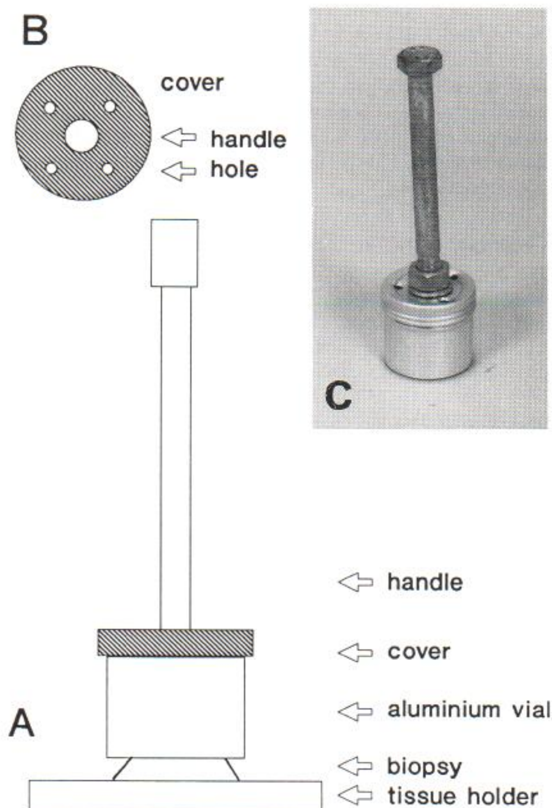

Fig. 1. Liquid nitrogen conduction freezing system. (*a*) lateral view. (*b*) view from the top. Note filling holes. (*c*) Insert: photograph of the system.

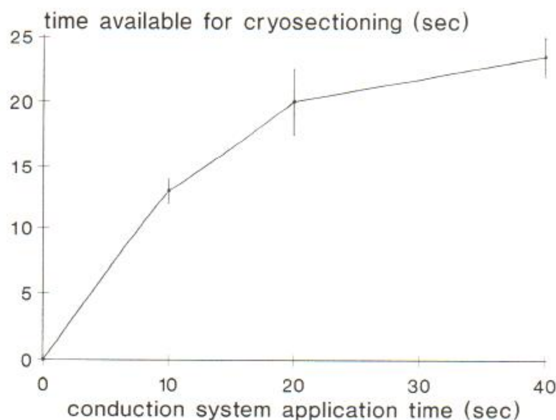

Fig. 2. Relation between conduction system application time and time available for cryo-sectioning.

MATERIALS AND METHODS

Liquid nitrogen conduction freezing

The conduction system consists of an aluminium vial (diameter 30 mm, height 29 mm) normally used as a specimen vial for the storage of frozen biopsies (Sanbio, Uden, The Netherlands). It is closed with a screw cap, in which some holes of approx. 2 mm are drilled. For better handling, a long screw is fixed in the middle of the top (Fig. 1). The entire system can be filled by dipping it in a reservoir with liquid nitrogen. The bottom of the vial is pressed on the tissue specimen lightly, in order to cool it to the appropriate temperature.

Calibration procedure

The optimal conditions for the conduction freezing process were determined experimentally, using a temperature-sensitive resistor (thermistor YSI 409 A, Tamson, Zoetermeer, The Netherlands) embedded in a block of Tissue-Tek (approx. 1×1×1 cm), mounted on the tissue holder of the cryostat (set at −25°C). The thermistor had first been calibrated in the range from -20°C (18 kΩ) to −115°C (19 MΩ): a linear curve was obtained by plotting the logarithm of the electrical impedance against the temperature. An application time of 20 s was found to be sufficient to equilibrate the block of Tissue-Tek with the liquid nitrogen. After removing the liquid nitrogen vial from the block, the time required to reach −40°C (80 kΩ) was recorded, since this is the highest temperature that allows proper sectioning of adipose tissue (Fig. 2). Following this method it was found that sections could be obtained up to 25–30 s after removal of the liquid nitrogen. This allows 5 to 7 high quality sections to be taken. Excessive pressure of the aluminium vial on the tissue should be avoided to prevent damaging of the first section.

Biopsies

Excisional scalp biopsies were taken from controls and from the margins of active lesions of patients affected with alopecia areata for routine HE-staining and immunophenotyping with the monoclonal antibodies K8.12 (specific for cytokeratin 13 and 16) (3, 4) and Tll (anti-CD2 antibody). All biopsies were immediately embedded in Tissue-TEK II OCT Compound (Miles Inc. Diagnostics Division, Elkhart, Ind., U.S.A.), snap frozen in liquid nitrogen and stored at −80°C until use. The specimens were sectioned at 5 μm in a 2800 Frigocut-N cryostat (Reichert-Jung, Cambridge Instruments GmbH, Nußloch, FRG). Routine hematoxylin-eosin (H.E.) staining was performed.

Immunohistology

For immunofluorescence, an indirect immunofluorescence technique according to Ramaekers et al. (5) was used. Acetone-fixed sections 5 μm thick, were investigated for the presence of cytokeratin 16 using K8.12 (dilution 1:20, Sigma Chemical Company, St. Louis, Mo., USA) and FITC-conjugated goat anti-mouse IgG (dilution 1:25, Tago, Burlingame, Calif, USA).

Immunostaining for T-lymphocytes was performed using a monoclonal anti-CD2 antibody (Tll dilution 1:100, Dakopatts, Glostrup, Denmark) with the peroxidase – antiperoxidase (PAP) technique (6). The sections were examined using a Zeiss photomicroscope.

RESULTS

Evaluation of the conduction freezing system

Fig. 3 illustrates the results of different staining procedures on sections obtained by the method described above. In Fig. 3*a, b,* the results of H.E. staining of sections obtained by the method described above are shown. The left hair follicle in Fig. 3*a* is cut in the lower third of the hair bulb. The connective tissue of the papilla is clearly visible, surrounded by matrix cells separated from the papilla by mucinous stroma. The hair follicle on the right is cut above the line of Auber, just at the top of the papilla. The most proximal part of it can just be seen. The matrix cells do not show differentiation at this stage. Both hair bulbs are surrounded by a thin sheath of connective tissue, entirely located in fatty tissue, which is well preserved. Fig. 3*b* shows the hair follicle sectioned in the transition zone between fatty tissue and dermis. Differentiation is already advanced.

In order to analyse an inflammatory infiltrate, sections of the active margin of alopecia areata lesions were stained with the monoclonal antibody Tll, which recognizes the CD2-antigen present on the majority of T cells. Fig. 3*c* demonstrates a well preserved hair follicle, at the level of the matrix, located in fatty tissue. A dense peribulbar infiltrate shows membrane staining for CD2.

To show that the detection of structural proteins remains unchanged, indirect immunofluorescence

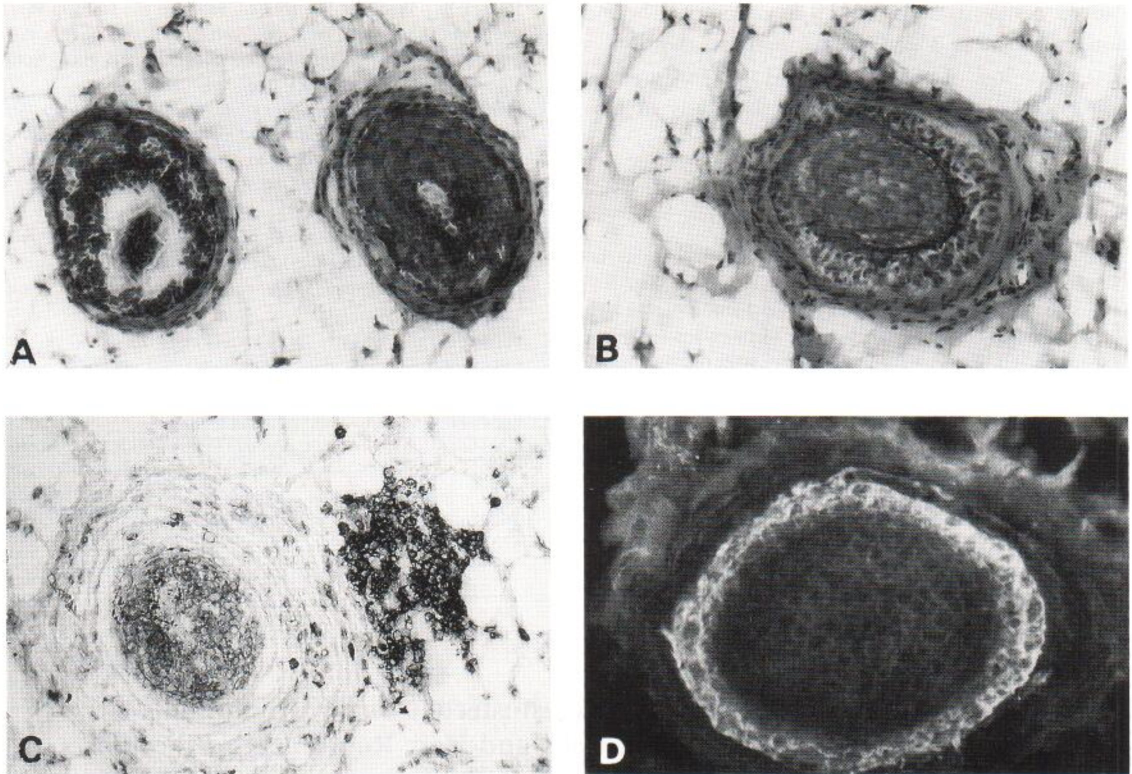

Fig. 3. Staining of frozen sections of hair follicles (*a*) H.E. staining of transverse sections through the hair bulb. Note connective tissue papilla in the left bulb, surrounded by mucinous stroma and hair matrix cells. Follicle on the right cut just at the top of the hair bulb. (*b*) H.E. staining of hair follicle located at the border between fatty tissue and dermis. The following structures can be differentiated: in the centre the cornified cells of the hair, already without nuclei, can be seen. This layer is followed by the inner root sheath, even the subdivision in different layers can be distinguished. The outer root sheath is fully differentiated and surrounded by a layer of connective tissue. (*c*) PAP staining of the peribulbar infiltrate in alopecia areata with the anti-CD2 antibody Tll. (*d*) Indirect immunofluorescence with the monoclonal antibody K8.12 showing positive immunofluorescence of the not yet differentiated root sheath at the level of the hair matrix.

staining cytokeratin 16 using the monoclonal antibody K8.12 (with specificity for cytokeratin 13 and 16) was performed. Fig. 3 *d* illustrates that the application of the conduction technique allows immunostaining of cytokeratin-16 without problems. The section is cut at the level of the matrix. The matrix cells are negative, whereas the epithelial root sheath, which is not yet differentiated at this level, shows marked immunofluorescence. Controls were negative.

DISCUSSION

Immunohistological staining of hair follicle structures in general requires the use of frozen sections. Since in many hair diseases the hair follicles may be reduced in number or size, serial sections are needed to obtain an overall view. Furthermore, as hair follicles are not orientated perpendicular to the skin surface, but at various angles, sections containing follicles throughout their length are difficult to obtain. Due to the different angles, a proper orientation is only possible for one or few follicles, the others being orientated in another direction. Horizontal sectioning of scalp tissue overcomes most of these problems. However, this approach is limited by the fact that the hair bulbs are usually located in the fatty tissue, which causes difficulties in obtaining cryosections due to the differences in the physicochemical properties of fatty tissue and hair bulb keratinocytes. With the conduction system described above, these problems can be overcome with minimum expense and without the need to adapt standard protocols for immunohistochemical staining. We could show that different types of antigens like intracellular polypeptides such as the cytokeratins

can be demonstrated, as well as membrane-bound antigens such as T cell surface markers, without a detectable loss of quality. We feel that this method will become a valuable tool for the study of hair diseases.

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