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Immunohistological demonstration of lymphocyte surface antigens in postmortem lymphoid tissues

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Summary. The postmortem stability of cell antigens has hardly been studied. Using monoclonal antibodies (mabs) we examined the postmortem detectability of lymphocyte surface antigens in different lymphoid organs by comparing two sensitive, immunohistological staining procedures.

To quantify the probable degree of autolysis of the tissues a score system was applied by taking into consideration the postmortem age as well as the core temperature of the corpses.

The antigens examined generally proved to be very resistant to autolytic influences. Differences were found when comparing different mabs and with regard to the type of lymphoid tissue. The loss of immunohistological reactions was most extensive in the spleen whereas tonsils showed almost no qualitative alterations in staining patterns. Reactivity of mabs with postmortem tissues decreased in the following order: Dako CD22 and anti-Leu 4, anti-Leu 3a, anti-Leu 7, Dako T8. The mabs anti-Leu 7 and Dako-T8 frequently failed to demonstrate their respective antigens but no correlation between the loss of staining and the degree of autolytic decomposition (our score) could be detected.

In general, postmortem tissues as well as tissues shock frozen after delay are suitable for qualitative immunohistology of those cells characterized by the mabs applied.

The APAAP-method proved unequivocally to be the superior staining technique.

Key words: Postmortem changes – Lymphoid tissues – Lymphocyte antigens – Immunochemistry – Immunoenzyme techniques

Introduction

When compared with all other blood cell types, lymphocytes appear to be the most resistant to autolytic influences. Penttilä and Laiho (1981) demonstrated peripheral blood lymphocytes in corpses stored at 4° C up to 270 h postmortem (p.m.) by light microscopy as well as by scanning electron microscopy. In blood samples taken intra vitam and stored up to 18 days under sterile conditions at different temperatures Oehmichen and Pedal (1983) observed morphologically well-conserved lymphocytes in blood smears prepared after 18 days of storage, even at 37° C. Henke et al. (1981) performed HLA-typing of lymphocyte suspensions from lymph nodes of cold-stored cadavers up to 36 h p.m. demonstrating postmortem preservation of the lymphocyte membrane.

The development of monoclonal antibodies (mabs) against lymphocyte surface antigens has facilitated the characterization of lymphocyte subpopulations as well as their tissue distribution. In spite of the extensive application of these mabs in immunocytology and immunohistology little is known about the stability of lymphocyte surface antigens, especially concerning the influence a delay in tissue preservation might have on the interpretation of immunohistological staining of postmortem tissues.

Gatter et al. (1984) observed generally good immunohistological staining results in autopsy tissues up till 4 days p.m. Specimens sampled intra vitam and stored up to 48 h in saline solution or cell culture medium showed preservation of antigens in the majority of cases. Pallesen and Knudsen (1985, 1986) applied immunohistology to demonstrate the postmortem stability of leucocyte antigens as well as non-haematopoietic antigens. The majority of the antigens examined proved to be persistant even 3 days p.m. These authors classified their tissues into rather crude categories of 24 h steps p.m. without defining the interval between death and cold-storage exactly.

In this investigation, we have attempted to gain more detailed information on the preservation of lymphocyte surface antigens by including: a longer

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than 72 h postmortem period, a more accurate classification of the postmortem age and by taking into consideration the estimated course of cooling of the cadavers as another main factor influencing autolysis.

For immunohistological staining we used commercially available mabs against distinct lymphocyte subpopulations. In addition, we compared the sensitivity of two common immunohistological staining procedures, i.e. the ABC(Avidin-Biotinylated Peroxidase-Complex)- and the APAAP(Alkaline Phosphatase-Anti-Alkaline Phosphatase)-method.

Materials and methods

Tissue samples of lymphoid organs (spleen, bronchial hilar lymph nodes, tonsils) were taken during forensic medical autopsies. The whereabouts of the cadavers between their death and autopsy was checked by means of the police protocols. By help of the table published by Prokop and Göhler (1976) and knowing the main variables (the temperature of environment, clothing, air humidity, weight, time of death) the course of cooling could be estimated. The postmortem age of the corpses, as well as the decrease of their core temperature under known conditions, were selected to quantify the probable degree of autolytic decomposition of the tissues. All specimens were grouped according to a score system based on the sample storage temperature of −75° C. For every hour which had passed since the time of death until the sampling of tissue specimens, the difference in temperature between the corpse core temperature and the storage temperature of −75° C was determined. Every degree Celsius of the core temperature above −75° C was equivalent to one point in this scoring system. Subsequently, the scores for every hour p.m. were added in the following way: For example, in the first h p.m. the core temperature is 37° C = 112 points, in the second h p.m. the core temperature is 36° C = 111 points..., at the eighth h p.m. the core temperature is 30° C = 105 points; total score = 112 + 111 + ... + 105.

Thirty samples of tonsils, 42 hilar lymph nodes and 48 spleen samples were examined, obtained within a postmortem range of 870 to 21294 points which corresponds to approximately 8 h to 6 days p.m. The specimens came from cases with a distribution of mortality and causes of death typical for forensic pathology. The age of the individuals ranged from 1 day to 89 years (median age, 39.8 years).

All tissue samples were snap frozen in liquid nitrogen and stored at −75° C. Cryostat sections were cut at 6 µm, air dried, fixed in acetone and stored at −20° C. Sections were routinely stained according to the ABC-method in the following way:

Sections were first incubated with the specific mouse mab (for details see Table 1) for 30 min at room temperature, and subsequently with the second biotinylated horse anti-mouse IgG (Vector Laboratories) for 30 min at room temperature and with ABC (Vector Laboratories) for 45 min at room temperature. Peroxidase was visualized by incubation with 3.3-diaminobenzidine (DAB) in 0.03% hydrogen peroxide. The DAB staining was intensified by incubation with 0.5% CuSO4 in PBS. Between the incubation steps the slides were rinsed in 0.04 M phosphate buffered saline (PBS, pH 7.4).

The appropriate dilutions of mabs have been determined previously (Berling et al. 1986).

Only if staining results were negative or uncertain was the staining repeated or controlled using the APAAP-method, since random tests showed that sections which were positive with the ABC-method also were positive with the APAAP-method (but not vice-versa). APAAP-staining was performed in the following sequence:

Sections were first incubated with the specific mab in the same dilution as used in the ABC-method for 30 min at room temperature followed by an incubation with a rabbit anti-mouse Ig (Dakopatts) and the APAAP-complex (Dianova) for 30 min each at room temperature. Subsequently the incubation of the second antibody and the APAAP-complex was repeated for 15 min also at room temperature. The alkaline phosphatase was developed by new fuchsin and naphthol- AS-bi-phosphate. Washings were done in 0.05 M Tris buffered saline, pH 7.4. Antibodies and the APAAP-complex were diluted with RPMI (Seromed) supplemented with 10% fetal calf serum (Boehringer). Endogenous alkaline phosphatase was blocked by addition of 0.04% of levamisole (Sigma).

Sections were regarded negative if no specific precipitation of substrate was visible or no difference in frequency, distribution or intensity of the precipitation in the test slide as compared to control sections was detected. Interpretation of APAAP stained sections was always unequivocal, i.e. background staining was completely lacking, hence the identification of single positive cells presented no difficulties.

Results

The postmortem demonstration of the examined lymphocyte surface antigens was generally preserved for a long period of time. Depending on

Table 1. Specifity and source of the monoclonal antibodies applied and the CD nomenclature of the antigens recognized

Antibody	CD nomenclature of antigen	Specifity	Source and reference
Dako CD22	CD 22	B-cells	Dakopatts (Nadler LM, 1986)
Dako T8	CD 8	suppressor/cytotoxic T-cells	Dakopatts (Reinherz et al. 1980)
Anti-Leu 4	CD 3	T-cells	Becton-Dickinson (Ledbetter et al. 1981)
Anti-Leu 3a	CD 4	T-helper cells	Becton-Dickinson (Evans et al. 1981)
Anti-Leu 7		subgroups of T-cells and NK (natural killer)-cells	Becton-Dickinson (Abo et al. 1981)

the kind of lymphoid organ, differences in detectability were apparent.

The demonstration of B-cells by mab Dako CD22 (Figs. 1a and 2a) and T-lymphocytes by anti-Leu 4 (Figs. 1a and 2b) proved these antigens to be the most resistant to autolytic influences. In all three lymphoid tissues with all scores both B- and T-cells could be demonstrated by these mabs using the ABC-method.

The antigen (or epitope) detected by mab anti-Leu 3a (Figs. 1b, 2c and d) also proved to be a p.m. well preserved lymphocyte marker. While helper T-cells could always be identified in tonsils, for the lymph nodes this was different, that is to say the one with the highest p.m. score (20930 points) did not stain using the peroxidase-technique. This specimen remained negative even when the APAAP-method was applied.

Six of the spleen samples, all with different scores, were negative with the ABC-technique (Fig. 1b). However, only the specimen with the highest score (21194) could not be stained using the APAAP-method.

The postmortem detectability of cells reactive with mab anti-Leu 7 (Fig. 1c, 2e and f) also depended on the organ examined:

Three ABC-technique negative samples of tonsil proved to be positive using the APAAP-method (Fig. 1c). ABC-technique negative staining results of nine spleen tissues were positive when the APAAP-technique was employed (Fig. 1c). In lymph nodes the results of the majority of samples (13/16) which were initially negative with the ABC-method stayed negative with the APAAP-technique (Fig. 1c). In those sections which included neuronal elements, but which did not show any Leu 7-positive cells, cross-reactivity of the mab anti-Leu 7 to neuronal structures could still be seen.

When comparing the two staining procedures the positivity obtained with this mab was inverted in just one sample scoring 3887 points. While the ABC-method demonstrated Leu 7-positive cells in one germinal centre the APAAP-method produced no staining.

The lymphocyte marker visualized by mab Dako T8 (Fig. 1d, 2g and h) turned out to be the antigen which was the least well preserved postmortally. However, it was positive in all tonsils with the ABC-method.

Regarding all five mabs and comparing the three lymphoid organs examined, it were the spleen's T8 reactive lymphocytes that showed the most extensive loss of antigen preservation. The majority of spleen samples (30/48) demonstrated no CD 8 staining according to the ABC-method. Samples up to the score of 8656 points were characterized by variable results. Spleen tissue from that score upwards remained negative. On checking these results by the APAAP-method, only 8/30 specimens, mainly samples with medium scores, were found to be positive (Fig. 1d). Cross-reactivity of Dako T8 to endothelial cells of sinusoids as demonstrated in fresh, surgically-removed spleen tissue was not demonstrable in any of the postmortem samples.

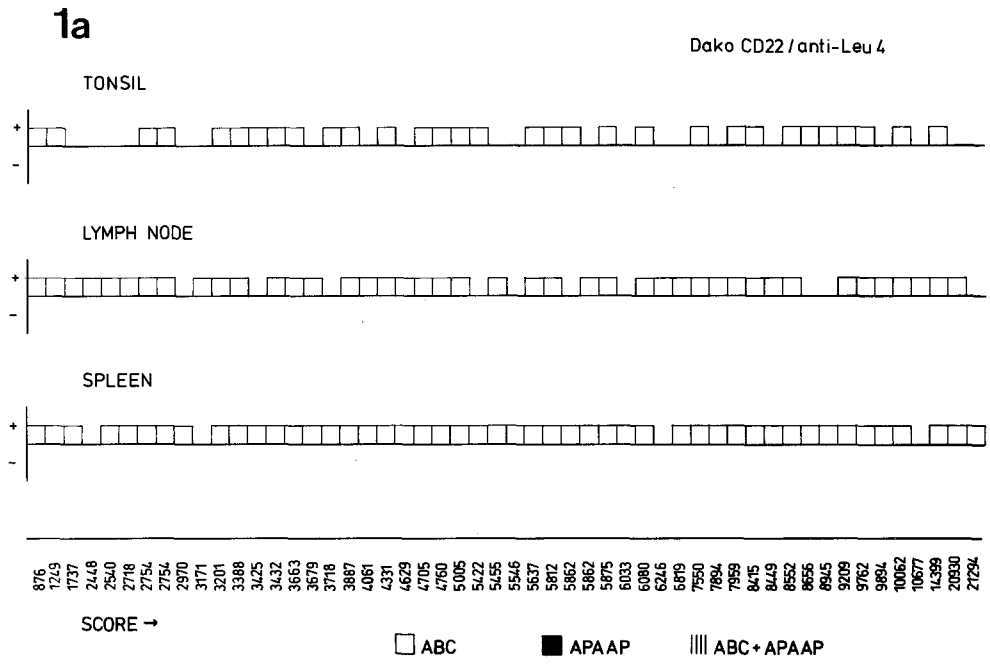
The rate of CD 8-negative specimens (14/42) in lymph nodes was lower than in spleens, but negative samples were equally scattered over the total range of tissues examined (Fig. 1d). After repeating the staining by the APAAP-technique more than half of the priorly negative samples proved to be positive (8/14). The remaining 6 negative specimens all were characterized by a higher postmortem score.

The typical membraneous staining pattern characteristic for fresh lymphoid tissues of all lymphoid organs examined, was lost increasingly with higher p.m. scores. In cases of very extensive autolysis (in spleen or lymph nodes), substrate precipitations were visible in areas which showed no or almost no counter staining of cell nuclei by haemalaun (Fig. 2a). The loss of nuclear staining usually occured sooner in T-cell areas as compared to B-cell regions (Fig. 2b).

In general, tonsillar tissues were best preserved both morphologically and immunohistologically, whereas autolysis reduced the morphology and immunohistological reactions of lymphocyte antigens in bronchial hilar lymph nodes, and particularly in spleen with increasing p.m. scores.

Discussion

The topographical distribution of immunohistological stainings always corresponded to the localization of B-lymphocytes, T-lymphocyte subpopulations and Leu 7-positive cells, as detected in sections of equivalent fresh tissues and as already described in the literature (McMillan et al. 1981; Poppema et al. 1981, 1983; Banerjee and Thibert 1983; Grogan et al. 1983; Hsu et al. 1983a; Ritchie et al. 1983; Si et al. 1983; Si and Whiteside 1983; Hsu and Jaffe 1984; Pizzolo et al. 1984; Swerdlow and Murray 1984; van der Valk et al. 1984; Timens and Poppema 1985; Berling et al. 1986; van Krieken and te Velde 1986). Therefore, the possibility of cross-reactivity of mabs to epitopes which might have originated or have been unmasked during autolysis does not seem very likely.



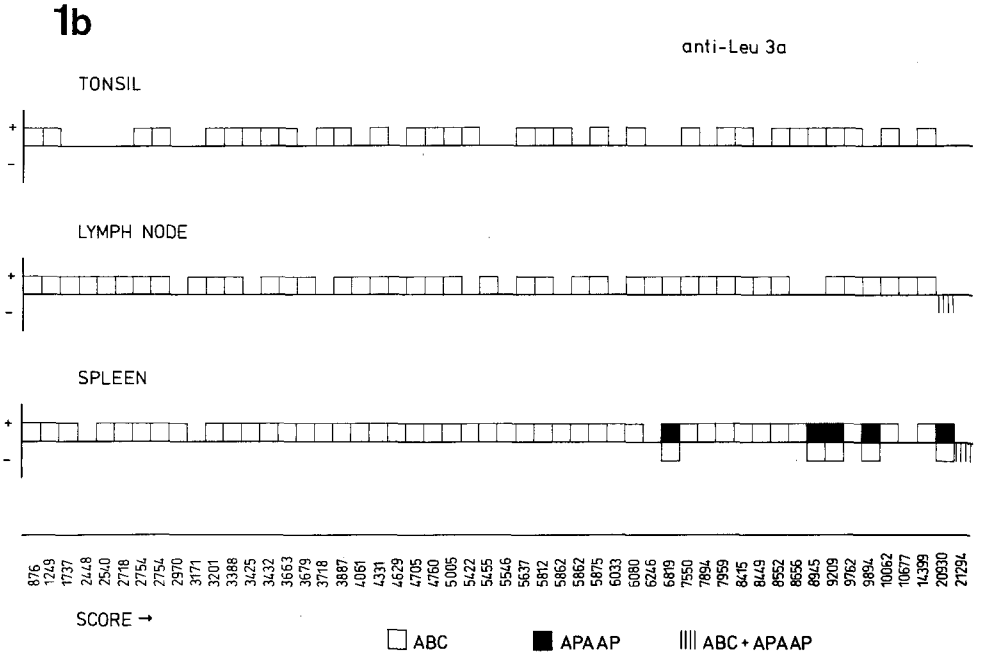
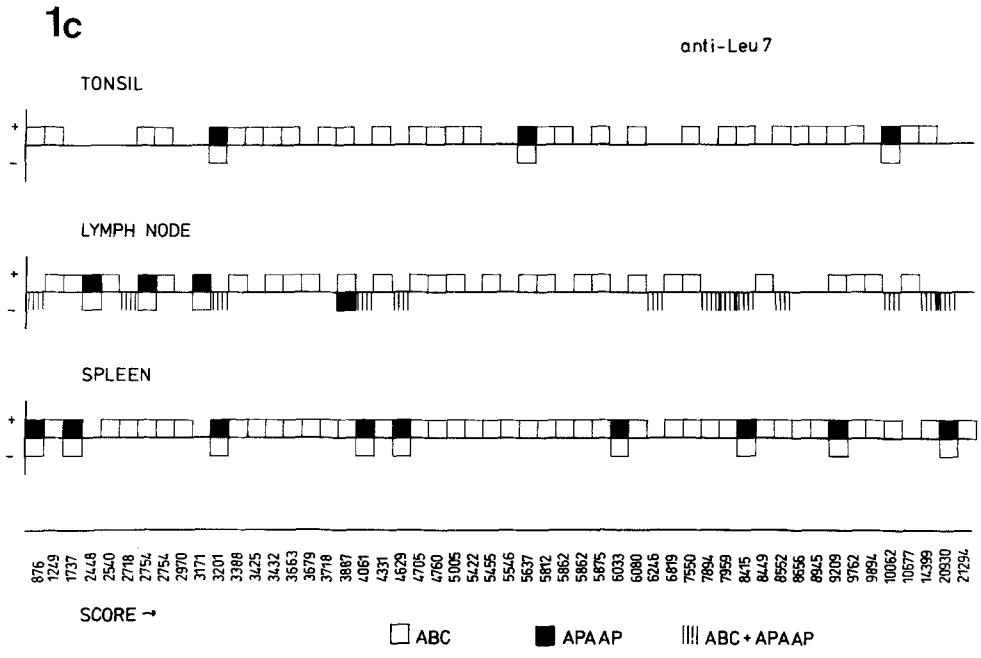


Fig. 1. Staining results with mabs Dako CD22, anti-Leu 4 (**a**), anti-Leu 3a (**b**), anti-Leu 7 (**c**) and Dako T8 (**d**) are plotted in a linear manner along the x-axis according to the scores of samples and the type of lymphoid organ. *Every square* represents one sample. As indicated by the y-axis, positive results are demonstrated above the x-axis, negative results below. *White squares* represent positive results obtained by the ABC-method, *black squares* those by the APAAP-method. *Hatched squares* demonstrate concordant results achieved by both methods



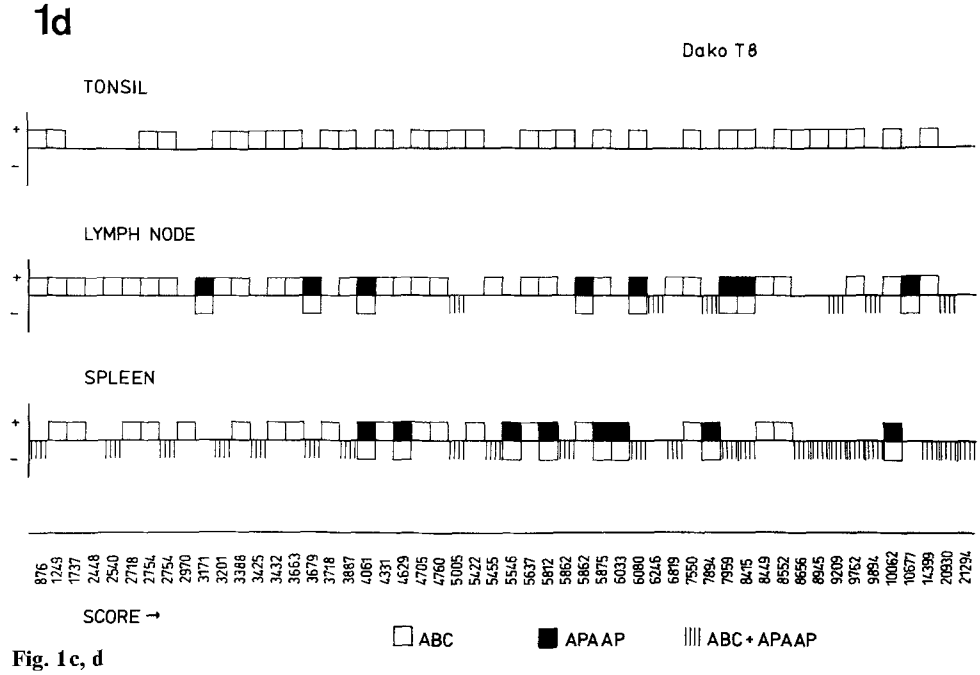
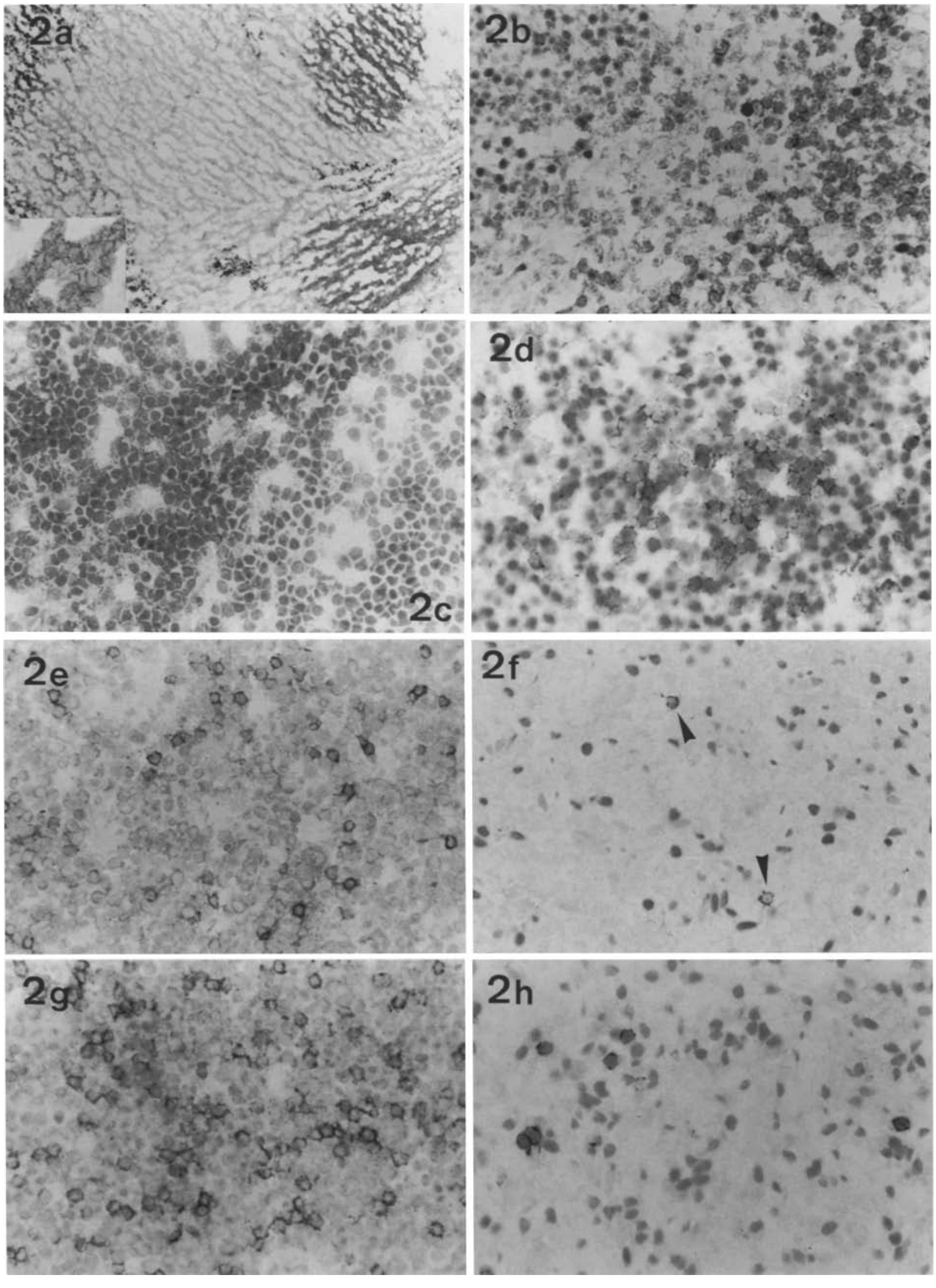


Fig. 1c, d

The detectability of lymphocyte surface antigens is maintained for a long period of time p.m. Using mabs Dako CD22 and anti-Leu 4 the immunohistological demonstration of B- and T-lymphocytes outlasts the purely morphological demonstration of these cells in cryostat sections. Like Pallesen and Knudsen (1985) we observed that the preservation of lymphocyte antigens depended on the mabs applied as well as the type of lymphoid organ examined.

Postmortem antigen preservation proved to be most extensive in tonsils, less in hilar lymph nodes, and especially low in splenic tissue samples. Apart from the fact that superficially positioned organs


2a
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cool down sooner (Pallesen and Knudsen 1985), this difference in immunohistological staining might be due to the high number of neutrophilic granulocytes present in the spleen. The low postmortem stability of these cells (Oehmichen and Pedal 1983) furnishes an additional autolytic potential by force of the granulocytic lysosomal enzymes.

We studied hilar lymph nodes situated less superficially than the cervical lymph nodes examined by Pallesen and Knudsen (1985). Therefore, our results from tonsils can be compared with their findings in lymph nodes and postmortem antigen preservation is apparently identical. Moreover, assuming the most unfavourable p.m. conditions in respect to temperature (24° C) and time (72 h) the tissues studied by Pallesen and Knudsen would still get a score of not more than 7000 points according to our system. This means that antigen preservation in superficially situated lymphatic tissue is maintained for an even longer period of time than reported by Pallesen and Knudsen.

Considering the five mabs applied the reactivity of these mabs with lymphoid tissues decreased in the following order: Dako CD22 and anti-Leu 4, anti-Leu 3a, anti-Leu 7, Dako T8. Mab Dako T8 reacted only irregularly with postmortem tissues. This characteristic was most obvious in spleen. No correlation between the demonstration of CD8 positive cells and our scoring system could be determined. Even in two pairs of specimens with identical scores, i.e. 2754 and 5862 respectively, originating from corpses with identical postmortem conditions and identical cooling of the core temperature, showed different results. In lymph nodes with higher scores, the staining results for the CD8 antigen also varied considerably.

Leu 7-positive cells could not be detected in a few of the spleens with low scores, applying the ABC-method. The ABC-technique often produced a distinct background staining in the red pulp as well as occasional membrane staining in the white pulp, due to the second antibody of the staining sequence. Therefore reliable detectability of Leu 7-positive cells which are mainly scattered in the red pulp was frequently hampered. Evidence for the stability of the Leu 7-antigen came from the application of the APAAP-method, confirming the results of Pallesen and Knudsen in spleen.

In contrast with the findings in spleen, mab anti-Leu 7 gave poor results in lymph nodes. The search for Leu 7-positive cells was often negative, even when using the APAAP-method. The results were unrelated to our scale. In normal fresh lymph nodes Leu 7-positive cells are situated almost exclusively in the germinal centres of lymphoid follicles and the number of these cells is much lower when compared with that in spleens (Banerjee and Thibert 1983; Poppema et al. 1983; Ritchie et al. 1983; Si and Whiteside 1983; Pizzolo et al. 1984). Nevertheless, the detectability of Leu 7-positive cells in some samples was not guaranteed despite the preservation of germinal centres. The low number of Leu 7-positive cells even in fresh lymph nodes may explain the discrepancy of the (qualitative) postmortem demonstration of these cells in lymph nodes compared with splenic tissue.

Most studies applying different immunohistological staining procedures have been restricted to peroxidase mediated methods (Hsu et al. 1981; Hsu et al. 1983b; Sternberger and Sternberger 1986). Only Cordell et al. (1984) compared the APAAP-method to a peroxidase-technique, i.e. the PAP(Peroxidase-Anti-Peroxidase)-method, and they found that staining of cell smears as well as cryostat sections (if incubation of second antibody and APAAP-complex was repeated) was better by the APAAP-method.

We also found far superior staining results using the APAAP-method when compared with the ABC-method. This may result from the absence of any background staining since apart from the

Fig. 2. (**a**) Lymph node with highest score (20930 points) stained by mab Dako CD22. Demonstrated on the right are two relics of follicles which were stained by the APAAP-method and contrast well to the unstained paracortex. The artificial, grid-like appearance of the tissue is due to mechanical influences during sectioning. The *inset* presents a detail out of a follicle demonstrating the precipitation of the product of alkaline phosphatase reaction while counter staining of nuclei by haemalaun is missing (×65), inset (×375). (**b**) Spleen (20930 points), ABC-method, anti-Leu 4. The *right part* of the figure demonstrates T-cells but with loss of precise membrane staining. Nuclei are difficult to discern compared to the fairly well preserved nuclei of B-cell area in the *left upper part* of figure (×320). (**c**) Lymph node with highest score (14399 points) being positive by anti-Leu 3a, ABC-method. Transition from follicle (*right part*) to interfollicular area (*left*) with demonstration of T helper cells. Here, typical membrane staining is also lost (×320). (**d**) Spleen (6819 points), anti-Leu 3a, APAAP-method. Distinct staining of T helper cells in the white pulp of a sample negative with the ABC-method (×320). (**e**) Tonsil with highest score (14399 points), anti-Leu 7, ABC-method. Good preservation of Leu 7-positive cells in the germinal centre of a tonsil follicle (×320). (**f**) Spleen (8415 points), anti-Leu 7, APAAP-method. Scattered Leu 7-positive cells discernible in red pulp (*arrows*) which could not be detected by the ABC-method (×320). (**g**) Tonsil with highest score (14399 points), Dako T8, ABC-method. Good preservation of the CD8 antigen in the interfollicular area (×320). (**h**) Spleen (10062), Dako T8, APAAP-method. Scattered suppressor/cytotoxic cells situated in red pulp which were undetectable by the ABC-method (×320)

tendency of the avidin-biotin-peroxidase-complex to bind to necrotic tissue and to connective tissue components, a distinctive and disturbing background staining, especially in the red pulp of the spleen, is caused by the peroxidase-like activity of haemoglobin (Cordell et al. 1984) from disintegrated erythrocytes. However, the APAAP-method still demonstrated lymphocyte antigens in tissues which were negative by the ABC-method, that is to say it proved to be more sensitive.

The long postmortem preservation of the majority of the lymphocyte antigens examined renders the use of postmortem tissues possible, for the identification of cells characterized by these antigens. Shock frozen tissue is also usable after delayed preservation. These conclusions relate to qualitative investigations only, since there is obviously an increasing loss of tissue components while autolysis proceeds.

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