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EFFECT OF FIXATION AND AUTOLYSIS ON IMMUNOHISTOCHEMICAL DETECTION OF CD ANTIGENS

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A b s t r a c t

To assess the effect of fixation and autolysis on immunoreactivity of commonly examined CD antigens (CD20, CD45RO, CD5, CD10, CD23, CD43 and CD 68), we studied biopticaly and autopticaly obtained lymphoid tissues (tonsils, appendices, spleens). They were fixed in different dilutions of formalin (1:9, 1:3, 1:1) (i.e. concentrations 4%, 9,5% and 19%, respectively), either buffered (pH 7.0) or unbuffered (pH 5.5, 4.7 and 4.0, respectively). The fixation time ranged from 24 hours to 5 days. Some of the samples were exposed to autolysis up to 24 hours at room temperature. We found out that for all tested antigens there were no important differences after 24-hour and 48-hour fixation at all tested dilutions of formalin. Only after prolonged fixation, decrease in immunoreactivity of some antigens (CD5, CD10) appeared, especially in samples fixed in formalin diluted 1:1. Also pH of the formaldehyde solution did not show to be a critical factor. As for the autolysis, we surprisingly did not observe significantly worse results of immunohistochemical detection of above-mentioned antigens even after 24-hour autolysis at room temperature.

K e y w o r d s

Fixation, Immunohistochemistry, CD antigens, Autolysis

INTRODUCTION

Immunohistochemistry has become a very useful tool in bioptic as well as autoptic diagnostics. Especially for diagnosis of neoplastic and inflammatory conditions, the results of immunohistochemical examination of the tissue are extremely important. During the processing, the tissue is influenced by many accompanying factors, which can affect the structure of antigens and epitopes. The fixation plays cardinal role between them.

The ideal fixative should prevent loss or damage of tissue components during the subsequent processing of the tissue. From the immunohistochemical point of view, it is necessary to preserve antibody binding sites (epitopes), their accessibility and their ability to bind antibodies (*1, 2, 3*). The fixative can mask epitopes and cause loss of their immunoreactivity. Therefore, the choice of suitable fixation fluid can influence the success of immunohistochemical examination.

Not only the choice of the fixative, but also its concentration and duration of the fixation are very important factors (*4 ,5*). During short fixation, the penetration of the fixative is insufficient and cross-linking of polypeptide chains (formaldehyde) or protein coagulation (alcohols) is incomplete, i.e. the tissue is underfixed. Prolonged fixation time, in contrast, leads to overfixation, because the fixative binds many epitopes of the examined tissue. This is a common problem especially during several day fixation, e.g. at weekends.

The quality of immunostaining is also dependent on duration of autolysis, i.e. the time period between the extirpation of the tissue (or death of an individual) and its immersion into the fixation fluid (*6*). But immediate fixation of voluminous tissues that have not been cut up also results in autolysis of their central parts.

From the above-mentioned follows that the result of immunohistochemical examination depends on many factors, which influence the investigated tissue during the processing before the detection of antigens is performed. The aim of this study is to observe the effect of the fixation and autolysis on immunoreactivity of some commonly used CD antigens in lymphoid (lymphoreticular) tissue. We focused our attention on the most widely used fixative – formalin (i.e. aqueous solution of formaldehyde), to assess the range of suitable concentration, pH and fixation time.

MATERIALS AND METHODS

Specimens of biopticaly obtained appendices (n=3) and human tonsils with mild chronic tonsillitis (n=12), and autopticaly obtained spleens (n=2) were investigated.

First, effect of formaldehyde concentration and fixation time was examined. Four specimens of human tonsils (2 cases, 10 blocks) were fixed within 1 hr after excision in buffered formaldehyde at a dilution of 1:9 and 1:1 (i.e. one volume part of commercial formalin, which is 38% solution of gaseous formaldehyde in water, and one volume part of water), pH 7.0, fixation times 24 hrs to 5 days. After tissue processing, immunohistochemical staining was performed with CD5, CD10, CD23, CD68, CD43, CD20 and CD45RO antibodies. Moreover, detection of CD2O and CD45RO after 48-hour fixation was tested.

Then, effect of pH of the fixative was studied. Six human tonsils were fixed (within 1 hr of excision) with phosphate buffered formaldehyde (pH 7.0) and with unbuffered formaldehyde (pH 4.7) at a dilution of 1:3. CD20 and CD45RO were demonstrated. Besides the tonsils, effect of pH and formaldehyde concentration was examined on small tissue samples – 3 appendices without macroscopic inflammatory changes were processed within 1hr after excision. Each of them was divided into 6 sections 2–3 mm thick and fixed for 24 hrs with formaldehyde at a dilution of 1:1, 1:3 and 1:9 (pH 4.0, 4.7 and 5.5, respectively) without use of phosphate buffer and with buffered formaldehyde (pH 7.0) of the same dilutions. After tissue processing, CD20 and CD45RO were stained.

In the third step, effect of tissue autolysis was investigated. 2 tonsils were processed – 4 representative tissue blocks were cut at 5-mm thickness and fixed after 0 hr, 3 hrs at 5°C, 24 hrs at 5°C and 24 hrs at 25°C. As a fixation fluid, unbuffered formaldehyde at a dilution of 1:3 was used. The fixation time was 24 hrs. In these samples, CD20 and CD45RO were demonstrated.

Finally, effect of the tissue volume on the penetration of the fixation fluid was studied. A spleen (of normal size and histology) obtained at autopsy was used. Two blocks 20x20x20 mm were cut and fixed for 24 hrs with unbuffered formaldehyde at a dilution of 1:3. Then, 2 excisions from the central and peripheral part of each block were cut. Immunohistochemical detection of CD20, CD45RO,

CD10 and CD68 was done after the tissue processing. To test the penetration of the fixative (and consequently to evaluate tissue autolysis), another spleen (120 g, 100x60x40 mm) obtained at autopsy was examined. It was fixed in toto in unbuffered formaldehyde at a dilution of 1:3. After a 48-hour fixation, blocks from superficial, middle and deep parts of the spleen were cut and CD20, CD45RO, CD10 and CD68 were demonstrated. The aim of this test was to imitate a common situation, when a large, biopticaly obtained spleen is fixed for several days (e.g. during weekend) and the fibrous capsule and the volume of the tissue limit the penetration of the fixative.

Tissue Processing and Method of CD Antigens Detection

Standard tissue processing was performed after the fixation: dehydration in a graded series of alcohol, clearing in xylene, infiltration with paraffin, paraffin embedding, sectioning.

Immunohistochemical reactions were performed using antibodies to CD20, CD45RO, CD5, CD23, CD43 and CD68 (Dako) and CD10 (Novocastra). The antibodies were used in dilutions according to recommendation of the manufacturer: CD20 1:100, CD45RO 1:1000, CD5 1:50, CD23 without dilution (ready-to-use), CD43 1:100, CD68 1:50 and CD10 1:20.

In the first step, for CD20, CD45RO, CD43 and CD68, microwave based antigen retrieval was used; tissue sections were heated up in a citrate buffer (0,01M, pH 6.0) for 20 minutes at 98°C. For CD5, CD10 and CD23, tissue sections were heated up in a citrate buffer (0,01M, pH 6.0) for 5 minutes at 120°C in a pressure cooker.

Then the primary antibodies were incubated for 1 hour at room temperature (CD20, CD45RO, CD43 and CD68) or overnight in a fridge (CD5, CD10 and CD23). Envision (Dako) containing secondary antibodies was used as a detection system. To visualise the reaction, diaminobenzidin was used as a chromogen.

CD23 was detected with use of amplification system with tyramide.

Antibody reactions were graded semiquantitatively (- to 3+):
– negative reaction
+ weakly positive reaction
++ satisfactory, distinct positivity
+++ very intense reaction

RESULTS

Evaluation of examined parameters was summarized in the form of tables.

Effect of Formaldehyd Concentration and Fixation Time (Table 1, 2)

CD5: the immunoreactivity decreased markedly after the prolonged fixation (5days) at both concentrations. CD10: the epitope probably does not tolerate fixation well, the weak positivity after short fixation time (24 hrs) at both concentrations becomes negative during prolonged period of fixation (5 days).

CD23 and CD68: neither fixation time, nor considerable differences in formaldehyde concentration influence distinct positive reaction – formaldehyde fixation, in this case, is the method of choice.

The reactivity was not significantly altered for CD68 and CD23 at both concentrations, for CD20 at 1:9, for CD45RO at 1:9 and for CD43 at 1:1, while for CD20 at 1:1, CD45RO at 1:1 and CD43 at 1:9 it was decreased after the prolonged (5-day) fixation.

In the only examined case the immunoreactivity increased after prolonged fixation: for CD45RO at 1:9, but not constantly – in 1 case out of 2. Here we can

Table 1
Effect of formaldehyde concentration and fixation time on immunoreactivity of CD5, CD10, CD23, CD68 and CD43

		CD5		CD10		CD23		CD68		CD43	
		24 hrs	5 days	24 hrs	5 days	24 hrs	5 days	24 hrs	5 days	24 hrs	5 days
Case 2	1:9	+++	+/–	+	–	+++	+++	+++	++	+++	+/++
	1:1	+++	+/–	+/++	+/–	+++	+++	+++	+++	++	+/++

Table 2
Effect of formaldehyde concentration and fixation time on immunoreactivity of CD20 and CD45RO

	Case 1			Case 2	
	CD20				
	24 hrs	48 hrs	5 days	24 hrs	5 days
1:9	++	+++	++	++	++
1:1	+++	++	+	++	+/++
	CD45RO				
	24 hrs	48 hrs	5 days	24 hrs	5 days
1:9	++	+++	++	+	++/+++
1:1	+++	++	++	+++	+/++

Table 3
Effect of pH (formaldehyde 1:3) on immunoreactivity of CD20 and CD45RO

	CD45 RO pH 7,0	CD 45 RO pH 4,7	CD 20 pH 7,0	CD 20 pH 4,7
Case 1	++	++	++	++/+++
Case 2	+++	+++	+++	+++
Case 3	+++	+++	+++	+++

speculate that at low formaldehyde concentration, 24-hour fixation does not always have to be quite sufficient.

Conclusions for the fixation time:

When comparing 24 and 48-hour fixation, the differences in immunoreactivity for CD20 and CD45RO are irrelevant at both concentrations.

Prolonged (5-day) fixation results in either constant (CD23, CD68, CD20 at 1: 9, CD45RO at 1:9, CD43 at 1:1) or decreased (CD5, CD10, CD20 at 1:1, CD45RO at 1:1, CD43 at 1:9) immunoreactivity. Nevertheless, this decrease usually (CD20, CD45RO) involves concentration 1:1, which is rarely used routinely. At the concentration 1:9, a positive reactivity for CD43 after 5-day fixation remains, although decreased. Only the immunoreactivity for CD5 and CD10 becomes negative. So that only in these cases prolonged formaldehyde fixation brings incorrect, false negative results.

Concentration of the fixation fluid:

At the concentration 1:9, the results are comparable to high concentration 1:1, in some cases (CD20) the immunoreactivity is weakly decreased, but not constantly. Only in 1 case out of 2, the reactivity of CD45RO in formaldehyde 1:1 was strongly (+++), whereas in diluted formaldehyde 1:9 only weakly (+) positive.

Effect of pH (Formaldehyde 1:3) on the Result of Immunohistochemical Examination (Table 3)

The differences in immunoreactivity of material fixed with unbuffered (pH 4,7) and buffered (pH 7,0) formaldehyde are in this set of cases insignificant and demonstrate that in examined cases, pH of the fixation fluid is not a critical factor.

Examination of appendices fixed in various formaldehyde concentrations (1:1, 1:3, 1:9) with adequately different pH (4.0, 4.7 and 5.5, respectively) shows (*Table 4*) that the reactivity of tested antigens is not influenced by considerable differences in pH of the fixative solutions.

We did not find an important difference in immunoreactivity of tested antigens at concentrations 1:3 a 1:9 in either buffered or unbuffered formaldehyde. In concentrated solutions (1:1) compared with diluted ones, the immunoreactivity of CD20 is slightly decreased.

Effect of Autolysis on the Immunoreactivity of CD Antigens (Table 5)

Immunoreactivity of tested antigens remained constant even after 24 hour autolysis at room temperature, which indicates that time of transport of native tissue does not influence the result of immunohistochemical detection of tested antigens. Of course, the histological finding is altered markedly after 24-hour autolysis at room temperature.

Table 4
Effect of pH at different formaldehyde concentrations on immunoreactivity of CD20 and CD45RO

		CD 20 pH 7,0	CD 20 unbuffered	CD 45 RO pH 7,0	CD 45 RO unbuffered
Case 1	1:1	++	++	++	++
	1:3	+++	+++	++	+++
	1:9	0*	++	++	++/+++
Case 2	1:1	+++	++	+++	++
	1:3	+++	+++	+++	+++
	1:9	+++	+++	+++	+++
Case 3	1:1	+++	++	+++	+++
	1:3	+++	+++	+++	+++
	1:9	+++	+++	+++	+++

* evaluation impossible

Table 5
Effect of autolysis on the immunoreactivity of CD antigens

	0 hr	3 hrs	24 hrs/5°C	24 hrs/25°C
CD 20	++/+++	+++	+++	+++
CD 45 RO	++/+++	+++	+++	+++
CD 5	+++	+++	+++	+++
CD 10	+++	++/+++	++/+++	++/+++
CD 23	+++	+++	+++	+++
CD 43	+++	+++	+++	+++

Effect of the Size of Tissue Sample and Location of the Examined Block on Immunohistochemical Reaction

The result of immunohistochemical investigation was very good, independent of the excision of the tissue from the centre or the periphery of the tissue block. When the spleen was fixed in toto, the immunostaining according to the depth of excision was also of the same intensity. Irrelevant differences in immunoreactivity of variously situated antigens may have been caused by quite small size of the investigated spleen (120 g). In case of splenomegaly, it is possible to expect markedly worse results in investigation of the tissue from the central part.

DISCUSSION

Choice of the Fixative:

Formaldehyde is the most popular fixative for its low cost, excellent preservation of morphology and adequacy for the vast majority of histologic diagnoses. Unfortunately, formaldehyde is arguably the least desirable fixative for the preservation of antigens. Moreover, its use has come under attack from several sources because of its undeniable toxicity and probable carcinogenicity (*1*). There is no universal fixative (magic juice) for immunohistochemistry (*7*). While some molecules, e.g. small peptides, are relatively well preserved in formaldehyde, they may be solubilized and lost by alcohol-containing fixatives. Conversely, coagulating fixatives are superior to preserve filamentous structures (proteins) and lymphocyte CD markers. For the fixation of membrane CD molecules, Bouin solution, B5, PLP (periodate-lysin-paraformalehyde) or zinc salts (*8,9*) were suggested.

Formaldehyde Concentration and Time of the Fixation:

The effect of formaldehyde fixation on antigens can be explained by taking into account the chemistry of formaldehyde fixation. When gaseous formaldehyde is dissolved in water, it quickly becomes hydrated to methylene glycol $CH_2(OH)_2$ and its polymers, polyoxymethylenes $HO(CH_2O)_nH$ (10).

Thereafter, equilibrium is greatly in favour of methylene glycol (and polymers). Free formaldehyde amounts to less then 0,1 % of the solution! When a piece of tissue is placed into 10% buffered formaldehyde, methylene glycol, the predominant molecule, quickly penetrates – but does not fix – the tissue. Free formaldehyde binds covalently with aminoacids, releasing space for methylene glycol to hydrate to free formaldehyde , and so on, until all reactive sites have been bound. The most important molecular change is the formation of cross-links between proteins (polypeptides), or between proteins and nucleic acids, involving hydroxymethylene bridges (*10*). An additional role for the formation of co-ordinate bonds has been proposed for calcium ions, which are the most abundant ions in tissues (*11*). The cross-links are responsible for the masking of epitopes by changing the three-dimensional structure of proteins. Studies using ^{14}C labelled formaldehyde

have shown that normally at least 24 hours are required to achieve complete fixation with formaldehyde (*12*). Shorter fixation times will interrupt the fixation process, which will continue during tissue dehydration by alcohol. However, this results in an irreproducible mixture of formaldehyde (cross-linking) and alcohol (coagulation) induced changes in the antigens (fixation schizophrenia) (*1*). Prolonged fixation times, in contrast, may lead to the formation of excessive cross-links. Delay of fixation effects in autolysis of the tissue. Diffusion of the fixative in animal tissues and the dependence on the size of tissue sample will be discussed later.

According to the presented test outcomes, formaldehyde solution 1:9 did not result in weaker reactivity than more concentrated solutions 1:1 or 1:3, and fulfils the demands (criteria) of the optimal formaldehyde fixation. This correlates well with conclusions of several authors that microwave antigen retrieval technique ameliorates problems resulting from variations in fixation (by formol-saline, buffered formalin or zinc-formalin) and section preparation as well as storage (*2,13,14*).

Effect of pH of the Formaldehyde Solution:

The pH of the fixing solution is a controlling factor in the reaction of formaldehyde with proteins in aqueous media. In unbuffered formalin (approximately pH 4) many amino and guanidyl groups are protonated (NH_3^+), hence formaldehyde can react only with relatively few uncharged groups ($R{\cdot}NH_2 + CH_2O = R{\cdot}NH{\cdot}CH_2OH$). As the reaction proceeds, other groups give off their protons and combine with formaldehyde. Consequently, the reaction proceeds slowly. In buffered neutral formalin (pH 7.0) amino groups are discharged and react avidly (*10*). Maximum tissue fixation (cross-linking) occurs in the pH range 4 to 5.5, no increase in tissue stabilization was observed above pH 5.5. The increased amount of formaldehyde bound at higher pH levels only blocks numerous reactive groups. Thus, fixation in buffered neutral formaldehyde inactivates more tissue groups than unbuffered formalin without improving fixation (cross-linking). Some authors (*15*) still recommend buffered neutral formaldehyde as universal formol fixative for immunohistochemistry. According to the presented results, there is a relatively broad pH interval where immunoreactivity of the tested epitopes remains unchanged, which correlates well with the above mentioned theoretical data.

Autolysis:

The influence of autolysis on immunohistochemical reactivity has been tested repeatedly. Antibodies to several CD antigens, as well as to cytoskeletal proteins were applied after 0 to 72 hours of autolysis and subsequent formalin-paraffin procedure (*6*). Out of CD antigens, maximal preservation was obtained with CD45 and CD45RO (72 hours). Other antigens showed remarkable variations, which must be taken into consideration: for final diagnosis, antigens less resistant to autolysis in the tissue sample may be necessary. The presented results – preservation of reactivity

of CD20, CD45RO, CD5, CD10, CD23 and CD43 after 24-hour autolysis – must be regarded from this point of view.

Penetration of the Fixative and the Size of the Tissue Sample:

The velocity of penetration of the fixative in the tissue depends on properties of the disperse phase (i.e. formalin) as well as disperse milieu (i.e. tissue) and is determined by the diffusion coefficient (δ) according the Einstein equation:

$\delta = kT/f = RT/Nf$,

where k is Boltzmann constant, and f frictional coefficient (*16*). This is different in different tissues, (e.g. lymph node, bone marrow or tonsil) and this difference must be respected.

Diffusion of the fixative may be limited even by the size of the tissue sample The peripheral parts may be fixed well by formalin, while the inner parts may be underfixed and exposed to alcohol during the dehydration. If autolytic decomposition of the central parts of the tissue has started, a mixture of irregularly fixed areas with differently blocked antigen determinants and artificial neoepitopes results. In this case application of proteases or heating for antigen retrieval has different effects on the tissue sections (*1*). The resistance to autolysis was discussed before, and the results in the corresponding part of this report are valid only for tested tissues and antibodies.

CONCLUSIONS

For formaldehyde, fixation time 24–48 hours does not decrease immunoreactivity of studied antigens. Even formaldehyde fixation during weekend does not influence the result of immunohistochemical examination.

Effect of formaldehyde concentration: results of immunohistochemical reactions are more stable at a dilution of 1:3 and 1:9. Dilution 1:1 decreases the immunoreactivity of tested antigens only after prolonged (5-day) fixation.

Effect of pH on formaldehyde fixation is not critical: use of neutral formaldehyde is not essential for the detection of tested antigens. If a clinician sends bioptic material fixed with unbuffered formaldehyde, we do not have to be afraid of decreased immunoreactivity.

Short autolysis (30–60 minutes from extirpation of the tissue from the body) does not modify either histological, or immunohistochemical finding. Transport of the native tissue from the operating room to department of pathological anatomy in the mentioned interval can be considered safe.

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VLIV FIXACE A AUTOLÝZY NA IMUNOHISTOCHEMICKOU DETEKCI CD ANTIGENU

S o u h r n

Cílem této práce bylo posoudit vliv fixace a autolýzy na imunoreaktivitu běžně vyšetřovaných CD antigenů (CD20, CD45RO, CD5, CD10, CD23, CD43 a CD68). Byla použita biopticky (tonsily, apendixy) i nekropticky (sleziny) získaná lymfatická tkáň, která byla fixována formolem o různých koncentracích (1:9, 1:3, 1:1), pufrovaným (pH 7.0) či nepufrovaným, po dobu od 24 hodin do 5 dní. Některé vzorky byly vystaveny autolýze až 24 hodin při pokojové teplotě. U žádného z testovaných antigenů a v žádné ze zkoumaných koncentrací nebyl nalezen podstatný rozdíl v imunoreaktivitě po 24-hodinové fixaci ve srovnání s fixací 48-hodinovou. Pouze po 5-denní fixaci docházelo k poklesu reaktivity některých antigenů (CD10, CD5), zejména při vysoké koncentraci formolu 1:1. Rovněž pH se u sledovaných antigenů neukázalo být kritickým faktorem. Pokud jde o autolýzu, nebyl zaznamenán významný pokles reaktivity studovaných antigenů dokonce ani při 24-hodinové autolýze při pokojové teplotě.

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