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Application of the critical molar concentration concept to heat-mediated antigen retrieval in immunohistochemistry

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Summary

Previously we have applied Scott's critical molar concentration concept to show that divalent cations, especially Mg^{2+} may be used to measure the affinity of a known monoclonal antibody for its antigen. In this paper we report the application of this same procedure to a study of a series of antigens (three globular proteins and three intermediate filaments). The concept was applied to samples without any previous treatment or after the application of heat-mediated antigen retrieval (using a pressure cooker). Our findings suggest that heat-mediated antigen retrieval sets free protein side-chain(s) that have been masked by formaldehyde fixation. This is reflected in a higher affinity of the antibody for the antigen in question.

Introduction

It is generally accepted in the immunohistochemical diagnosis of biopsies that fixation is the primary cause for most failures to demonstrate tissue proteins. Formaldehyde is the most important fixative that has been used, as illustrated by the studies of Nitschmann & Hadorn (1944), Fraenkel-Conrat *et al.* (1945), Fraenkel-Conrat & Olcott (1948a,b), Berod *et al.* (1981), Brandtzaeg (1982), Fox *et al.* (1985), and Montero (1997), to quote just a few, that have attempted to clarify the mechanism of formaldehyde fixation. The general inference of these investigations is that fixation masks the antigenicity of proteins in some way.

More recently, however, a series of methods have appeared that unmask or reveal this masked antigenicity: microwaves, pressure cooker, autoclaving, and conventional hot-water treatment. All these procedures have in common the use of heat (Shi *et al*. 1997). Temperatures as high as 100 °C are preferred for unmasking. The unmasking takes place by the breaking of the many bonds between protein side-groups and formaldehyde created during fixation (Shi *et al*. 1991).

The antigen–antibody reaction takes place by the almost perfect fit of the epitope in the antigen and the paratope in the antibody. This fit is maintained by electrostatic bonds, dipoles, hydrogen bonding, van der Waals forces, charge transfers and hydrophobic bonds (Braden & Poljak 1995). In some situations, there may be a certain degree of induced fit (Rini *et al.* 1992).

In 1965, Scott & Dorling established the concept of Critical Electrolyte Concentration (CEC), which is a measure of the affinity between a reagent and its corresponding substrate. We have applied this concept to the study of the influence of salts of a monovalent or divalent cation on the antigen–antibody reaction (Montero *et al.* 1996) in an attempt to establish a similar procedure in the field of immunohistochemistry. In addition, we have studied what we call the critical molarity concentration (CMC) in substitution of the CEC abbreviation of Scott (1967).

In this paper we have studied the effect of increasing the molarity of magnesium chloride salt, added to dilutions of different antisera, on the demonstration of an antigen, both in sections treated with an antigen retrieval procedure, using the pressure cooker method, and in sections not treated by this method. Our results support the conclusion that heat treatment of sections leads to an unmasking of some of the protein side-groups participating in epitope–paratope binding, thus causing an apparent increase in affinity between the antigen and the antibody.

Materials and methods

Tissues

Tissue blocks of routine biopsy specimens from our Pathology Department were used. The tissues had been fixed overnight (10% buffered formalin) and processed following the procedure for the Tissue-Tek VIP (Miles Scientific). Sections, 5 μm-thick, were mounted on glass slides coated with chromalum-gelatin (Pappas 1971), and dried either at 37 °C overnight or in a 60 °C paraffin oven for 30 min.

Reagents

Three filamentous and three globular proteins were selected as antigens using commercial monoclonal antibodies.

The intermediate filaments were desmin (antiserum from BIOGENEX, Ab No. 072 N), vimentin (antiserum from DAKO, clone V9) and glial fibrillary acidic protein (GFAP) (antiserum from DAKO, clone 6F2). The globular proteins were S100 (BIOGENE, MUo058-UC), prostate specific antigen (PSA) (DAKO, clone ER-PRS) and carcinoembryonic antigen (CEA) (BIOGENEX, clone TF3H8-1).

Procedures

The experiments were performed at room temperature. The sections were deparaffinized in xylene and taken through decreasing ethanol concentrations to distilled water. Endogenous peroxidase was inhibited with 3% hydrogen peroxide for 30 min. The sections were rinsed thoroughly with water and Tris-buffered saline (TBS) and covered with the primary antisera solution for 30 min, followed by a 10 min wash with TBS. The incubation with the biotin-labelled antibody (LSAB, DAKO kit) took 10–15 min, followed by reaction with the streptavidin–peroxidase complex and the final development of peroxidase activity with 3,3′-diaminobenzidine (DAB).

A series of tissue sections were incubated with the primary antisera in presence of ascending molarities of magnesium chloride (0.03, 0.06, 0.12, and 0.25 M) in order to determine the CMC value. This value was the molarity at which the immunohistochemical reaction first became negative. When necessary, higher molarities were tested (0.5, 1.0, 1.5, 2.0 and 4.0 M, which is the solubility limit of $MgCl_2$).

The sections treated for antigen retrieval followed an identical procedure, except for the treatment in the pressure cooker, after the inhibition of endogenous peroxidase. Three liters of 0.01 M sodium citrate (pH 6.0) were brought to the boil in a 5-litre capacity stainless-steel pressure cooker. The sections mounted on silanized slides were lowered into the boiling solution and allowed to boil for 2.5 min at full pressure before placing the pressure cooker under cold running water. After reaching room temperature, the slides were taken out from the pressure cooker and rinsed with TBS.

Results

The results obtained for the six antigens studied are given in Table 1, together with the parameters assessed.

DESMIN. A monoclonal antibody from BIOGENEX was used for this antigen. After trying a range of dilutions, 1 : 200 was selected, based on the best result obtained in a dilution series. The time of incubation with primary antibody was 30 min, at room temperature. The data were collected using the smooth muscle cells of the muscularis mucosae and the muscularis propiae as the test cells. The intensity of the reaction with DAB decreased with increasing molarity of magnesium chloride. The CMC values are given in Table 1.

VIMENTIN. A monoclonal antiserum from DAKO diluted 1 : 500 with an incubation time of 60 min at room temperature was used. The stained cells were those in the connective tissue known to give a positive reaction for this antigen, namely fibroblasts and endothelium. The CMC values for this antigen are given in Table 1.

Glial fibrillary acidic protein (GFAP). A monoclonal antibody from DAKO (clone 6F2) was used at a dilution of 1 : 200. Sections were incubated for 30 min with primary antiserum at room temperature. Cases of tumours of the central nervous system (ependymomas, and glioblastoma multiforme) were studied as the test tissues. The CMC values obtained are given in Table 1.

S100. A monoclonal antibody from BIOGENEX, was used. After a dilution study, a 1 : 1000 dilution was finally selected. Sections of the large intestine where the Meissner–Auerbach plexus shows an intense positive reaction were used as the test tissues. Table 1 lists the CMC values.

CEA. A monoclonal antibody from BIOGENEX (clone TF3H8-1) was used at a dilution of 1 : 200 and 30 min of incubation with primary antiserum at room temperature. The colonic epithelium in a normal colon adjacent to an

Table 1. CMC of $MgCl_2 \cdot 6H_2O$ obtained with the use of an antigen retrieval procedure and without it for three globular proteins and three intermediate filaments.

Antigen	Dilution	Incubation time[a] (min)	CMC	
			Without Ag retrieval	With Ag retrieval[b]
Desmin	1 : 200	30	0.8 ± 0.5	$1.4 \pm 0.7^{***}$
Vimentin	1 : 500	60	2.3 ± 0.3	$3.6 \pm 0.2^{***}$
GFAP	1 : 1600	30	2.6 ± 0.2	$4.0 \pm 0.1^{***}$
S100	1 : 1000	30	0.4 ± 0.3	$1.9 \pm 0.5^{***}$
CEA	1 : 200	30	0.7 ± 0.3	$2.2 \pm 0.4^{***}$
PSA	1 : 200	30	0.08 ± 0.04	$0.6 \pm 0.3^{**}$

Values represent mean ± SD ($n = 7$, except GFAP, $n = 5$). Ag = antigen, CEA = carcinoembryonic antigen, GFAP = glial fibrillary acidic protein, PSA = prostatic specific antigen, [a]incubation time with the primary antibody, [b]antigen retrieval procedure used was 'pressure cooker' (see Materials and methods). $^{**}p < 0.01$, $^{***}p < 0.001$, paired samples Student's t-test for each row.

adenocarcinoma and in the adenocarcinoma itself was used as the experimental model. The corresponding CMC values are given in Table 1.

PSA. A monoclonal antibody from DAKO (clone ER-PR8) was used at a dilution of 1 : 200 and 30 min of incubation with primary antiserum at room temperature. The test tissue was hyperplastic prostate tissue. The CMC values for the two series in this case are shown in Table 1.

Discussion

Two preliminary considerations have to be taken into account. First, the dilution selected varied considerably among the different antisera, perhaps due to the different protein content of the employed sera, which is not always provided by the suppliers. Second, the heterogeneity of the material obtained from the files of the pathology department may also have contributed to the variable results of the antigen–antibody reactions.

Theoretical and practical antecedents

Scott & Willett (1966) state in their paper on the binding of cationic dyes to biological polyanions that the 'Affinity [of dyes] is the sum of all forces acting between the participants. Coulombic and van der Waals forces, hydrogen bonds and charge transfers may all be involved'. Despite the important differences between a substrate–stain reaction versus an antigen–antibody reaction, the CMC concept later developed by Scott (1973) was the inspiration behind our previous and present studies (Montero *et al*. 1996).

In addition to the binding forces listed by Scott & Willett (1966), Braden & Poljak (1995) highlighted the role that the high degree of complementarity plays in protein–protein binding. The existence of an induced fit when the complementarity is not absolute is also important (Rini *et al*. 1992).

Antigen retrieval procedures

Any antigen retrieval procedure acts by loosening or breaking the cross-linkages between proteins and formaldehyde, a process induced by high temperatures through the pressure cooker treatment (Shi *et al*. 1991). We assume that the antigen retrieval procedure sets free many binding forces previously masked by the fixation process. Therefore, the higher values of the CMC parameters obtained after antigen retrieval are related to this demasking.

We obtained fairly low levels of CMC for some antisera (0.5 M for desmin, 0.25 M for S100, and 0.06 M for PSA). These findings were unexpected; they are similar to those reported by Scott and Dorling (1965) for the substrate–dye reaction in tissue, but the affinity between antigen and antibody is much stronger, and thus higher values of CMC were expected. We propose that the low values obtained may be partially due to the considerable masking of binding forces by formalin fixation. Therefore, the rise in CMC values after antigen retrieval supports the hypothesis of the liberation of protein side groups masked by fixation. Whether the restoration to a normal status is complete or not is something that cannot be stated here, although we are inclined to believe that full restoration is not achieved.

Another point to be considered is the diversity of the CMC values for different antigens. This diversity is also apparent in the literature, where a large heterogeneity of results have been reported after the use of diverse antigen retrieval procedures, such as microwave, pressure cooker and others having in common the increase of temperature.

The CMC values presented here are averages for the employed dilution of the antiserum. These values may change with dilution, since the dilution factor depends on the protein content of each lot (occasionally provided by the supplier). Nevertheless, the statistics performed with these data show that the CMC values obtained for each antigen are within the normal range of a series of random experiments.

We have found only one report contrary to the intensity-increasing effect of heat: Lan *et al*. (1995) reported that microwave treatment reduces the intensity of immunostaining for some surface antigens. On the other hand, some authors have suggested a prolonged treatment with plain water for antigen retrieval (Puchtler and Meloan 1985), as well as reagents such as urea or borohydride, proposed by others, for the improvement of immunostaining.

All these reports call for a closer attention to the problems associated with the interpretation of the chemical mechanism of antigen–antibody reactions in immunohistochemistry. There are no fixed rules to apply to each and every case in the field of immunohistochemistry when dealing with fixed, paraffin-embedded material. However, we are of the opinion that there may be some general rules which are of value in the interpretation of the problems arising in this field. Our results confirm that the fixation procedure affects the binding of two proteins in tissues. Therefore, we observed an increased affinity after antigen retrieval. We interpret this change of affinity as an increase in the number of free protein side chains in the epitope which are able to bind to their corresponding counterpart in the paratope. This offers new possibilities for studying the changes introduced by heat in the physicochemical status of epitopes, 'frozen' by chemical fixation, through the release of protein side-chains from their binding with formaldehyde.

It is worth noting that immunostained control sections showed some background staining that always dissappeared after treatment with the lowest concentration of calcium chloride. We interpret this as the rupture through the ionic strength of the electrostatic attractions responsible for unspecific binding. In some cases, we observed a slight increase of immunostaining, compared to the control sections, after treatment with the lowest salt concentration. This effect may have the same explanation, but in this case the disappearance of the electrostatic charges helped to improve the specific signal.

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