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PERSPECTIVE

Heat-induced Antigen Retrieval: What Are We Retrieving?

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SUMMARY Of all molecular aspects lost and recovered during formalin fixation and antigen retrieval, respectively, electrostatic charges have probably received the least attention. This review will focus on our work during the past 7 years. It strongly supports the tenet that electrostatic charges, i.e., net negative on antigens and net positive on antibodies, play an important part in immune reactions and therefore should be given greater attention when focusing on quality control in immunohistochemistry. **(J Histochem Cytochem 54:961–964, 2006)**

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IT IS WELL KNOWN that for the greater majority of tissue antigens, formalin fixation (FF) will lead either to a reduction of their functional affinity for the antibody (avidity) or completely erase the antigen's intrinsic affinity for the same. The ultimate measurable effect that FF had on most tissue antigens was either reduced or absent staining, respectively. Antigen retrieval (AR), frequently also named epitope or target retrieval, is the term used to describe the restoration of the antigen's optimal immune reactivity after FF.

At present, the most probable and widely promoted concepts regarding the underlying chemistry of FF and AR are loss and retrieval, respectively, of the antigen's epitopes. The following review of our work during the past 5–6 years is intended to promote another concept that we hope will form the basis for a better understanding of this chemistry and contribute to quality assurance in immunohistochemistry (IHC).

At first, a brief review of certain tenets regarding some basic IHC (van Oss and Absalom 1984) is intended to help in establishing the basis for what is to follow.

There is a general agreement today that proteins function as antigens through their discrete molecular entities known as antigenic determinants or epitopes. On proteins, these determinants may consist of three to eight amino acids arranged either in a linear sequence along the primary and secondary structure of peptides or distributed spatially across the tertiary and quaternary structures of the protein molecules. On glycoproteins and polysaccharides, the extensive three-dimensional branching of glycosidic bonds can also be the basis for these antigenic determinants. If foreign to the host, these antigenic determinants will elicit an immunological response and thereby lead to the formation of specific antibodies.

The forces and bonds that participate in immune reactions are electrostatic (coulombic) charges, van der Waals forces, hydrogen bonding, and hydrophobic bonding. Electrostatic charges can of course be either positive or negative and exist on the polar side groups of soluble proteins (e.g., on carboxyl and amino groups). It is important to keep in mind that these electrostatic charges occur on the entire hydrophilic surface of the protein molecule, including on the amino acids that form the antigenic determinants of the antigen (epitopes) as well as on the antigen-binding site of the antibody (paratope). The initial attraction between antigens and antibodies is the result of their total complementary net electrostatic charges (− or +), regardless of whether they are located within or outside the epitopes and paratopes.

van der Waals forces are mutual dipole attractions that exist within atoms (nucleus and electrons) as well as between atoms and molecules. Between immunoreactive reactants, van der Waals forces become stronger the closer to the reactants and the better the steric fit between the epitope and the complementary paratope. This clearly implies, however, that van der Waals forces do not contribute at all to the necessary initial attraction between antigens and antibodies. Hydrogen bonding is another form of dipole attraction and therefore also does not play a major role in the initial im-

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mune attraction. Hydrophobic bonding may also occur between proteins but is not a very well-defined mode of interaction and therefore has been alluded to by many reviewers as yet another variety of van der Waals interaction. While the latter three, van der Waals forces, hydrogen bonding, and hydrophobic bonding, are important in the strengthening of any formed immune bonds and, ultimately, in the precipitation of the immune complex, in IHC we are dealing mainly with firmly bound tissue antigens; hence, precipitation is irrelevant.

Thus, of the four reactive elements listed, it would appear that electrostatic charges are by far the dominant force of any initial attraction between antigens and antibodies as well as their interactions as they pertain to IHC. For IHC, I would therefore like to postulate the following series of events that occur during the incubation with the primary antibody.

The net negative electrostatic charges of the tissue-bound antigens will attract the positively charged, soluble and, therefore, highly mobile antibodies. The initial strength of this attraction between the reactants is controlled by the total of their complementary net surfaces charges, including those existing within the immunoreactive epitope as well as within the paratope of the antibody. Ultimately, this initial general molecular attraction will terminate in the formation of specific bonds between the antigen's relevant epitope and the antibody's paratope, provided of course they are indeed of opposite charges. Any of the earlier-described van der Wals forces may subsequently strengthen these bonds.

It is important to differentiate between the initiating general electrostatic attraction between antigens and antibodies and the subsequent specific immunochemical interaction between the epitope(s) and the paratope. Thus, when the primary antibody we use in IHC is monoclonal and, therefore, binds only to a specific epitope, a multitude of non-immunoreactive sites will of course also contribute to the overall electrostatic charges of antigens and thereby will all contribute to the previously mentioned initial electrostatic attraction of the antibody.

A recall of the electrophoretic distribution of serum proteins (and tissue proteins) should help to remind us that, at near-neutral pH, most proteins, excluding antibodies, are indeed predominantly anodic and do carry the net negative charge that we expect of most antigens. A well-known tenet states that negatively charged antigens, as a rule, will elicit antibodies with a net positive charge. And, as we remember, most antibodies do indeed migrate towards the cathode. An equally well-established tenet tells us that the strongest electrostatic attraction between an antigen and its antibodies will be obtained at a pH that is intermediate between the isoelectric points of the two (van Oss and Absalom 1984).

On a theoretical basis, at least, it would appear that we have eliminated the other three factors as insignificant in IHC. However, we have performed several experiments that were designed to, and indeed do, support our claims regarding the importance of electrostatic charges. They were based on two well-known and widely accepted factors that specifically influence the strength of the electrostatic attraction between antigens and antibodies, namely, pH and ions. As they pertain to IHC, they consist of the pH of the antibody diluent as well as the type and concentration of cations in the diluent buffer (e.g., Na^+).

To explore and demonstrate the influence that these two factors have in IHC, we stained 15 antigens both with and without the use of AR at the diluent pH of 6.0 and 8.6, respectively, and with increasing concentrations of cations (Boenisch 1999,2001). Without the use of AR, we found that antigens reactive with monoclonal antibodies of subtype IgG1 stained most intensely at the slightly acidic pH, whereas antigens reacting with monoclonal antibodies of subtypes IgG2a and IgG3 stained strongest at the alkaline pH of 8.6. When we did use AR, the IgG2a antibodies now also stained strongest at pH 6, whereas optimum staining with a subclass IgG3 antibody remained at pH 8.6. We believe that this reversal of optimal staining from pH 8.6 to 6 with antibodies of subclass IgG2a is an indication of the antigen's recovery of lost negative charges during AR. (It might be of interest to speculate how the different immunoglobulin subclasses relate to the use of AR and environmental pH for achieving optimal staining.) These pH-dependent optimal staining intensities could be observed at antibody dilutions higher than those recommended by the vendor but were totally abolished when the primary antibody was used instead at the higher recommended concentration. Prolonging the incubation time with the higher dilutions, however, did not change this pH dependence. Thus, although an increase in antibody concentration can compensate for an unfavorable environmental pH, prolonging the incubations with the more diluted antibody will not.

Varying the concentration of Na^+ ions (in the form of NaCl) can markedly influence staining intensities at all antibody concentrations. Larsson (1988) referred to the presence of inorganic cations as forming a "shield" around the negatively charged antigens, thereby reducing or obstructing the attraction of the positively charged antibodies. (I might add that adding an identical concentration of K^+ ions had an even stronger detrimental influence, but it is not known why.) It is also noteworthy to mention that phosphate-buffered saline, a widely used antibody diluent, had one of the most pronounced negative effects on staining intensities. This makes sense because this diluent not only contains Na^+ ions from NaCl but also the monosodium (NaH_2^+) and

disodium (Na_2H^+) cations of the phosphate buffer. Increasing additions of NaCl to Tris buffers also had an increasingly suppressive effect on staining intensities (Boenisch 1999).

Thus, although these observations are really nothing new, they do eloquently support our claim that electrostatic attractions are indeed at work here.

In order to further underscore the important role electrostatic attractions play, we studied 40 tonsil antigens by the prolongation of the incubation time with their primary antibodies instead of the application of the vendor-recommended (or "mandated") AR (Boenisch 2002). Of these 40 antigens investigated, 35 were found to stain equally intense simply by prolonging the antibody incubation time from the recommended 10 min to 1 hr. Two additional antigens could also be stained successfully by prolonging the antibody incubation time to 15 hr at 4C. In summary, of the 40 investigated antigens recommended or mandated for AR by the vendor, only three failed to show a positive response to the longer antibody incubation and, therefore, require AR. We believe that this absence of any immunoreactivity provides evidence that only 3/40 antigens tested (or <10%) were deprived of their epitopes by FF. This, in turn, is likely due either to the loss of the negative charges within the epitope and/or the loss of the structural formation within spatially distributed component amino acids spanning quaternary and tertiary structures. Both cases would result in the loss of the antigen's intrinsic affinity for the antibody. Whereas these non-functional epitopes will of course remain so regardless of how long we incubate with the primary antibody, any reduction in the electrostatic charges located outside the epitopes can be readily compensated for by the described prolongation of the antibody incubation.

We frequently read about the so-called formalin-induced "masking" of epitopes as a cause for poor staining. First, it is not well understood what this masking means. Second, whereas prolongation of the antibody incubation can readily compensate for a reduced electrostatic charge, it is not likely to unmask epitopes.

It is important to keep in mind that any reductions in the electrostatic charge (a reduction of the functional affinity) and/or the loss of the epitope (lost intrinsic affinity) most likely represent only the ultimate and measurable immunochemical expression of what is otherwise a highly complex formalin-induced chemical denaturation process. The same may consist of one or several changes, including loss of charges due to the formation of methylene bridges, loss of the quaternary and tertiary structures, and/or the steric inversion of the hydrophilic and hydrophobic sites.

Nevertheless, our investigations strongly support the concept that the formalin-induced damage of most tissue antigens ultimately manifest themselves in the progressive loss of their electrostatic charges, and that these losses are likely more severe the longer the fixation process.

Therefore, I sincerely hope that the data I have shared will contribute to a better understanding of the immunochemical forces at work when we stain tissue antigens, but they are not intended to detract from or belittle the importance of AR in IHC!

For some time now, we have known and accepted—even though we frequently complained about it—that inconsistent lengths of FF of tissue was the cause of variable damage to antigens. (In most hospital labs today, uncontrolled lengths of FF appear to be the norm rather than the exception.) Especially when AR was omitted, excessive FF was found to either render some of the investigated antigens to stain weaker or not at all.

This has recently led us to examine the usefulness of AR for the sole and specific purpose of compensating for inconsistent lengths of FF. I would like to give credit to Shi's group for their work in this field (Shi et al. 1998). His group investigated three particular antigens for their variable survival during inconsistent incubations in formalin, a finding that inspired us. We therefore investigated 30 tonsil antigens by deliberately subjecting the tissue blocks to variable lengths of FF for periods of between 12 hr and up to 8 days (Boenisch 2005). This was followed by AR with the use of citrate buffer, pH 6, for 20 min at 97C. We found that 27/30 antigens were consistently retrieved, with the emphasis on "consistent," regardless of how long they had been in FF within these periods. Only three of the studied antigens could no longer be retrieved after 2 days of FF. For this reason alone, consistent application of AR is highly recommended, particularly as it will further the many ongoing efforts towards the quality assurance of IHC. Furthermore, the consistent application of AR to all tissue sections has recently been shown to also allow for a much simpler pretreatment, i.e., the simultaneous removal of paraffin, rehydration, and AR (Boenisch in press).

In summary, I am convinced that in the greater majority of antigens, FF reduces and AR retrieves—not epitopes— but the electrostatic charges necessary for attracting and reacting with the antibody. We also emphasize that these electrostatic charges occur throughout the hydrophilic surfaces of antigens including the targeted epitope(s). Only in <10% of antigens investigated was there any evidence that FF caused the loss of the intrinsic affinity, that is, the epitope. However, we also wish to again stress the importance of AR as a means for alleviating staining inconsistencies that frequently result from variable lengths of FF.

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