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RAPID COMMUNICATION

Reversing the Effects of Formalin Fixation with Citraconic Anhydride and Heat: A Universal Antigen Retrieval Method

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SUMMARY Formalin is a commonly used fixative for tissue preservation in pathology laboratories. A major adverse effect of this fixative is the concealing of tissue antigens by protein cross-linking. To achieve a universal antigen retrieval method for immunohistochemistry under a constant condition, we developed a new method in which the effects of formalin fixation were reversed with citraconic anhydride (a reversible protein cross-linking agent) plus heating. Formalin-fixed, paraffin-embedded tissues from various organs were examined for immunohistochemical localization of a wide variety of antigens. Deparaffinized tissue sections were placed in an electric kitchen pot containing 0.05% citraconic anhydride solution, pH 7.4, and the pot was set at "keep warm" temperature mode of 98C for 45 min. This mode allowed heating the sections at a constant temperature. The sections were then washed in buffer solution and immunostained using a labeled streptavidin–biotin method using an automated stainer. In general, formalin-fixed tissues demonstrated specific immunostainings comparable to that in fresh frozen tissues and significantly more enhanced than after conventional antigen retrieval methods. In particular, even difficult-to-detect antigens such as CD4, cyclin D1, granzyme β, bcl-6, CD25, and lambda chain revealed distinct immunostainings. Different classes of antigens such as cellular markers and receptors, as well as cytoplasmic and nuclear proteins, consistently produced enhanced reactions. This method provides efficient antigen retrieval for successful immunostaining of a wide variety of antigens under an optimized condition. It also allows standardization of immunohistochemistry for formalin-fixed tissues in pathology laboratories, eliminating inter-laboratory discrepancies in results for accurate clinical and research studies.

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KEY WORDS
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Antigen retrieval (AR) methods using heat have been widely applied as significantly effective pretreatment for immunohistochemistry (IHC) on routine light microscopic preparations (Shi et al. 1991). The application of AR methods included treatment of deparaffinized sections with microwaves, exposure to combined action of heat and pressure in a pressure cooker, and combined action of enzyme digestion and microwaves (Gown et al. 1993; Elias 2001).

The current AR methods include the use of citrate buffer, Tris-HCl containing 5% urea, and EDTA solutions each combined with heating in a microwave oven or autoclave. The effectiveness of AR–IHC may be influenced by the pH values of AR solutions (Evers and Uylings 1994; Shi et al. 1995). While the most commonly used protocol, i.e., heating in citrate buffer, pH 6.0, for 20 min is effective for many antigen/antibody reactions (Shi et al. 1999), variations in the solution media, buffered equilibrium, temperature, and heating source have been used to adjust for successful AR–IHC of other antigens by different laboratories. However, AR–IHC method under a constant condition could not be universally applied to various antigens (Shi et al. 1997,1999). A major drawback could be generation of discrepancies in the results among different pathology laboratories that render data comparisons unreliable.

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During the course of testing reversible protein cross-linking agents for AR in formalin-fixed, paraffin-embedded tissues, we found that heating the tissue sections in citraconic anhydride solution constantly produced specific immunostainings comparable to that in fresh frozen tissues and significantly more enhanced than by the standard AR methods. Formaldehyde reacts predominantly with the protein amino groups and forms intra-molecular cross-links. Dixon and Perham (1968) showed that citraconylation by citraconic anhydride provided an easy method for the reversible blocking of protein amino groups, in which both the introduction and removal of the citraconyl groups might be carried out under suitably mild conditions that most proteins could withstand.

We report, for the first time, the use of citraconic anhydride solution as a medium in heating AR for formalin-fixed tissues. The mechanism behind its function is based on the favorable effect of the reagent of reversible blocking of protein amino groups (Dixon and Perham 1968). The method provides efficient AR for successful immunostaining of a wide variety of antigens under an optimized condition.

Materials and Methods

Tissue Specimens

Human tissues from various organs (tonsil, ovary, skin, lymph node, stomach, breast, colon, lung, and thymus) obtained as surgical biopsy specimens that had been routinely fixed in 10% buffered formalin and embedded in paraffin by standard methods were studied after obtaining informed consent. The fixation time ranged from 18 hr to 24 hr. A series of these tissues also had duplicate frozen samples available. Serial paraffin sections, 3-μm thick, were cut, mounted on silane-coated glass slides (Muto Pure Chemicals; Tokyo, Japan), and dried at 60C in an oven for 3 hr before use. The sections were deparaffinized in xylene, rehydrated in graded ethanol series, and endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in distilled water. Sections of the same thickness were also prepared from the duplicate frozen samples. Tissue sample from Hodgkin's disease was used for latent membrane protein-1 (LMP-1) antigen localization.

Titration Study of Citraconic Anhydride Solution

Citraconic anhydride (molecular weight = 112.08) in liquid form (25 g) was obtained from Tokyo Kasei Co. Ltd.; Tokyo, Japan. To determine an optimal concentration for obtaining a maximal immunostaining result, different concentrations of citraconic anhydride in distilled water (1%, 0.5%, 0.1%, 0.05%, 0.01%, and 0.001%) each at pH 2.0, pH 7.4, and pH 10 were prepared and used in AR methods performed for 45 min at room temperature vs heating at 98C in an electric kitchen pot, capable of maintaining a constant temperature (980 W, Model NC-ET22, National; Matsushita Electric Co., Osaka, Japan). Serial tissue sections from lymph node, breast, or lung were immunostained for CD4, cyclin D1, granzyme β, or PAR4 antigenic determinants.

New AR Method

For the universal AR, deparaffinized sections on slides in stainless steel staining racks were placed in an electric kitchen pot (Matsushita Electric Co.) filled with ~800 ml of 0.05% citraconic anhydride solution, pH 7.4 (Immunosaver; Nissin EM Co. Ltd., Tokyo, Japan) so that the solution would cover the entire staining racks, and the pot was set at "keep warm" temperature mode of 98C for 45 min. This mode maintains the inside temperature of the pot at a constant level (temperature variability, ±2C). The needed volume of the solution is determined according to the capacity of the electric pot used. The sections were then washed in buffer solution (APK Solution; Ventana Medical Systems, Tucson, AZ). Since citraconic anhydride is considered a toxic agent, it is necessary to use a hood and to prevent contact with skin and eyes when performing the AR procedure, although the concentration needed was very low.

Conventional AR Methods

Conventional AR method for each antigen was performed at its optimal condition in parallel according to the data sheet instructions accompanying each antibody. Serial sections from the same blocks were used for comparison purpose. After deparaffinization, rehydration, and blockade of endogenous peroxidase by 0.3% hydrogen peroxide in distilled water, the sections were placed in stainless steel racks and subjected to high temperature antigen unmasking technique in 0.01 M citrate buffer, pH 6.0, in a pressure cooker for 1 min after reaching operating temperature and pressure (~5 min), or in Coplin jars containing 0.05 M citrate buffer solution, pH 6.0, or 0.1 M Tris-HCl buffer containing 5% urea, pH 9.0 and heated in a microwave oven for 10 min (two times) according to the standard methods described previously (Shi et al. 1996,1999; Elias 2001).

Immunohistochemistry

After the respective AR methods, formalin-fixed, paraffin-embedded sections, as well as fresh frozen sections, were processed using a labeled streptavidin–biotin (LSAB) method (Kristiansen et al. 2003) according to the Ventana DAB Universal Kit and an automated stainer (NexES IHC, Ventana Medical Systems; Yokohama, Japan). The primary antibodies examined and their sources and corresponding dilutions are listed in Table 1. In addition, antibodies to several hormones [growth hormone (GH), adrenocorticotropic hormone (ACTH), human chorionic gonadotropin (HCG), prolactin, human placental lactogen (HPL), and insulin], diffusible proteins (lactoferrin and S-100) and enzymes (prostatic acid phosphatase, PAcP and placental alkaline phosphatase, P-ALP) obtained from DAKO, Japan were also examined. Briefly, nonspecific binding sites in tissues were blocked with 10% normal goat serum for secondary goat anti-mouse and anti-rabbit antibodies or normal horse serum for secondary horse anti-goat antibody followed by the applications of primary antibodies at the specified dilutions and then corresponding biotinylated secondary antibodies (Ventana). After washings in phosphate-buffered saline, pH

Table 1 Comparison of immunostainings using different antigen retrieval methods

Antibody	Clone/species	Source	Antibody dilution	Citrate buffer	Tris-HCl + 5% urea	Citraconic anhydride
bcl-2	123	DAKO	50	+/W	±/W	+++/M
bcl-6	Rabbit	DAKO	25	—	—	++/M
CA125	M11	DAKO	50	++/M	++/M	+++/S
Calretinin	Rabbit	Zymed	Original	++/W	++/W	+++/M
CD1a	O10	Immunotech	Original	+/W	+/W	+++/M
CD2	AB75	Novocastra	100	++/W	+/W	+++/M
CD3	Rabbit	DAKO	100	+/W	+/W	+++/M
CD4	1F6	Novocastra	20	++/M	—	+++/S
CD5	4C7	Novocastra	50	++/W	±/W	+++/M
CD8	C8/144B	DAKO	100	+/W	±/W	+++/M
CD10	56C6	Novocastra	50	±/W	+/W	+++/M
CD15	Leu-M1	Becton Dickinson	50	++/M	++/M	+++/S
CD20	L26	DAKO	4000	+++/W	++/M	+++/S
CD21	2G9	Novocastra	50	+/W	+/W	+++/M
CD23	1B12	Novocastra	200	+/W	—	+++/M
CD25	4C9	Novocastra	100	±/W	—	++/S
CD30	Ber-H2	DAKO	100	+/W	+/W	+++/S
CD34	NU-4A1	Nichirei, Japan	100	+/W	+/W	+++/S
CD35	RLB25	Novocastra	100	+/W	+/W	+++/M
CD38	SPC32	Novocastra	1000	++/M	++/W	+++/M
CD43	MT-1	Bio-Science	100	+++/W	+++/W	+++/M
CD45	2B11+PD7/26	DAKO	1000	+++/W	++/M	+++/S
CD45RO	UCHL-1	DAKO	200	++/W	±/W	++/S
CD56	1B6	Novocastra	50	++/W	++/W	+++/M
CD57	Leu-7	Becton Dickinson	50	++/M	++/W	+++/S
CD68	KP-1	DAKO	5000	++/W	±/W	+++/M
CD68	PGM-1	DAKO	200	+/W	±/W	+++/M
CD79a	JCB1117	DAKO	200	++/M	++/W	+++/S
CD99	12E7	DAKO	500	+/W	+/W	++/S
CK5/6	D5/16 B4	DAKO	500	+/W	++/M	+++/S
CK7	OV-TL 12/30	DAKO	50	++/M	+/W	+++/S
CK10/13	DE-K13	DAKO	200	++/W	++/W	+++/M
CK20	Ks20.8	DAKO	200	+/W	++/M	+++/S
CyclinD1	P2D11F11	Novocastra	20	+/W	—	++/M
Desmin	D33	DAKO	1000	+/W	++/W	+++/M
ER	1D5	DAKO	25	±/W	+/W	+++/M
Factor VIII	F8/86	DAKO	2000	++/W	++/M	+++/M
Granzyme β	11F1	Novocastra	100	+/M	—	+++/S
IgA	Rabbit	DAKO	5000	++/W	+/M	+++/M
IgG	Rabbit	MBL	50000	++/W	+/W	+++/M
IgM	Rabbit	DAKO	20000	++/W	+/W	+++/M
MDR-1	JSB-1	Nichirei, Japan	50	±/W	+/W	+++/
Keratin, wide	Z622	DAKO	1000	++/W	±/W	+++/S
Ki-67	MIB-1	DAKO	500	++/M	+/W	+++/S
κChain	Rabbit	DAKO	20000	+/W	±/W	+++/M
LMP-1	CS1-4	DAKO	400	+/W	±/W	+++/M
CD74	LN2	Nichirei, Japan	10	++/M	—	+++/S
λChain	Rabbit	DAKO	200000	+/W	±/W	+++/M
Melan-A	A103	DAKO	20	±/W	—	+++/M
Anti-leukocyte B cells	MB-1	Seikagaku Kogyo, Japan	500	+/W	+/W	+++/M
NSE	BBS/NC/VI-H14	DAKO	5000	++/W	+/W	++/S
NF	2F11	DAKO	2000	++/M	++/W	+++/M
P53	Rabbit	Novocastra	5000	+/W	+/W	+++/S
P80	5A4	Novocastra	100	+++/W	—	+++/S
PAR-4	Goat	Santa Cruz	50	—	—	+++/S
PCNA	PC10	DAKO	500	++/W	±/W	+++/S
SP-A	PE-10	DAKO	100	±/W	±/W	+++/S
PgR	PgR636	DAKO	100	++/W	++/W	+++/S
TIA-1	2G9	Immunotech	100	±/W	+/W	+++/S
TTF-1	8G7G311	DAKO	200	±/W	±/W	+++/S
VEGF	Rabbit	Santa Cruz	500	++/W	+/W	+++/S
Vim	V9	DAKO	10000	±/W	++/W	+++/S

"Original" dilution means using the prediluted reagent without further dilution. −, 0%; ±, <5%; +, 5–25%; ++, 26–50%; and +++, >50%. W, weak; M, moderate; and S, strong.

7.4, streptavidin–biotin–peroxidase complex was applied, and the reactions were visualized with diaminobenzidine reaction. Nuclear counterstaining was with Mayer's hematoxylin. For each antigen, a negative control section was included in which the primary antibody was substituted with corresponding normal serum from mouse, rabbit, or goat (DAKO). Likewise, for each primary antibody, a previously known positive control section was used.

Evaluation of Immunostainings

Evaluation of immunostained sections was done independently by three observers experienced in immunohistochemical assessment of tissue sections (SN,MG,YS). This evaluation was performed blindly without the knowledge of AR methods used. Both the extent and intensity of immunostainings were considered. In each tissue, the extent of staining was assessed by the mean percentage of immunostained area and classified as 0%, −; <5%, +; 5−25%, 1+; 26−50%, 2+; and >50%, 3+. The intensity of staining was judged as weak, moderate, and strong. The results in agreement with two or more observers were considered as final.

Results

To determine what concentration of citraconic anhydride would result in the maximal AR level in tissue sections, we first performed a preliminary study in which various concentrations of citraconic anhydride were used in AR for 45 min at room temperature vs heating at 98C. Serial tissue sections from lymph node, breast, or lung were immunostained for CD4, cyclin D1, granzyme β, or PAR4 antigenic determinants. The results uniformly showed no staining with the AR performed at room temperature. The strongest intensity of immunostaining for the antigens tested was achieved with AR in 0.05% citraconic anhydride at pH 7.4 and heating at 98C. In addition, AR in 0.01% citraconic anhydride at pH 7.4 and heating at 98C also revealed frequent positive stainings for the antigens but at a lower intensity. The other concentrations and pH ranges examined gave no, or significantly weaker, results. Based on these findings, a concentration of 0.05% citraconic anhydride was considered optimal.

Subsequently, formalin-fixed, paraffin-embedded tissue sections from various human organs were universally subjected to the AR method with 0.05% citraconic anhydride and heat followed by immunostaining with a battery of more than 60 different primary antibodies using a single standard automated immunostaining method. Serial sections from the same tissues were also subjected to the conventional AR methods for the respective antigens and immunostained in parallel.

In all instances, the extent and intensity of immunostainings after AR with our method showed significant enhancement over the conventional methods (Table 1). There was no apparent effect on morphology. Several repeated immunostainings gave consistent results. In a series of tissues with available fresh frozen and formalin-fixed, paraffin-embedded materials, the immunostaining results of formalin-fixed tissues were comparable to those of the counterpart fresh frozen tissues. Figure 1 illustrates several representative micrographs showing comparison among immunostainings of formalin-fixed, paraffin-embedded serial tissue sections using the conventional AR methods and citraconic anhydride method as well as immunostainings in frozen sections. The stainability for CD4 in an example of formalin-fixed lymph node tissue section (first row) was found to be 2+ with moderate intensity by citrate buffer (first column), negative by Tris-HCl + 5% urea (second column), 3+ with strong intensity by citraconic anhydride (third column), and 3+ with moderate intensity in frozen section (fourth column). Detection of cyclin D1 in formalin-fixed breast cancer tissue (second row) was 1+ with weak intensity by citrate buffer, negative by Tris-HCl + 5% urea, 2+ with moderate intensity by citraconic anhydride, and 2+ with weak intensity in frozen section. Granzyme β immunostaining in formalin-fixed lymph node sections (third row) was 1+ with moderate intensity by citrate buffer, negative by Tris-HCl + 5% urea, and 3+ with strong intensity by citraconic anhydride or in frozen section. Protease-activated receptor 4 (PAR4) in formalin-fixed lung tissue (fourth row) showed negative staining by citrate buffer or Tris-HCl + 5% urea, and 3+ staining with strong intensity by citraconic anhydride or in frozen section. The status of several hormones (GH, ACTH, HCG, prolactin, HPL and insulin), diffusible proteins (lactoferrin and S-100) and enzymes (PAcP and P-ALP) was also assessed after AR with our method. The extent and intensity of immunostaining for these substances were found to be similar to those obtained before the application of AR method (Figure 2).

Discussion

Formalin is a commonly used fixative for tissue specimens in pathology laboratories. Moreover, archival formalin-fixed, paraffin-embedded tissue specimens provide a principal source of human tissues for diagnostic and retrospective research studies. Diagnostic pathology procedures frequently require the use of IHC. However, when formalin-fixed tissues are encountered, localization of many antigens needs at least one type of AR to make them accessible for reaction with their specific antibodies. The diversity of AR methods may be a significant source of introducing discrepancies in the immunostaining results among different laboratories, making inter-laboratory data less reliable for comparison. Thus, developing a gen-

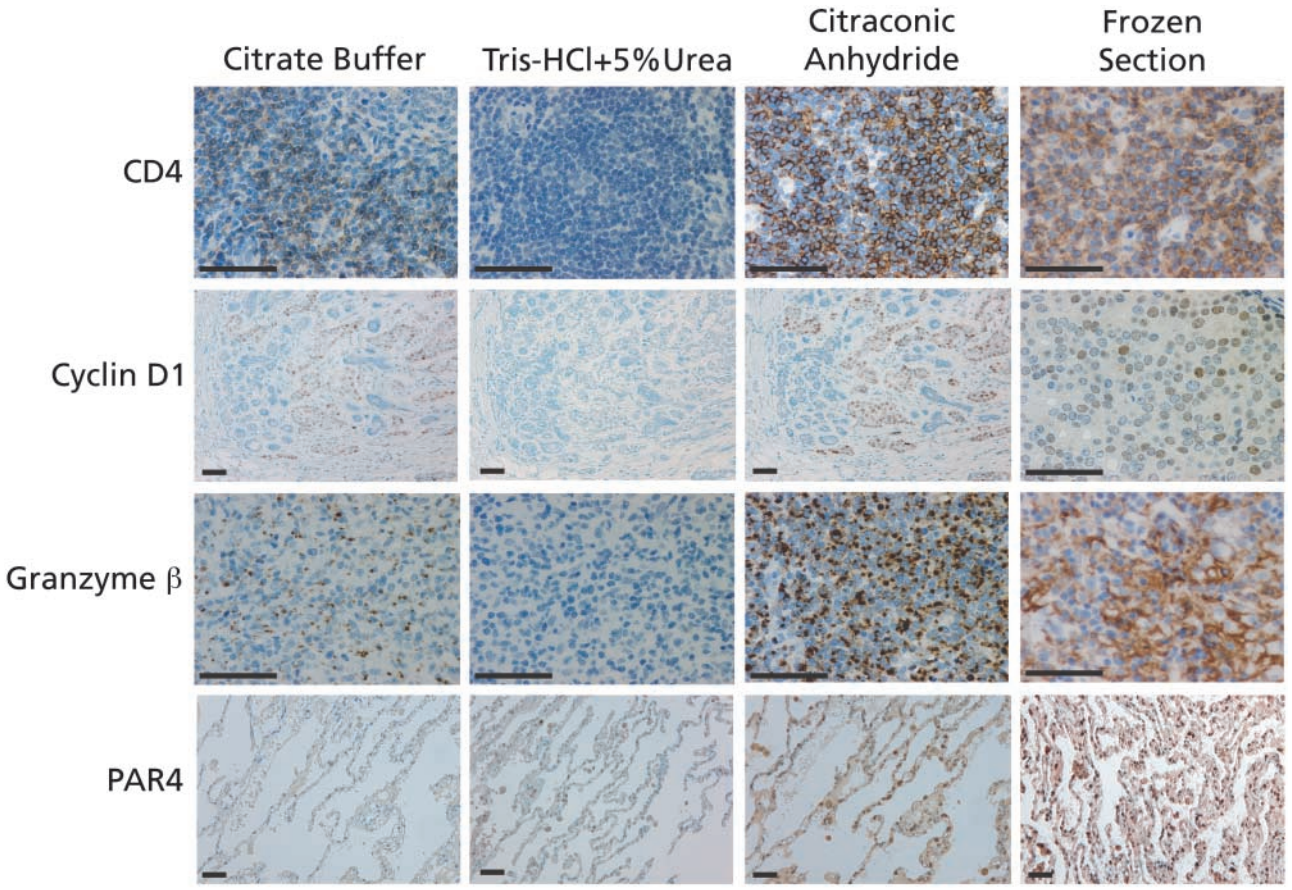

Figure 1 Representative micrographs showing comparison among immunostainings of formalin-fixed, paraffin-embedded serial tissue sections using the conventional AR methods and citraconic anhydride method as well as immunostainings in frozen sections. In all instances the extent and intensity of immunostainings after AR with citraconic anhydride showed significant enhancement over the conventional methods and was comparable to that in frozen sections. First row: CD4 in a lymph node tissue; 2+ with moderate intensity by citrate buffer, negative by Tris-HCl + 5% urea, 3+ with strong intensity by citraconic anhydride and 3+ with moderate intensity in frozen section. Second row: cyclin D1 in a breast cancer tissue; 1+ with weak intensity by citrate buffer, or negative by Tris-HCl + 5% urea, 2+ with strong intensity by citraconic anhydride, and 2+ with weak intensity in frozen section. Third row: granzyme β in a lymph node tissue; 1+ with moderate intensity by citrate buffer, negative by Tris-HCl + 5% urea, and 3+ with strong intensity by citraconic anhydride or in frozen section. Fourth row: PAR4 in a lung tissue; negative by citrate buffer or Tris-HCl + 5% urea, and 3+ with strong intensity by citraconic anhydride or in frozen section. Bars = 50 μm.

eral strategy for antigen retrieval provides a critical step toward standardization of IHC for formalin-fixed tissues.

Currently, AR comprises an essential part of IHC. In the present study, we showed that heating formalin-fixed tissue sections in 0.05% citraconic anhydride solution, pH 7.4, at 98C for 45 min restores the immunostaining of a wide variety of antigens. The immunostaining results in formalin-fixed, paraffin-embedded sections after AR with our method were comparable to those in fresh frozen sections from the duplicate tissues, indicating that the formaldehyde cross-links had been efficiently released.

The major factors which influence the intensity of immunostaining following AR included buffered equilibrium, pH, heating temperature, and heat source (Cattoretti et al. 1993; Munakata and Hendricks 1993; Evers and Uylings 1994; Kawai et al. 1994; Taylor et al. 1994; Shi et al. 1995,1999). It has been noted that high-temperature heating is the most important factor for retrieval of antigens concealed by formalin fixation (Shi et al. 1991). Different heating methods have been used for AR, such as microwaving (Shi et al. 1991), autoclaving (Bankfalvi et al. 1994; Igarashi et al. 1994), pressure cooking (Norton et al. 1994), water bath (Kawai et al. 1994), and steam heating (Pasha et al. 1995). In the routine method, the number of sections treatable at the same time in a microwave oven is restricted. When the bulk sections are processed, irradiation condition setting of microwave oven is variable. Autoclave process from heating to cooling is time-consuming and inconvenient. Thus, we

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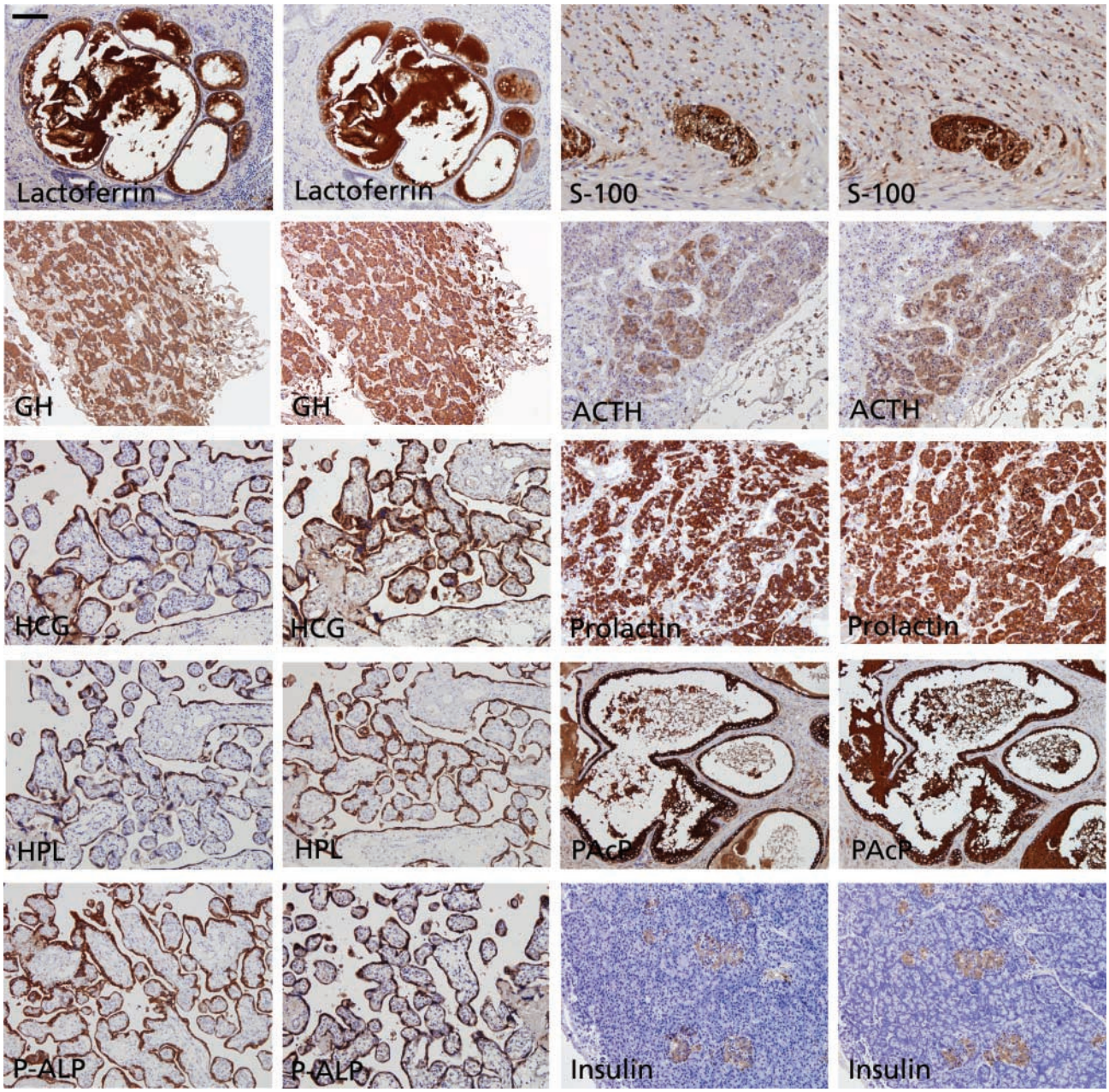

Figure 2 Representative micrographs showing immunostainings of comparable areas from formalin-fixed, paraffin-embedded serial tissue sections with no AR (first and third columns) or AR with citraconic anhydride method (second and fourth columns). Diffusible proteins (lactoferrin in breast, S-100 in colon), hormones (GH and ACTH in hypophysis, HCG in placenta, prolactin in hypophysis, HPL in placenta, and insulin in pancreas) and enzymes (PAcP in prostate, P-ALP in placenta) did not show apparent loss of staining after AR method with citraconic anhydride. Bar = 50 μm for all.

took advantage of using a conventional electric kitchen pot equipped with a "keep temperature mode" capable of maintaining a constant temperature.

In the present study, AR with citraconic anhydride solution and heat under an optimal condition was able to satisfactorily retrieve a wide variety of antigens for IHC. Several repeated immunostainings gave consistent results, indicating that the method was highly reproducible.

Although several AR methods combining heat with a variety of solutions have been reported, our method appears to be novel in that its mechanism of function may be more readily defined. We used citraconic anhydride solution as a medium in heating AR. The

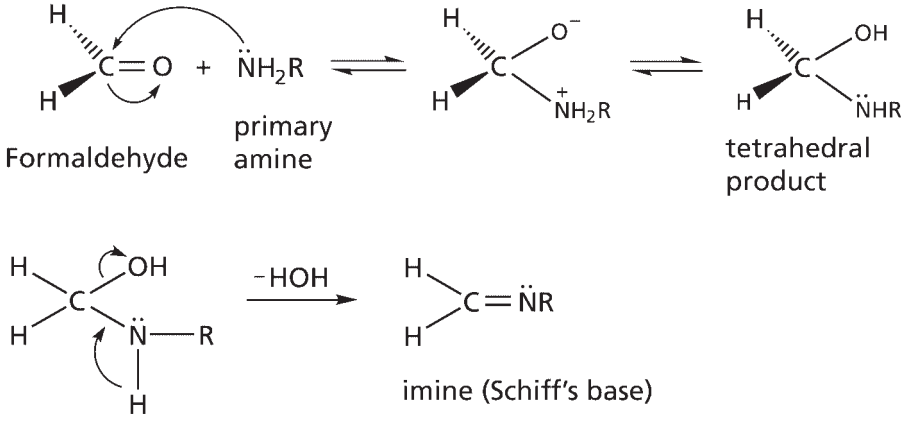

Figure 3 Schematic representation of reaction between formaldehyde and a primary amine for imine (Schiff's base) formation via a tetrahedral intermediate. The reaction involves nucleophilic attack by the primary amine on the carboxyl group of formaldehyde.

mechanism behind its function is based on the reagent's favorable effect of reversible blocking of protein amino groups. We observed a significant increase in the degree of immunostaining in virtually all tissue specimens examined. Critical support for this observation could be derived from the existing literature as well. In fact, citraconic anhydride had been successfully used in the past as a reversible inactivator for the inactivation of biologically active surface glycoproteins without interference with their immunochemical and antigenic activities (Habeeb 1997).

The reaction of formaldehyde has been extensively studied in the past, dating back to 1948 (Fraenkel-Conrat and Olcott 1948). Formaldehyde reacts predominantly with the lysyl residues and forms intramolecular cross-links (Fraenkel-Conrat and Mecham 1949). The first step in the reaction involves the free amino groups of proteins with the formation of aminomethylol groups that then condense with other functional groups (e.g., phenolic, imidazole, and indole groups) to form methylene bridges. Citraconic anhydride reacts with the free amino groups of proteins and replaces the positively charged NH^{3+} groups of lysyl residues with negatively charged carboxyl groups (Dixon and Perham 1968; Habeeb and Atassi 1970). Consequently, an explanation for the mechanism underlying successful AR in our method could be that formaldehyde cross-links originate as imines (Schiff's bases). Moreover, the amines that do not have convenient neighbors to react with will probably remain as Schiff's bases in the stored formalin-fixed, paraffin-embedded tissues (Figure 3). Theoretically, citraconic anhydride can react with these adducts and with the next-step products of cross-linking, which will result in reversibly modified amines. At pH 7.4 in hot water, adducts of citraconic anhydride and amines will slowly hydrolyze, which will liberate the original (desired amines). Thus, it is hypothesized that citraconic anhydride acts as a "shuttle" that attacks the cross-links in the sample, converting them into protected amines (and liberated formaldehyde quickly dissipates into solution, so that adducts cannot re-form), and then allows the amines to be liberated during the second, slower, stage of the process via hydrolysis. Apart from the effect of citraconic anhydride solution used in the present study, both a high-temperature heating and pH of the buffer solution have been shown to be critical factors in the success of AR methods. In this regard, our AR method showed uniformly negative immunostainings when performed at room temperature and no, or significantly weaker, immunostainings when pH ranges other than 7.4 (2.0 or 10.0) were used. These observations indicated the importance of heat and pH for successful AR.

Some studies have described the denaturating effect of citraconylation, reversed by subsequent acid treatment (Bindels et al. 1985; Batra 1991). In these studies, high-fold molar excesses (100-fold or 960-fold) of citraconic anhydride per lysine were needed to allow a significant (more than 90%) modification of lysine residues of the proteins leading to the denaturation. To address whether treatment with citraconic anhydride in our AR method might have caused "denaturation" or "renaturation" of proteins in the tissues, we elaborated on several antibodies in our series such as bcl-6, CD99, and PAR4 that were working mainly on frozen sections. In this regard, our results showed closely comparable intensities of immunostaining between frozen sections and the AR-treated paraffin sections. In fact, most antibodies used in our study were unreactive on formalin-fixed, paraffin sections prior to AR treatment. Furthermore, we examined a number of hormones (GH, ACTH, HCG, prolactin, HPL, and insulin) and diffusible proteins (lactoferrin and S-100) that might be vulnerable to loss after renaturation. Likewise, comparable intensities of immunostaining for these substances were observed after our AR method that suggested the lack of a significant loss. To assess endogenous enzymatic survival, two enzymes, PAcP and P-ALP, were examined after our

AR method that revealed no significant decrease or loss of immunostaining. Indeed, the concentration of citraconic anhydride used in our AR method was significantly lower (0.05%: ~5 mM) and beyond the molarities reported to have denaturating effect (Bindels et al. 1985; Batra 1991). Together, these observations indicated that our AR treatment method may likely cause "renaturation" of proteins in tissue sections, resulting in re-exposure of antigenic determinants, a hypothesis suggested by Shi et al. (1999).

Some advantages of our method over the existing AR methods include: a) it is simple, reliable, and reproducible, b) it can be applied universally for AR, thus highly promising for optimization and standardization of IHC, and c) it allows AR of a significant number of slides in one batch, eliminating inter-batch variability factor. Apart from these advantages, a drawback of our AR method could be potential for introducing background staining, although this might be applied to any AR method. To address this issue, we performed a comparative study in a selected group of tissues with available fresh frozen and formalin-fixed, paraffin-embedded materials. Compared with the fresh frozen tissues, we did not observe changes in the background stainability or the extent and intensity of specific immunostainings using a predetermined dilution of a given primary antibody after AR of formalin-fixed, paraffin-embedded tissue sections by the present method.

Currently, we are examining the use of citraconic anhydride as a reversible blocker of protein amino groups during formaldehyde and glutaraldehyde tissue fixation processes, since this can allow chemical modification of such amino groups that are the main targets of these fixatives and can be reversed later for immunoreactions. Like formaldehyde, glutaraldehyde also reacts mainly with the lysyl residues, and some reaction occurs with tyrosyl, histidyl, and sulfhydryl residues (Habeeb and Hiramoto 1968). It may be that this approach could enable preservation of both ultrastructure and immunoreactivity for successful immunoelectron microscopy.

In summary, we have reported a novel method that can be universally applied for AR and successful IHC in clinical pathology laboratories. Continued efforts to refine methods of antigen preservation or retrieval in tissues fixed with aldehyde-based fixatives will ultimately allow standardization of IHC for such tissues and elimination of inter-laboratory discrepancies in results for accurate clinical and research studies. Further utilization of our method is warranted to incorporate it into routine pathology laboratory practice.

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