Thiol-reactive compounds prevent nonspecific antibody binding in immunohistochemistry

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Nonspecific antibody binding is the primary source of confounding background in immunohistochemistry (IHC). Based on observed patterns of background staining, and the known spontaneous reduction of immunoglobulin disulfide bonds *in vivo* and *in vitro*, we tested the hypothesis that nonspecific antibody binding in IHC is mediated by sulfhydryl interactions. Coincubation of primary antibodies with reduced glutathione (GSH), L-cysteine, iodoacetic acid, Ellman's reagent and other thiophilic reagents in pH 8 tris-EDTA (TE) buffer inhibited background staining. In contrast, oxidized glutathione (GSSG) exerted no effect. When empirically optimized, coincubation of GSH with primary antibodies significantly improved IHC signal:noise ratio. Tissue preincubation with mercaptans, soft and borderline metals, and other sulfhydryl-reactive reagents also inhibited background staining, but at the expense of target sensitivity. ELISA results confirmed direct binding between murine serum antibodies and GSH in a nonantigen-dependent manner. In summary, thiol-reactive compounds prevent nonspecific antibody binding in IHC. We propose a mechanism whereby nonspecific background resulting from formation of disulfide bridges and other sulfhydryl bonds between primary antibodies and tissue side groups is interrupted by prior exposure to thiol-reactive reagents such as GSH. These findings provide a molecular basis to improve the specificity of IHC and other immunoassays, and hold implications for antibody-based clinical diagnostics and therapeutics.
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Immunohistochemistry (IHC) is an important laboratory tool used to demonstrate specific molecules in tissue sections. Unfortunately, nonspecific antibody binding in IHC can produce high background staining, resulting in inconclusive target elucidation that hinders interpretation.[1] Implicated causes of nonantigenic antibody adherence include hydrophobic protein interactions, ionic and electrostatic forces, aldehydes in overfixed specimens, endogenous Fc receptor binding and antigen diffusion.[2] Endogenous biotins and insufficiently quenched tissue enzymes are additional potential sources of inappropriate signaling in IHC.[3–5] Strategies to reduce background include tissue pretreatment with serum from the secondary antibody or an unrelated species, or application of protein-rich solutions containing bovine serum albumin, casein or skim milk.[5] However, these approaches frequently fail to adequately suppress nonspecific staining. Success of IHC depends on many variables such as method of tissue fixation and antigen retrieval, target quantity and availability, avidity of the primary antibody for its cognate antigen, assay kinetics like temperature and fluid dynamics, and visualization strategy. Critically, IHC requires antibody reactivity with antigenic motifs that may be altered or rigidly conformed by fixation, all in a spatially and biochemically complex 3-dimensional environment. For these reasons, primary antibody concentrations used in IHC are necessarily high. Concentrated antibody solutions incubated under static conditions may result in off-target labeling, producing the unwanted background staining familiar to all experienced immunohistochemists. Here, we demonstrate that thiol-reactive compounds can prevent the nonspecific antibody binding that produces background, and describe a technique that significantly improves IHC signal-to-noise ratio.

Materials and methods

Tissue Processing and Histologic Evaluation

From our in-house laboratory rodent colony, tissues from healthy sentinel mice were collected at

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scheduled necropsy, fixed overnight in 10% neutral-buffered formalin or 100% ethanol, and routinely processed and embedded. Additional specimens were collected in OCT compound (Sakura Finetek USA, Torrance, CA, USA), flash frozen in liquid nitrogen, and stored at -70°C. Tissues evaluated included pancreas, liver, kidney, thymus with surrounding mediastinal connective tissues, skin and gastrointestinal tract. In addition to routine hematoxylin and eosin (H&E), selected tissues were stained with Sudan black B or periodic acid-Schiff (PAS; with or without diastase digestion) by routine histologic methods.[6]

Immunohistochemistry

For all IHC experiments we followed a standard operating procedure, varying only the pretreatment of the primary antibody solution or tissue section before the assay. Epitope recovery for formalin-fixed tissues was accomplished by heating slides in pH 6 citrate-buffered Target Retrieval Solution (DAKOCytomation, Carpinteria, CA, USA) in a rice steamer for 20 min followed by benchtop cooling, or by incubating in a commercial trypsin protease digestion solution (DAKO) at 37°C for 20 min. On an automated immunostainer (i6000, Biogenex; San Ramon, CA, USA), deparaffinized and rehydrated tissue sections were circumscribed with a PAP pen and sequentially overlaid with the following reagents: 3% H_2O_2 in PBS $\times 5$ min (repeated), egg white avidin (1 egg white in 100 ml PBS, coarsely filtered)[7] $\times 5$ min, 0.2% biotin (Sigma, St Louis, MO, USA) $\times 5$ min, primary antibody $\times 1$ h, biotinylated species-appropriate secondary antibody (H&L; Sigma) $\times 30$ min, 1:100 streptavidin-peroxidase (DAKO) $\times 10$ min, 3,3'-diaminobenzidine (DAB; DAKO) $\times 5$ min, and Gill's hematoxylin counterstain $\times 1$ min. Slides were washed $5 \times$ with PBS/Tween-20 (Sigma) between steps. For fluorogenic IHC, AlexaFluor 488-conjugated secondary antibodies (Molecular Probes/Invitrogen, Carlsbad, CA, USA) were applied and slides mounted with antifade Vectashield plus DAPI (Vector Laboratories, Burlingame, CA, USA). When mouse primary antibodies were applied to mouse tissues, we used the ARK kit (DAKO). Photomicrographs were taken with a Nikon DXM1200 camera (chromogenic) or Zeiss Axioskop system (fluorogenic). We tested a variety of primary antibody sources including rabbit anti-human CD3 and pan-cytokeratin (DAKO), iNOS (NOS2, Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti-human c-myc (Novocastra, Newcastle-upon-Tyne, UK) and goat anti-*Treponema pallidum* (Novocastra; used on archival rabbit tissue with *T. paraluis-cuniculi*). During initial screening, primary antibody concentrations were increased $2-5 \times$ over empirically determined laboratory optimums in order to assure moderately intense background staining in the absence of blocking.

Tissue and Antibody Blocking

For background blocking experiments, deparaffinized and rehydrated tissues were incubated for 1 h before IHC with a variety of test compounds (all Sigma or Pierce Biotechnology, Rockford, IL, USA) including methyl and ethyl mercaptan (15% v:v in H_2O), iodomethane, formaldehyde (2% v:v) and paraformaldehyde (0.2% v:v), iodoacetic acid and iodoacetamide (0.5 M), N-ethyl maleimide (0.04 M), and the heterobifunctional crosslinking reagent 4-(N-maleimidomethyl) cyclohexanecarboxylic acid N-hydroxysuccinimide ester (SMCC; 3 mM). In another series of experiments, tissues were incubated for 20 min with log dilutions (ranging from ~ 8 nM to 40 mM) of metal chloride salts solubilized in ethanol. These included cobalt(II), copper(II), iron(II) and (III), lead(II), and mercury(II) chloride. For antibody blocking studies, test reagents (3–90 mM) were coincubated with primary antibodies for 1 h on ice. Compounds evaluated (all Sigma) included reduced glutathione (GSH) and oxidized glutathione (GSSG), L-cysteine, n-acetyl-L-cysteine, iodoacetic acid, and 5,5-dithiobis(2-nitrobenzoic acid) (DTNB; Ellman's reagent). Buffers used for antibody dilution and blocking reagent coincubation were phosphate-buffered saline (PBS; pH 7.4) and 10 mM tris-HCl pH 8, 1 mM ethylenediaminetetraacetic acid ($1 \times$ tris-EDTA; TE buffer). Buffer pH was verified with a meter and adjusted as needed before use.

Glutathione ELISA

In order to evaluate antibody behavior directly in a sulfhydryl-rich compartment, we added to a standard in-house murine parvovirus (MPV) enzyme-linked immunosorbent assay (ELISA) microplate wells coated only with GSH (Sigma). Sera from mice in our surveillance colony, and from known positive and negative controls, were diluted 1:800 and applied to wells coated with MPV antigens or GSH only. Additional wells were treated with fetal calf serum (FCS) or PBS in place of murine serum. After primary incubation and washing, wells were incubated sequentially with biotinylated monoclonal anti-mouse immunoglobulin (BD Pharmingen, San Diego, CA, USA), extravidin-peroxidase (Sigma), and 2,2-azino-bis (3-ethylbenzothiazoline-6-sulfunoic acid) diammonium salt (ABTS substrate, KPL, Gaithersburg, MD, USA) for color development. Optical density (OD) development at 405/592λ was recorded by an ELISA plate reader (Dynatech MR7000, Dynatech Laboratories Inc., Chantilly, VA, USA). Results were reported as mean absorbance from duplicate measurements.

Results

Tissue Fixation and Histologic Evaluation

We first reviewed the effects of tissue fixation on the intensity of IHC target labeling and nonspecific background staining. In agreement with prior experience, we found that the degree of background positively correlated with target sensitivity (not shown). The highest level of both specific and nonspecific staining was evident in frozen sections, followed in decreasing order by ethanol-fixed tissues and antigen-retrieved FFPE sections. In the absence of antigen retrieval, staining for most antigens (c-myc being a notable exception) was weak or absent in FFPE tissues. In order to generate hypotheses regarding potential sources of nonantigenic antibody adherence, we inventoried specific cellular sites of spurious labeling in our test battery of tissues. Irrelevant antibody binding occurred repeatedly within specific cells and subcellular cytoplasmic compartments. Exocrine pancreatic acinar cells exhibited very high nonspecific antibody binding properties, with signal concentrated in the protein-rich (eosinophilic) outer portion of the cytoplasm. In the liver and kidney, nonspecific labeling of cytoplasmic granules (coalescing into a diffuse pattern at higher antibody concentrations) was heaviest in periportal hepatocytes and proximal convoluted tubules, respectively. In the gastrointestinal tract, nonspecific labeling was most evident in gastric chief and parietal cells, surface epithelial enterocytes, myenteric ganglion neurons, and as fine stippling in the sarcoplasm of smooth muscle cells. In fatty connective tissues, inappropriate staining of adipocytes was concentrated in the thinly marginated cytoplasm rimming the large central fat vacuole. Adipocyte staining persisted even when other sources of background were alleviated by antibody dilution (not shown). Nonspecific labeling of collagen-rich connective fibers and other tissue elements became apparent at higher antibody concentrations. Background was lost when primary antibody was omitted from our protocol, demonstrating this to be the source of nonspecific staining and not secondary antibody, endogenous biotins or unquenched tissue enzymes.

Special Stains

Based on cellular and subcellular nonantigen-mediated tissue staining patterns, we postulated that enzymes or enzyme products might account for nonantigen-mediated antibody adherence. In cells with high oxidative enzyme activity, accumulated lysosomal breakdown products, including lipofuscin, represented one potential source of antibody binding. In order to determine whether lipofuscins contributed to IHC background, we stained tissues with Sudan black B. However, there was no overlap between Sudan black and the IHC chromogen DAB,

nor did Sudan black appreciably reduce immunohistochemical background. To test whether glycogen and other carbohydrate moieties could be involved in indiscriminate antibody binding, we stained tissues by PAS with or without alpha-amylase (diastase; Sigma) digestion. Amylase digestion eliminated PAS staining in control liver sections, but had no effect on IHC background (not shown). We discounted mucins as a likely source of antibody binding because mucin-rich cells such as gastric foveolar epithelial cells and intestinal goblet cells were not common sites of inappropriate staining. Likewise, lipids were not considered molecular targets for nonspecific antibody binding because the organic solvents used for routine processing removed most hydrophobic molecules from tissue sections (confirmed by the loss of oil red O staining; not shown). Taking these observations together, we concluded that lipofuscins, glycogen, mucins and lipids represented unlikely sources of nonspecific antibody adherence in IHC.

Tissue Blocking

We next considered as potential antibody binding targets tissue enzymes and their associated factors and substrates. Specifically, we wondered whether sulfhydryl and other reactive chemical side groups could be forming thiol linkages with exposed SH groups on spontaneously reduced antibody molecules.[8–10] To test this possibility, we chemically inactivated functional side groups in tissue sections. Using parameters described previously, nonspecific background was abolished or significantly diminished by tissue pretreatment with methyl and ethyl mercaptan, formaldehyde and paraformaldehyde, iodoacetic acid, iodomethane, and the heterobifunctional crosslinking reagent SMCC. However, tissue pretreatment significantly impaired or abolished target signal along with background. Moreover, caustic reagents such as the mercaptans produced tissue damage that impaired morphologic detail. Under our experimental conditions, tissue pretreatment with iodoacetamide and *N*-ethyl maleimide did not alter background staining.

To more specifically address the role of sulfhydryl groups in nonspecific antibody binding, we preincubated tissues with ethanol-solubilized soft, borderline and hard metal chloride salts. Soft metals are highly thiophilic, whereas borderline metals are intermediate and hard metals do not bind sulfhydryl groups.[11] By our methods, the thiophilic soft metals Hg(II) and Pb(II) eliminated background at very low concentrations ($\leq 8\,nM$), whereas the borderline metals Fe(II), Co(II), Cu(II), and Zn(II) required higher concentrations ($\geq 8\,mM$), and the hard metal Fe(III) exerted no effect at any tested concentration. Specific target signal was weak following treatment with Fe(II) and lost with the other divalent metal cations at concentrations that reduced background

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Table 1 Effect of GSH concentration on target and background staining in tissues fixed by different methods

(GSH) (mM)	FFPE		Frozen and EtOH	
	Target (signal)	Background (noise)	Target (signal)	Background (noise)
0	+++	++	++++	+++
0.6	+++	++	++++	+++
3	+++	+/−	++++	+++
6	++	−	++++	+++
9	+	−	++++	+++
30	−	−	+++	+/−
60	−	−	+++	+/−
90	−	−	++	+/−
300	−	+	−	+

IHC target and background labeling in formalin-fixed paraffin-embedded (FFPE) *vs* frozen and ethanol (EtOH) fixed tissues following coincubation of primary antibodies with reduced glutathione (GSH) in pH 8 tris-EDTA buffer on ice for 1 h.
Table represents a composite of results; determination of optimal GSH concentrations required empirical determination for each IHC protocol.

(not shown). Taken together, these results strongly implicated thiol groups as important mediators of nonspecific antibody binding in IHC. As tissue pretreatment impaired target signal at concentrations that inhibited background, we discounted the utility of this approach with the above compounds as a practical IHC blocking strategy.

Antibody Blocking

Because tissue pretreatment negatively impacted target sensitivity, we next evaluated supplementation of thiol-reactive reagents directly into the primary antibody solution. GSH, L-cysteine, N-acetyl-L-cysteine, iodoacetic acid, and 5,5-dithio-bis(2-nitrobenzoic acid) (DTNB; Ellman's reagent) in the range of 3–90 mM inhibited or abolished non-specific background staining when coincubated with primary antibodies in pH 8 TE buffer (Table 1). Encouragingly, target signal was at least partially retained with all of these compounds. Coincubation with GSSG exerted no effect staining patterns, highlighting the importance of free sulfhydryl groups for blocking activity. For reasons not immediately clear, pH 7.4 PBS proved significantly inferior to pH 8 TE buffer as a coincubation medium.

In order to validate the practicality of this blocking approach, we performed a series of empirical optimization experiments using GSH as our model compound. We coincubated antibodies with GSH in concentrations ranging from 0.6 to 300 mM in TE buffer on ice for 1 h. Concentrations of GSH in the range of 30–90 mM usually were required to prevent nonspecific background in frozen and ethanol-fixed sections (Table 1). In contrast, concentrations in the range of 3–6 mM proved efficacious for blocking background in most FFPE tissues without losing target signal, presumably reflecting the lower inherent background staining in FFPE *vs* frozen and alcohol-fixed tissues. Suboptimal concentrations of GSH resulted in diminished

blocking effectiveness, whereas too high concentrations eliminated target signal along with background (Table 1). Very high concentrations of GSH (≥90 mM) sometimes produced tissue background staining with an altered pattern (eg goblet cell mucous vacuoles and lymphocytes). In a time course series, we found that incubation on ice for up to 4 h provided no significant benefit over a 1 h incubation, and that incubations for >48 h at 4°C resulted in loss of target signal and a return of high background (not shown), possibly due to excessive antibody reduction by GSH. Incubation times could be shortened to <1 h when performed at room temperature, but because of temperature fluctuations in our laboratory we encountered significant interassay variation. When optimized, GSH coincubation with primary antibodies could produce dramatic reductions in nonspecific background staining with excellent retention of target signal (ie increased signal:noise ratio; Figure 1a). Importantly, GSH did not interrupt labeling of mouse immunoglobulins by biotinylated anti-mouse IgG FAb in solution (Figure 1b), thus permitting use of this blocking strategy in mouse-on-mouse IHC protocols such as the ARK kit (DAKO). GSH blocking worked equally as well in fluorogenic applications (Figure 1c) as in chromogenic, and blocked nonspecific binding of primary mouse monoclonal (Figure 1b), rabbit polyclonal (Figure 1a and c) and goat polyclonal (not shown) antibodies. However, empirical determination of optimal GSH concentration was required for each IHC protocol in order to achieve maximal blocking without a loss of target signal.

To confirm the role of sulfhydryl interactions in nonspecific antibody binding, we partially reduced primary antibody solutions with 20 mM dithiothreitol (DTT) in PBS for 30 min, followed by spin-column concentration and resuspension in TE buffer. DTT pretreatment increased both specific and nonspecific tissue labeling (not shown). We attributed this to the liberation of aggregated immunoglobulins joined together in solution by
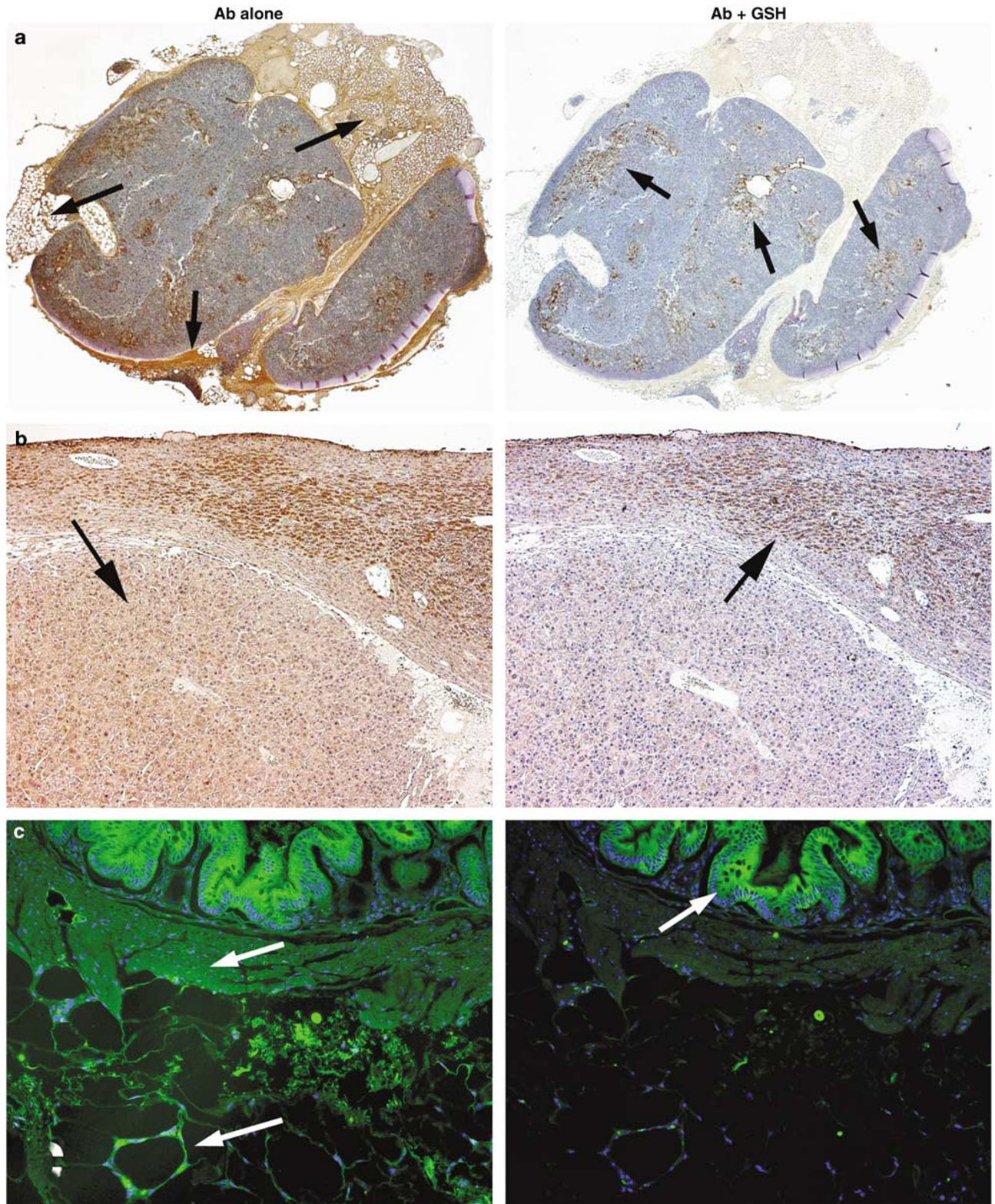
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Ab alone   Ab + GSH

Figure 1 Coincubation of reduced glutathione (GSH) with primary antibodies improves IHC signal:noise ratio. (**a**) Pan-cytokeratin, ethanol-fixed mouse thymus. Left: Perithymic mediastinal tissues (arrows) exhibit profound nonspecific background staining (brown). Right: GSH abolishes nonspecific background while retaining thymic epithelial cell labeling (arrows). (**b**) c-myc, formalin-fixed paraffin-embedded *alb-c-myc* transgenic mouse liver.[22] Left: Staining diffusely present in hepatocytes, including in tumor nodule (arrow). Right: GSH coincubation reveals that c-myc from the albumin-promoted transgene is concentrated in nontransformed hepatocytes (arrow). (**c**) Pan-cytokeratin, ethanol-fixed mouse colon. Left: Nonspecific fluorescent signal (green) in colon wall and mesenteric adipocytes (arrows). Right: GSH coincubation enhances specific fluorescent visualization of colonic epithelium (arrow).
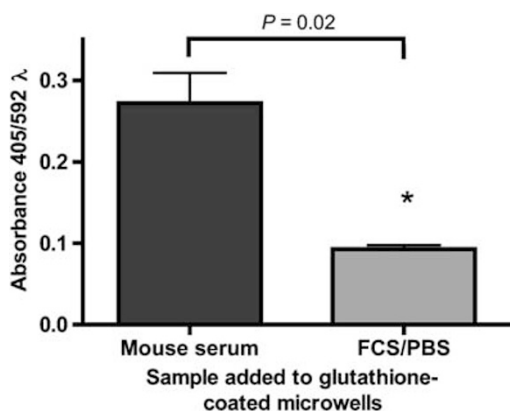
Figure 2 Murine serum antibodies are bound *in vitro* by GSH in a nonantigen-dependent manner. When run in tandem with a murine parvovirus (MPV) ELISA, microwells coated only with reduced glutathione (GSH) bound a significant fraction of murine serum antibodies (left column). Absorbance was weak in negative control wells exposed to fetal calf serum (FCS) or physiologic-buffered saline (PBS) in the first step (right column). Mean absorbance for GSH-coated microwells was only marginally lower than MPV positive control wells and significantly higher than negative control wells (not shown). *$P < 0.05$.

disulfide bonds. Specific target labeling was lost when DTT-reduced antibody mixtures were subsequently treated with GSH, possibly due to prevention of intrachain disulfide bond reformation and a return to the native folded state. Taken together, the DTT experiments provided further evidence that sulfhydryl groups are key mediators of nonspecific antibody binding in IHC.

Glutathione ELISA

To more directly evaluate antibody behavior in a sulfhydryl-rich compartment, we included microwells coated only with GSH (Sigma) in an ELISA-based serologic screen for MPV performed routinely in our animal diagnostic laboratory. Using parameters described above, the mean OD for GSH-coated wells exposed to normal murine serum was 0.27 (± 0.04), whereas the mean absorbance for wells where mouse serum was replaced by FCS or PBS was 0.09 (± 0.01; $P = 0.02$, two-tailed unpaired t test, $n = 16$; Figure 2). The mean absorbance for the GSH-coated wells alone met the positive cutoff value for this assay (0.2), and was three times the level of the negative MPV controls run in tandem (0.09; not shown). Thus, under static ELISA incubation conditions, GSH directly bound a significant fraction of murine serum antibodies in the absence of any specific antigen.

Discussion

In this report, we show that thiol-reactive compounds block nonspecific antibody binding, and describe a strategy to improve IHC signal-to-noise ratio. Coincubation of primary antibody solutions with GSH in the range of 3–90 mM (1–30 mg/ml) on ice for 1 h in pH 8 TE buffer can inhibit background staining with minimal impact on target labeling depending on tissue fixation and the IHC assay in question. We found that the lower end of this GSH concentration range generally worked well for FFPE tissues, whereas higher GSH concentrations were required for frozen and alcohol-fixed sections. Nevertheless, we encountered significant exceptions to this rule (eg high background in FFPE liver stained for c-myc). Thus, empirical optimization of GSH concentration by titrational series was required for each IHC protocol.

Our results implicate sulfhydryl interactions such as disulfide bridging (Figure 3) as an important mechanism of nonspecific antibody binding, although we did not test this question directly. The cysteine sulfhydryl group represents one of the most nucleophilic functional side chains found in proteins.[12] The disulfide bridge is the most labile covalent bond commonly found within and between proteins, and dynamic breaks and linkages occur spontaneously.[9] In the completely folded state, immunoglobulin cysteine residues participate in inter- or intrachain disulfide bonds. However, it is not uncommon to detect anywhere from 0.2 to 1 mole of free SH per mole immunoglobulin *in vivo* and *in vitro*.[8,12,13] Noncovalent mechanisms help stabilize the 3-dimensional structure of antibody molecules with reduced half-cystine residues.[13] Moreover, some immunoglobulin subtypes contain an odd number of cysteine residues, and therefore at least one SH group uninvolved in internal bridging.[10,14] Human IgG2 contains an unbridged cysteine SH group, and human IgG1 and rat IgG2b have S–S bonds particularly vulnerable to nucleophilic attack.[15,16] Rabbit immunoglobulins contain more disulfide bonds than mouse antibodies, and may be at greater risk for generation of reduced SH groups.[13] Moreover, when whole rabbit serum is used in immunoassays, nonspecific interactions between IgA and substrate may occur because of labile disulfide bridges linking IgA to secretory component.[17] Disulfide bridge formation has been shown to occur between IgG of multiple species and human C1q under physiologic conditions *in vitro*.[18] Additionally, antibody sulfhydryl groups may react with protein carbonyl moieties by Michael's addition.[19] Capping of reactive thiol groups and protection from nucleophilic degradation may explain why bovine serum albumin and other proteins added to commercial antibody solutions prolong their shelf life.

Tissue fixation is a critical determinant of IHC outcomes. Formalin fixation without subsequent antigen retrieval severely limits the number of antigenic targets detected by IHC. Concomitantly, FFPE tissues not subjected to antigen unmasking exhibit very low background staining when compared with ethanol-fixed and frozen sections.
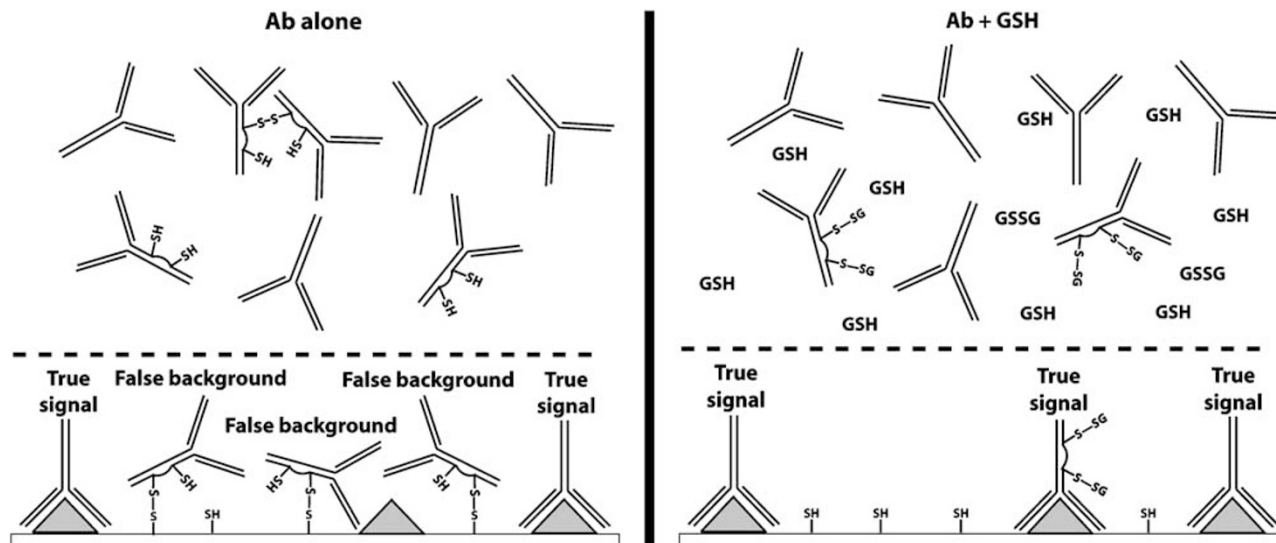
Figure 3 Proposed mechanism for thiol-mediated nonspecific antibody (Ab) binding and its inhibition by GSH. Left: Reduced intrachain S–S bonds liberate SH groups that can form disulfide bridges with other antibodies and tissue sulfhydryls. Right: GSH binds antibody SH residues, preventing unwanted disulfide bridges with substrate thiols.

The return of background in formalin-fixed tissues increases in direct proportion to the robustness of the antigen retrieval method.[20] Formaldehyde fixes tissues in part by forming methylene bridges between reactive ε-amino groups on lysine residues. However, formaldehyde also reacts with other chemical functional groups including thiols.[21] We postulate that the low level of inherent background staining in formalin-fixed tissues is attributable to the inactivation of endogenous thiols. However, the empirical experiments described in the present work were strictly performance-based. Directed chemical studies will be needed to identify the precise mechanisms by which antibodies react with non-target molecules in IHC and other immunoassays.

In summary, we report that thiol-reactive compounds prevent nonspecific antibody binding and background staining in IHC. We describe a technique, based on the supplementation of GSH and related reagents to primary antibodies in appropriate buffer, that can significantly improve assay specificity with minimal impact on sensitivity. The proposed mechanism is based on the inhibition of intermolecular disulfide bridge formation and/or other thiol linkages between antibodies and substrate chemical side groups. In support of this hypothesis, we have shown that tissue preincubation with thiophilic but not nonthiophilic metals inhibits background, that antibody treatment with GSH but not GSSG prevents nonspecific labeling, and by ELISA that murine serum antibodies bind glutathione directly in a nonantigen-dependent manner. Our thiol-based blocking strategy provides a new tool to address the problem of background staining in IHC, and to extend the technique to applications where traditional signal-to-noise ratios are unacceptably low. The presumptive identification of thiol interactions as the primary source of nonspecific binding in IHC opens avenues to improve the specificity of other immunoassays, and has implications for antibody-based clinical diagnostics and therapeutics.

Acknowledgements

Duality of interest

US patent application submitted (ABR).

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