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Immunohistochemical Artifact for Nitrotyrosine in Eosinophils or Eosinophil Containing Tissue

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Immunohistochemical artifacts for nitrotyrosine were investigated in eosinophils with regard to fixatives. Immunoreactivity for nitrotyrosine was revealed in separated eosinophils and in gastric mucosa fixed with periodate, lysine-paraformaldehyde (PLP). The increase in immunoreactivity by PLP was due to periodate itself, a component of PLP. Nitrotyrosine formed by peroxidase using NO_2^- and H_2O_2 or by peroxynitrite was not completely inhibited by 100 mM dithionite but the immunoreactivity for nitrotyrosine antibodies by PLP was completely inhibited by 5.7 mM dithionite. Although untreated eosinophils or ovalbumin (OVA) did not show protein tyrosine nitration in a standard Western blot, the treatment of the blotted membrane with PLP increased the reactivities of proteins from eosinophils with anti-nitrotyrosine antibodies. The increase in immunoreactivity of OVA with anti-nitrotyrosine antibodies by PLP did not change with pre-treatment with dithionite but was abolished by treatment with dithionite after PLP fixation. In HPLC assays, periodate did not generate nitrotyrosine from L-tyrosine and aminotyrosine. These results suggest that the treatment of eosinophils or eosinophil-containing tissues with PLP fixative augments the immunoreactivity of nitrotyrosine antibodies with eosinophils due to the formation of epitopes similar to nitrotyrosine by an oxidation reaction of periodate, which evokes an artifact in nitrotyrosine immunohistochemistry.

Keywords: Nitrotyrosine; Immunohistochemistry; Artifact; Eosinophils; PLP fixative

INTRODUCTION

Nitric oxide (NO) is produced by constitutive and inducible nitric-oxide synthase and has a variety of functions such as regulation of vascular tone, neurotransmission and modulation of inflammatory processes.[1] During inflammatory conditions, a rise in NO derived from NO synthase results in microbial toxicity and host injury.[2] NO reacts at a near diffusion-limited rate with $O_{2.}^-$ to yield peroxynitrite ($ONOO^-$), a powerful oxidizing species, and many toxic properties of NO have been attributed to the formation of $ONOO^-$.[2] In a metal-catalyzed reaction, $ONOO^-$ causes nitration of phenolic compounds, such as free and protein-bound tyrosine.[3–5] Nitration of specific tyrosine residues has been reported to affect protein structure and function,[6] suggesting that 3-nitrotyrosine formation may not only be a specific marker for the presence of $ONOO^-$ *in vivo*[6] but could be causally involved in the pathogenesis of several diseases such as neurodegenerative disease,[7] atherosclerosis,[8] allergic asthma[9,10] and inflammatory gastro-intestinal diseases.[11,12]

Recent studies have revealed an alternative mechanism of tyrosine nitration with potential *in vivo* relevance.[13,14] It has been shown that heme peroxidases such as myeloperoxidase or eosinophil peroxidase can nitrate protein tyrosine residues and other phenolic compounds.[15–17] High levels of nitrotyrosine in alveolar lavage fluid from human asthma patients was diagnostic of leukocyte infiltration of the lung in asthma.[10] In lipopolysaccharide-activated murine macrophages, protein tyrosine nitration was due to a nitrite-dependent peroxidase reaction rather than to peroxynitrite.[18]

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Immunohistochemical detection of protein-bound tyrosine nitration was evaluated in various diseases.[13,14] However, some studies have failed to detect nitrotyrosine in atherosclerotic lesions by HPLC.[19,20] Moreover, there were some discrepancies in the detection of nitrated protein by different methods.[14] As mentioned above, inflammatory cells such as neutrophils, eosinophils and macrophages are crucial candidates to generate reactive nitrogen species responsible for nitrotyrosine. However, there was no agreement on immunostaining for nitrotyrosine in these cells. Therefore, this study was designed to investigate the appropriate fixatives for nitrotyrosine immunohistochemistry using peroxidase-catalyzed tyrosine nitration of human eosinophils or tissue containing eosinophils.

MATERIALS AND METHODS

Chemicals

Dextran T-500 and Percoll were purchased from Pharmacia Biotec. Phorbol myristate acetate (PMA), bovine serum albumin (BSA), 3-aminotyrosine and ovalbumin (OVA) were obtained from Sigma. Catalase was obtained from Boehringer Mannheim GmbH. Anti-CD16-coated magnetic beads were obtained from Milteny Biotec. Diethylenetriaminepentaacetic acid (DTPA) was obtained from Dojindo. A rabbit polyclonal anti-nitrotyrosine was purchased from Upstate Biotechnology. Peroxynitrite, synthesized in a quenched flow reactor and with contaminating H_2O_2 removed by manganese dioxide, was purchased from Dojindo. 3-Nitrotyrosine was obtained from Aldrich Chemical Co. and superoxide dismutase (SOD) was purchased from Wako Pure Chemicals. All other reagents were of the highest purity commercially available.

Eosinophil Preparation

Human eosinophils were obtained from fresh, citrate-treated whole blood drawn from healthy volunteers. Informed consent was obtained from the volunteers after explaining the purpose and methods of this study. After removal of erythrocytes by Dextran sedimentation (4.5% Dextran T-500), leukocytes were isolated by Percoll density gradient ($\sim$1.080 g/l) at room temperature. After hypotonic lysis of residual erythrocytes from the collected granulocyte layer, neutrophils were removed by the magnetic bead selection system using anti-CD16-coated magnetic beads. Neutrophils bound to CD16 antibodies coupled to the beads were removed using a magnetic cell separation system (Vario MACS, Miltenyi Biotech GmbH). Pure eosinophils (purity $>$99%) were obtained by negative selection, because eosinophils are CD16-negative, as they passed through a column, and were suspended in HBSS.

Gastric Mucosal Specimens

Gastric mucosal samples were collected from surgical resection. The normal tissue was obtained from uninvolved stomach regions of five patients with gastric cancer. Samples were embedded in optimal cutting temperature (OCT) compound (Miles Inc.) and rapidly frozen in liquid nitrogen.

Immunocyto- or Immunohistochemistry for Nitrotyrosine

Cytocentrifuged slides of separated eosinophils and neutrophils (98% purity) or cryosections of gastric mucosa were fixed in ice-cold methanol for 5 min, ice-cold acetone for 5 min, 10% neutral-buffered formalin for 1 h, 4% paraformaldehyde for 1 h, Bouin's for 1 h, 4% glutaraldehyde for 1 h, Carnoy's for 1 h or periodate lysine-paraformaldehyde (PLP)[21] for 20 min or 1 h. Some slides were treated with periodate (10 mM) for 20 min and then fixed with neutral-buffered formalin for 20 min. Paraformaldehyde, glutaraldehyde and periodate were dissolved in 0.1 M phosphate buffered saline, pH 7.4. Some slides were incubated with a mixture of either 1 μM NO_2^- and 1 μM H_2O_2 or 5 μM NO_2^- and 5 μM H_2O_2 or 10 mM peroxynitrite in PBS containing 0.1 mM diethylenepentaacetic acid (DTPA) at 37°C for 30 min. To show the contribution of eosinophil peroxidase in tyrosine nitration, sodium azide (1 mM) was added to the incubation mixture of NO_2^- and H_2O_2. After incubation, slides were fixed with 10% neutral-buffered formalin. All fixed slides were washed with PBS containing 0.05% Tween 20 for 5 min, and then treated with 3% H_2O_2 in methanol for 20 min to inactivate endogenous peroxidase. After washing three times with PBS for 20 min, the slides were mounted with 2% normal goat serum in PBS to inhibit non-specific immunoreactions. Polyclonal antibody against nitrotyrosine was diluted 800-fold with PBS, and layered on the slides overnight at 4°C. The slides were washed three times with PBS and incubated with peroxidase polymer-conjugated Envision plus (Dako) for 30 min at room temperature. After washing three times with PBS, peroxidase activity was localized using 3,3′-diaminobenzidine tetrahydrochloride and 0.01% H_2O_2, and counterstained with hematoxylin. The slides of gastric mucosa were double-stained with Luxol-fast-blue to confirm the specificity of eosinophils.[22] The specificity of anti-nitrotyrosine antibodies was confirmed by their pre-incubation

with 10 mM 3-nitrotyrosine for 2 h and by incubation of slides with 5.7 or 100 mM sodium dithionite for 1 h after fixation or tyrosine nitration to reduce nitrotyrosine to aminotyrosine. A negative control study was performed using non-immune rabbit immunoglobulin. Microscopic observations were performed using an Olympus IX-70 light microscope.

Sodium Dodecyl Sulfate-polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

Eosinophils (5×10^5 or 1.5×10^6) were incubated with 1 μM PMA, 0–50 μM NO_2^-, 50 μg/ml SOD, and 30 μg/ml catalase, or a combination of these reagents in 500 μl of HBSS at 37°C for 30 min. Cells were immediately centrifuged. The cell pellets, 1 or 10 mg/ml BSA and 1 or 10 mg/ml OVA were added with non-reducing Laemmli sample buffer, then boiled for 5 min. Dithionite (final concentration, 5.7 mM) was added to a part of the samples dissolved in Laemmli buffer for 2 h. All these samples were subjected to 4–20% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked by overnight incubation at 4°C with 3% nonfat dry milk in 20 mM Tris HCl, pH 7.7, containing 137 mM NaCl and 0.1% Tween 20 (buffer A). The membranes were washed twice with buffer A for 5 min. Prior to washing, some membranes were incubated with PLP fixative for 1 h or ice-cold methanol for 5 min or 10% neutral buffered formalin for 20 min or 4% paraformaldehyde for 20 min. A PLP-fixed membrane was treated with 5.7 mM dithionite for 1 h. All fixed membranes were washed and then reacted with a polyclonal antibody (1:1000 dilution) in buffer A containing 1.5% nonfat dry milk for 2 h. After washing three times with buffer A for 10 min, the membranes were incubated with a horseradish peroxidase (HRP)-labeled anti-mouse IgG, which was diluted 2000-fold in buffer A containing 1.5% nonfat dry milk. The membranes were washed, and peroxidase was detected with enhanced chemiluminescence Western blot analysis (NEN Life Science Products). The specificity of nitrated proteins was evaluated by pre-incubation of an anti-nitrotyrosine antibody with 10 mM 3-nitrotyrosine for 2 h.

HPLC Assay

L-Tyrosine (1 mM) or 3-aminotyrosine (1 mM) was incubated with PLP fixative for 1 h or with 10 mM sodium periodate for 1 h in PBS. After incubation, the formation of nitrotyrosine was analyzed by HPLC using 5 μm Spherisorb ODS-2RP-18 column, with 93% of 50 mM potassium phosphate (pH 3.0), and 7% methanol as the mobile phase at 1 ml/min, and UV detection at 274 nm.[23]

RESULTS

Immunocytochemical Study of Eosinophils

Immunoreactivity for nitrotyrosine was detected in cytoplasm, cell membrane, granules, and the nucleus of eosinophils fixed with PLP (Fig. 1A). Nitrotyrosine immunostaining of PLP fixed-eosinophils was abolished by pre-incubation of anti-nitrotyrosine antibodies with 3-nitrotyrosine (Fig. 1B). Cytocentrifuged eosinophils fixed with ice-cold methanol and incubated with 1 μM NO_2^- and 1 μM H_2O_2 showed localized nitrotyrosine immunostaining in marginal areas of the cells (Fig. 1C). The nitrotyrosine immunoreactivity induced by NO_2^- and H_2O_2 was inhibited by the addition of sodium azide (Fig. 1D). The staining for nitrotyrosine incubated with NO_2^- and H_2O_2 was not detected in eosinophil slides reacted with anti-nitrotyrosine antibodies pre-incubated with 3-nitrotyrosine. A control study using non-immune rabbit immunoglobulin did not show the immunostaining.

Immunoreactivity with anti-nitrotyrosine antibodies was compared with several fixatives and periodate, a component of PLP fixative. Immunoreactivity with anti-nitrotyrosine antibody was not observed in fixatives such as ice-cold methanol (Fig. 2A), ice-cold acetone (Fig. 2B), neutral-buffered formalin (Fig. 2C), Bouin's (Fig. 2E) or Carnoy's (Fig. 2G). Paraformaldehyde and glutaraldehyde did not show the immunoreactivity when fixation time was 20 min. However, when fixation time was increased to 1 h in these fixations, a trace of

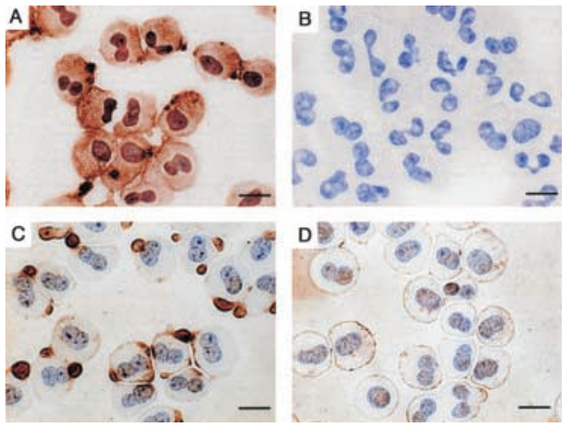

FIGURE 1 Immunocytochemistry of nitrotyrosine in cytocentrifuged eosinophils. Peroxidase staining for nitrotyrosine was detected in the cytoplasm, granules and nucleus in eosinophils fixed with periodate-lysine-paraformaldehyde (PLP) (A). With preincubation of anti-nitrotyrosine antibodies with 3-nitrotyrosine, the nitrotyrosine immunoreactivity was abolished (B). When cytocentrifuged eosinophils were incubated with 1 μM NO_2^- and 1 μM H_2O_2, localized immunostaining of nitrotyrosine was detected in the marginal area of eosinophils (C). The nitrotyrosine immunostaining was inhibited by the addition of 1 mM sodium azide (D). The bar length is 10 μM.

immunoreactivity was detected (Fig. 2D,F). The treatment of eosinophils with periodate, a component of PLP fixative, showed almost a similar immunoreactivity as that with the PLP fixative (Fig. 2H).

The inhibition of immunoreactivity with anti-nitrotyrosine antibodies by dithionite was compared with tyrosine nitrated eosinophils with 5 μM NO_2^- and 5 μM H_2O_2, peroxynitrite or that treated with PLP fixative. Tyrosine nitrated eosinophils by NO_2^- and H_2O_2 or peroxynitrite was partially but not completely inhibited by a high concentration of dithionite (100 mM) (Fig. 3B,D), respectively. However, the immunoreactivity of eosinophils fixed with PLP with anti-nitrotyrosine antibodies was completely inhibited by a low concentration of dithionite (5.7 mM) (Fig. 3F).

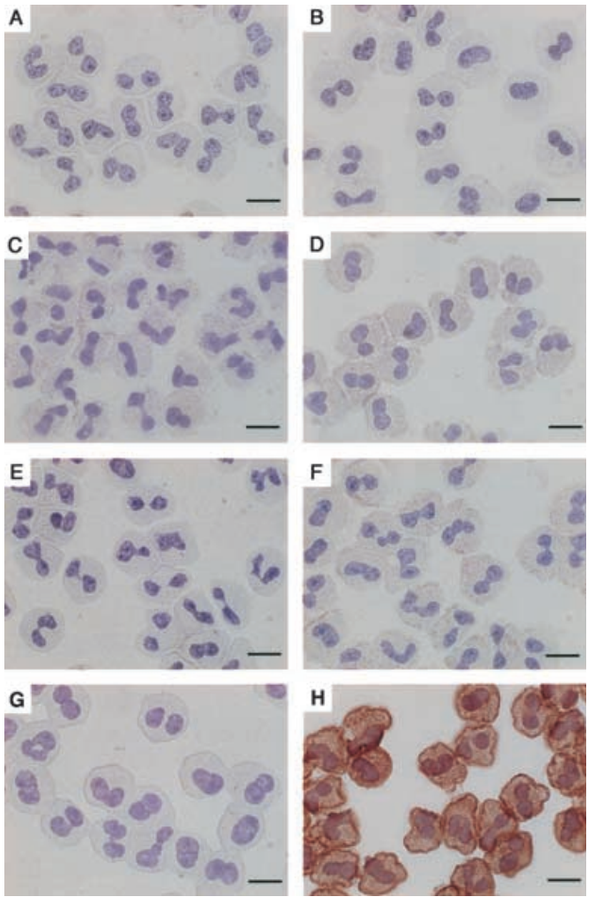

FIGURE 2 Immunocytochemistry of nitrotyrosine in cytocentrifuged eosinophils treated with various fixatives. Eosinophils fixed with ice-cold methanol (A), ice-cold acetone (B), 10% neutral-buffered formalin (C), Bouin's (E), and Carnoy's (G) did not show the immunoreactivity with anti-nitrotyrosine antibodies. However, paraformaldehyde and glutaraldehyde showed trace levels of immunoreactivity, respectively (D, F). Treatment with periodate before fixation with neutral-buffered formalin showed high immunostaining (H) and the staining pattern resembled the immunostaining with PLP fixative. The bar length is 10 μM.

Immunohistochemical Study of Gastric Mucosa

Immunostaining for nitrotyrosine was evaluated in the specimens of human gastric mucosa because it contains eosinophils in its tissue. Nitrotyrosine immunoreactivity was detected mainly in the cells of the basal lamina propria of the mucosal layer by fixation with PLP (Fig. 4A) but was not detected in the gastric mucosa fixed with ice-cold methanol, neutral buffered formalin and paraformaldehyde. Nitrotyrosine immunoreactive cells in PLP-fixed gastric mucosa were co-stained with Luxol-fast blue. Gastric mucosa incubated with 1 μM NO_2^- and 1 μM H_2O_2 showed the staining for nitrotyrosine in similar portions of the gastric mucosa as PLP-fixed specimens (Fig. 4B). The immunostaining cells for nitrotyrosine incubated with NO_2^- and H_2O_2 were stained with blue pigment of Luxol-fast blue (Fig. 4C). The immunoreactivity for nitrotyrosine of gastric mucosa fixed with PLP or incubated with NO_2^- and H_2O_2 was not detected in the gastric mucosal specimens treated with dithionite prior to incubating with anti-nitrotyrosine antibodies or using pre-incubated nitrotyrosine antibodies with 3-nitrotyrosine

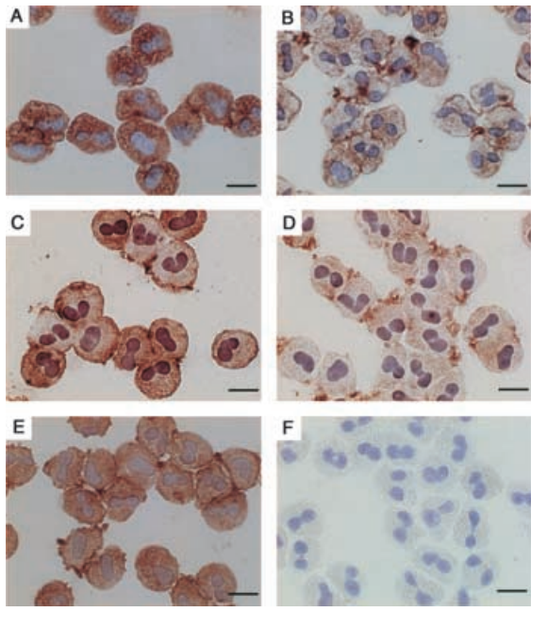

FIGURE 3 Effect of dithionite on immunocytochemistry of nitrotyrosine in cytocentrifuged eosinophils. Immunoreactivity for nitrotyrosine in eosinophils treated with 5 μM NO_2^- and 5 μM H_2O_2 (A) was partially inhibited by treatment with 100 mM of dithionite (B). The immunostaining of nitrotyrosine in eosinophils fixed with neutral buffered formalin for 5 min and incubated with 10 mM peroxynitrite (C) was partially inhibited by treatment with 100 mM dithionite (D). The immunostaining with anti-nitrotyrosine antibodies in PLP-fixed eosinophils (E) was completely inhibited by treatment with 5.7 mM dithionite (F). The bar length is 10 μM.

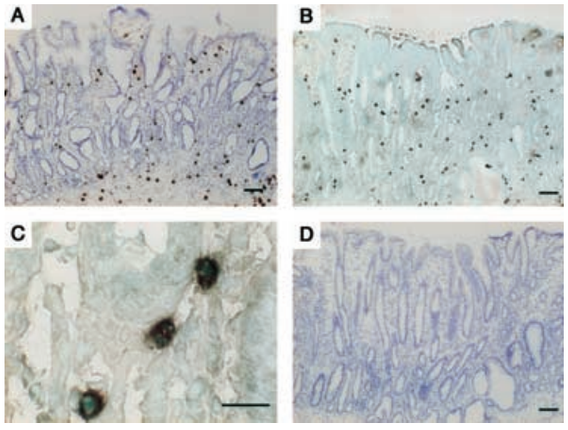

FIGURE 4 Immunohistochemistry of nitrotyrosine in human gastric mucosa. Nitrotyrosine immunoreactivity was detected in many cells from the basal lamina propria of the gastric mucosa fixed with PLP (A). Gastric mucosa incubated with 1 μM NO_2^- and 1 μM H_2O_2 shows nitrotyrosine immunoreactivity in cells of basal lamina propria from the gastric mucosa (B). The immunostaining cells for nitrotyrosine incubated with NO_2^- and H_2O_2 were co-stained with Luxol-fast blue (C). The immunostaining for nitrotyrosine incubated with NO_2^- and H_2O_2 was inhibited by pre-incubation of nitrotyrosine antibodies with 10 mM 3-nitrotyrosine (D). The bar length is 10 μM.

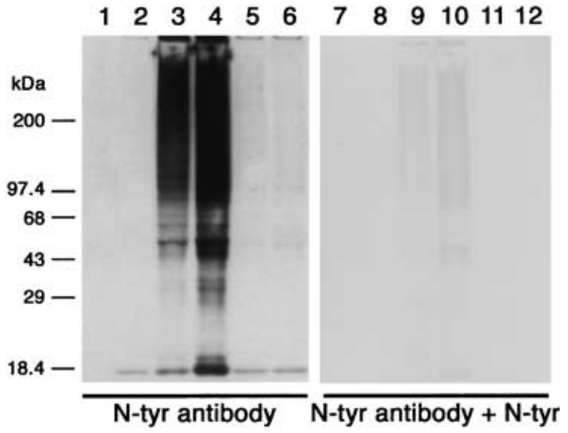

FIGURE 5 Immunoblot analysis of protein-bound nitrotyrosine in eosinophils. Lane 1 shows non-treated eosinophils. Eosinophils were incubated with 1 μM PMA (lane 2), with PMA and 50 μM NO_2^- (lane 3), with PMA, 50 μM NO_2^-, and 50 μg/ml SOD (lane 4), or with PMA, 50 μM NO_2^-, and 30 μg/ml catalase (lane 5). A sample incubated with PMA and 50 μM NO_2^- was treated with 1 mg/ml dithionite (lane 6). Each sample for electrophoresis contained 30 μg of the proteins. Lanes 1–6 were immunoblotted with anti-nitrotyrosine antibodies and lanes 7–12 with anti-nitrotyrosine antibodies pre-incubated with 10 mM 3-nitrotyrosine for 2 h.

(Fig. 4D). A control study using non-immune rabbit immunoglobulin did not show staining in the gastric mucosa fixed with PLP or that treated with NO_2^- and H_2O_2.

Immunoblot Analysis of Protein-bound Nitrotyrosine

Protein-bound tyrosine nitration in eosinophils and the reactivity of nitrated or non-nitrated proteins from eosinophils with anti-nitrotyrosine antibodies after the modification of these proteins with fixatives was investigated using SDS-PAGE and Western blotting (Fig. 5). Non-treated cells (lane 1) and cells incubated with 1 μM PMA (lane 2) did not show tyrosine nitrated proteins. However, cells incubated with 1 μM PMA and 50 mM NO_2^- (lane 3) showed many nitrated proteins corresponding to molecular weights of 14, 17, 31, 46 and 56 kDa at least. The intensity of the nitrated protein bands was augmented by SOD (lane 4), and diminished by catalase (lane 5) and dithionite (lane 6). These tyrosine nitrated protein bands were abolished by pre-incubation of anti-nitrotyrosine antibodies with 3-nitrotyrosine (lanes 7–10) (Fig. 5). The immunoreactivities of proteins of non-treated cells or PMA plus NO_2^--treated cells and OVA to anti-nitrotyrosine antibodies was augmented by treatment of blotted membranes with PLP. The augmentation of the reactivities of anti-nitrotyrosine antibodies with proteins from eosinophils and OVA was not changed by pre-treatment with dithionite (Fig. 6) but was abolished by pre-incubation of antibodies with 3-nitrotyrosine. An increase in the immunoreactivity for OVA with anti-nitrotyrosine antibodies was observed in the blotted membrane incubated with PLP and paraformaldehyde (Fig. 7A). The increase in immunoreactivity of PLP-treated OVA with anti-nitrotyrosine antibodies was not changed by pre-incubation of OVA with dithionite but was abolished

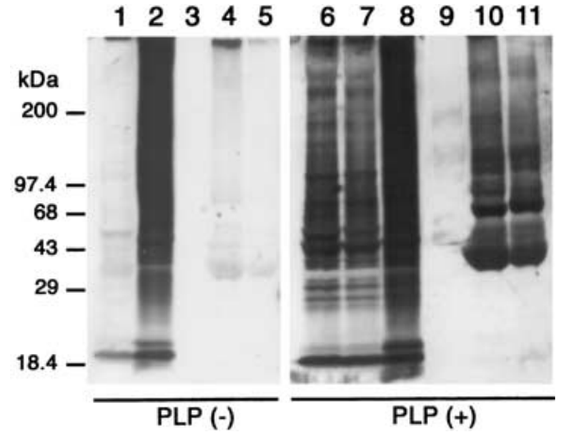

FIGURE 6 The effect of fixatives on immunoreactivity in immunoblot analysis. The blotted membranes of lanes 6–11 were treated with PLP fixative. Non-treated eosinophils (3×10^6), 90 μg proteins (lanes 1, 6); eosinophils incubated with 1 μM PMA and 50 μM NO_2^-, 30 μg proteins (lanes 2, 8); 50 μg BSA (lanes 3, 9); 50 μg OVA (lanes 4, 10); 30 μg proteins of non-treated eosinophil samples treated with 1 mg/ml dithionite (lane 7); 50 μg OVA treated with 1 mg/ml dithionite (lanes 5, 11).

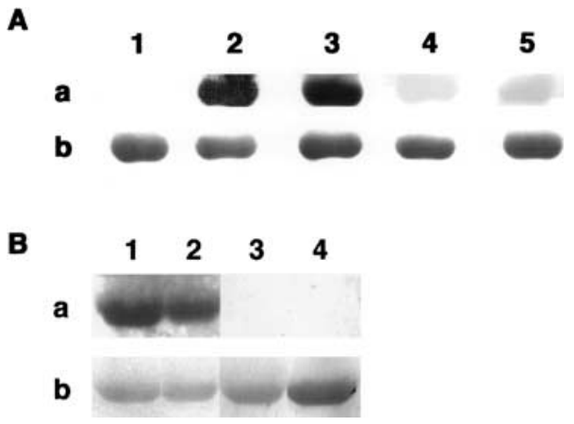

FIGURE 7 Effect of fixatives and dithionite on immunoreactivity of OVA in immunoblot analysis. (A) OVA (5 μg) was subjected to SDS-PAGE, transferred on nitrocellulose membrane and immunostained with anti-nitrotyrosine antibodies. The membrane was non-treated (lane 1), incubated with PLP for 1 h (lane 2), with 4% paraformaldehyde for 20 min (lane 3), with 10% neutral buffered formalin for 20 min (lane 4) and ice-cold methanol for 5 min (lane 5). (B) OVA (5 μg) or dithionite (5.7 mM)-treated OVA (5 μg) was subjected to SDS-PAGE, transferred on nitrocellulose membrane and immunostained with anti-nitrotyrosine antibodies. The OVA membrane was incubated with PLP for 1 h (lane 1); the dithionite (5.7 mM)-treated OVA (5 μg) membrane was incubated with PLP for 1 h (lane 2); the OVA membrane was treated with dithionite (5.7 mM) after incubation with PLP (lane 3); the dithionite (5.7 mM)-treated OVA (5 μg) membrane was treated with dithionite (5.7 mM) after incubation with PLP (lane 4). (a) Immunoblotting; (b) Comassie blue staining.

by incubation of PLP-treated membranes with dithionite (Fig. 7B). Moreover, the reactivities of blotted proteins from eosinophils with anti-nitrotyrosine antibodies increased by PLP and paraformaldehyde treatment but did not increase by ice-cold methanol and neutral buffered formalin treatment.

HPLC Assay

Generation of nitrotyrosine was not observed by the incubation of L-tyrosine or 3-aminotyrosine with PLP fixative or sodium periodate.

DISCUSSION

In the present study, we investigated the latent artifacts of nitrotyrosine immunohistochemistry in inflammatory cells or the inflammatory tissue by inappropriate use of fixative. Two tyrosine nitrating mechanisms were suggested to exist in inflammatory cells such as neutrophils, eosinophils and macrophages because these cells can produce the tyrosine nitrating agent, peroxynitrite, due to the simultaneous production of NO and $O_{2\cdot}^-$, and these cells have an alternative mechanism of tyrosine nitration by heme peroxidases such as myeloperoxidase[15,16] and eosinophil peroxidase.[17] Despite a chemical relation between peroxynitrite and tyrosine nitration, direct evidence for peroxynitrite-mediated nitration *in vivo* is still lacking.[13,24] There were several studies reporting that simultaneous generation of NO and $O_{2\cdot}^-$ *in vitro* did not cause significant nitration of free tyrosine[25–29] because of dityrosine formation at low steady-state concentrations of peroxynitrite.[27] In contrast, highly efficient tyrosine nitration by low fluxes of $NO/O_{2\cdot}^-$ was demonstrated by diminishing the effect of uric acid, a strong scavenger of peroxynitrite and a product of the hypoxanthine/xanthine oxidase system used for the generation of $O_{2\cdot}^-$.[30] With regard to the alternative mechanisms of tyrosine nitration, biochemical evidence has been accumulating. The tyrosine nitrating capacity of eosinophil peroxidase was several fold higher than that of myeloperoxidase.[17] In alveolar lavage fluid of human asthma, high levels of nitrotyrosine was diagnostic of leukocyte infiltration of the lung.[10] Protein tyrosine nitration in activated murine macrophages was caused by a nitrite-dependent peroxidase reaction rather than peroxynitrite.[18] However, the pathophysiological significance of the generation of tyrosine nitrating species via the alternative mechanisms of tyrosine nitration was not established.

Nitrotyrosine immunoreactivities of PLP fixed-specimens were evaluated in several studies.[10,11,31,32] The generation of nitrotyrosine was first demonstrated immunohistochemically in eosinophils of lung tissue fixed with PLP from asthma patients regarding the origin of tyrosine nitrating species. The present results showed the immunoreactivity for nitrotyrosine in almost all eosinophils and eosinophil-like cells in the gastric mucosa fixed by PLP, and then the immunoreactivity for nitrotyrosine emerged after the tyrosine nitration procedure in samples treated with other fixatives than PLP. From this point of view, PLP fixative is not a preferable technique to evaluate the immunostaining for nitrotyrosine in eosinophil containing tissue.

Immunolocalization of nitrotyrosine in tyrosine nitrated eosinophils by the peroxidase system was different from that due to the concentrations of NO_2^- and H_2O_2. The localization of nitrotyrosine in eosinophils incubated with 1 μM NO_2^- and 1 μM H_2O_2 was in a marginal area of the cells and was different from the site of eosinophil peroxidase, the matrix of the large crystalloid-containing granules.[33] It is clear that the proteins responsible for the generation of tyrosine nitrating species and the tyrosine nitrated proteins by the reaction with reactive nitrogen species are not similar and exist at a distant place. Actually, tyrosine nitration with 5 μM NO_2^- and 5 μM H_2O_2, which generate higher

concentrations of reactive nitrogen species than that with 1 μM NO_2^- and 1 μM H_2O_2, was diffused throughout the entire site of the cell. Therefore, it is difficult to distinguish the mechanisms from the immunolocalization.

The reduction of immunostaining in eosinophils by dithionite was obviously different between the treatment of eosinophils with PLP and peroxynitrite or the peroxidase system. It is not clear that tyrosine nitrated proteins in eosinophils were easily reduced by dithionite. With regards to tyrosine nitrated granulocytes containing eosinophils and neutrophils, dithionite reduction was complete in neutrophils but incomplete in eosinophils (data not shown).

Periodate can oxidize carbohydrates to aldehydes. Eosinophils fixed with paraformaldehyde and glutaraldehyde showed trace levels of immunoreactivity for anti-nitrotyrosine if the fixation time become a little longer. Although it is not clear that these immunoreactivities are an artifact, aldehyde groups may be associated with epitopes that have an affinity to anti-nitrotyrosine antibodies. However, it is true that periodate can form similar epitopes to nitrotyrosine in proteins of eosinophils or OVA and then tyrosine nitration cannot occur.

In conclusion, it is suggested that nitrotyrosine immunohistochemistry in PLP-fixed and eosinophils containing tissue is an artifact and may cause confusion in the pathogenesis of reactive nitrogen species related diseases.

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