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Species-specific immunostaining of embryonic corneal nerves: Techniques for inactivating endogenous peroxidases and demonstration of lateral diffusion of antibodies in the plane of the corneal stroma

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Abstract

Species-specific and species-common monoclonal antibodies (MAbs) to nerve-specific cell surface epitopes were used to compare pre-treatment techniques for nerve staining. Endogenous peroxidases were inactivated in four ways: (1) 0.3% hydrogen peroxide (H_2O_2); (2) 1% periodic acid (PA) (pH 1.85–1.95); (3) sodium *meta*-periodate (10–40 mM, pH 4.5); or (4) HCl (pH 1.80). Staining of chick and quail corneal nerves and dorsal root ganglia (DRG) nerves with the MAbs was species-specific. Staining of chick and quail corneal nerves was unaffected by pre-treatment with 0.3% H_2O_2, but was eliminated by pre-treatment with 1% PA. Chick and quail DRG nerve staining tolerated 0.3% H_2O_2, and at least one epitope also tolerated 1% PA. Corneal nerves of both chick and quail displayed concentration-dependent sensitivity to pre-treatment with sodium *meta*-periodate; DRG nerves were not sensitive to such pre-treatment. Corneal nerves tolerated pre-treatment with HCl (pH 1.80), whereas DRG nerves did not. These findings indicate sensitivity of corneal nerve epitopes to oxidation, in contrast with sensitivity of DRG nerve epitopes to low pH. Results also indicate that tissue trimming regulated whole-mount staining of corneal nerves, suggesting that antibodies cannot diffuse across corneal basement membranes, even after detergent extraction. However, antibodies are able to diffuse laterally into the stroma from any cut edge.

Keywords: Cornea; Nerves; Periodate; pH; Peroxidases; Chickens; Quail; Antibody diffusion

1. Introduction

A reproducible, reasonably rapid technique is needed to visualize the pattern of corneal nerves in whole-mount preparations. Past techniques to visualize corneal nerves have involved time-consuming, unpredictable methods, e.g. the use of silver and gold solutions in modified Golgi techniques (Bee, 1982; Bee et al., 1986), although new methods are appearing (Jacot et al., 1995, 1997). The latter techniques do not discriminate between the nerves of different species of animals, and thus cannot be used to locate nerves derived from grafted tissue in chimeric embryos, such as for example, those in quail/chick chimeras (Le Douarin, 1973). A new technique, therefore, is required to distinguish between the often intermingled nerves arising from both host and graft tissues in experimental chimeric embryos constructed to study the development of cornea innervation.

Monoclonal antibodies (MAbs) directed against species-specific and species-common epitopes of peripheral nerves offer an opportunity to discriminate between chick and quail peripheral nerves. As isolated by

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Tanaka et al. (1990), the CN antibody reacts specifically with chick nerves, the QN antibody reacts specifically with quail nerves, and the CQN antibody reacts with both chick and quail nerves. These MAbs appear to recognize distinct epitopes on a 116 kDa glycoprotein of nerve cell surfaces, although precisely which molecule(s) are recognized and whether the epitopes are carbohydrate or protein moieties have not been determined.

During the course of optimizing the whole-mount immunohistochemical staining procedure described below for use with diaminobenzidine (DAB) visualization of sites of antibody reactivity, alternative techniques of tissue pre-treatment were used to inactivate endogenous peroxidases. It is necessary to inactivate such enzymes to allow any subsequent nerve staining to be attributed to the peroxidase conjugated to the secondary antibody added at the end of the experiment. One commonly used technique inactivates peroxidases by incubation with a relatively high concentration of hydrogen peroxide (H_2O_2) (0.3%) (Shimizu et al., 1992; Yoshinaga et al., 1992); a common alternative technique utilizes 1% periodic acid (PA) (Ishikawa et al., 1986; Kuratani and Tanaka, 1990a,b; Kuratani et al., 1991; Kuratani and Aizawa, 1995). When these two techniques were utilized in the present study, radically different degrees of nerve staining were obtained, revealing differences between corneal and DRG nerves in susceptibility of epitopes to conditions of oxidation and pH. Finally, and unexpectedly, the degree to which chick corneal tissue was trimmed and radially slit determined whether the corneal nerves could be stained at all, whereas staining of quail corneal nerves was much less influenced by the extent and pattern of trimming.

2. Materials and methods

2.1. Embryos and tissue preparation

White Leghorn chicken (*Gallus domesticus*) embryos and Japanese quail (*Coturnix coturnix japonica*) embryos were incubated in a humidified, forced-draft incubator. Embryos were removed from their shells and euthanized immediately by severing the spinal cord in accord with the Recommendations for Euthanasia of Experimental Animals (Close et al., 1997).

Whole eyes were removed without puncturing, and dorsal body walls were dissected to expose the dorsal root ganglia (DRGs). All dissections were performed in Saline G (Puck et al., 1958). Eyes and body walls were fixed in 3.7% formaldehyde in Saline G at 0–4°C on a tumbling rotator for 1–2 h. Whole eyeballs were excised from Day 20 chick and Day 16 quail embryos, 1 day before hatching, in each case, whereas body walls containing DRG nerves were prepared from Day 9 chick and Day 7 quail embryos, the oldest ages at which consistent whole-mount staining of DRG nerves was obtained. After fixation, tissues were rinsed for 5–10 min in Saline G on a rotator at room temperature. Tissues, thereafter, were stored in Saline G + 30% sucrose + 0.1% Na azide at 0–4°C, and transferred into Saline G alone during dissections.

In preparation for immunostaining, corneas were dissected from eyeballs in one of three ways: (1) non-trimmed corneas: the surrounding scleral tissue and limbal rim were not removed and therefore none of the corneal stromal edge was exposed; (2) trimmed corneas: scleral and limbal rim tissue were removed, thereby exposing the edge of ~90% of the circumference of the corneal stroma (a small pigmented piece of scleral tissue was left attached to the cornea as a visible marker and handle); (3) trimmed and slit corneas: 2–3 radial cuts were introduced toward the center of trimmed corneas to allow increased penetration of antibodies into the center of the corneal stroma.

2.2. Inactivation of endogenous peroxidases and immunohistochemical staining

For whole-mount staining of chick and quail corneas and DRGs, all steps were performed at room temperature on a tumbling rotator, unless otherwise noted. Following trimming, tissues were rinsed in Saline G + 0.2% Triton (v/v) X-100 (TX-100) (3 × 10 min) to permeabilize the tissues. Endogenous peroxidases then were inactivated in one of four ways (on a rotator in the dark): (1) 0.3% hydrogen peroxide (H_2O_2) (3 × 20 min); (2) 1% periodic acid (PA) (pH 1.85–1.95) (3 × 10–15 min); (3) sodium *meta*-periodate (10–40 mM in 0.05 M sodium acetate, pH 4.5) (3 × 10 min); (4) HCl (pH 1.80) (3 × 10 min). Tissues then were rinsed in Saline G + 0.2% TX-100 briefly, and in Saline G + 0.2% TX-100 + 2% (w/v) powdered non-fat milk (4 × 15 min) before incubation overnight at 0–4°C with primary MAbs dissolved in the latter solution. Three types of MAbs were used as hybridoma cell culture supernatants (Tanaka et al., 1990): chick nerve-specific (CN), quail nerve-specific (QN), chick- and quail-nerve specific (CQN) MAbs. All were diluted 1:4 in Saline G + 0.2% TX-100 + 2% powdered milk just before use.

Following incubation with primary MAbs, tissues were rinsed with Saline G + 0.2% TX-100 + 2% powdered milk (4 × 15 min) and then incubated overnight at 0–4°C with secondary goat anti-mouse (GAM)-peroxidase-conjugated antiserum (Jackson ImmunoResearch Laboratories, Westgrove, PA) diluted 1:100 in Saline G + 0.2% TX-100 + 2% powdered milk. Tissues then were rinsed once in Saline G + 0.2% TX-100 + 2% powdered milk (1 × 15 min) and then with Saline G + 0.2% TX-100 in preparation for a 30-min incubation with DAB (0.24 mg/ml Saline G + 0.2% TX-100)

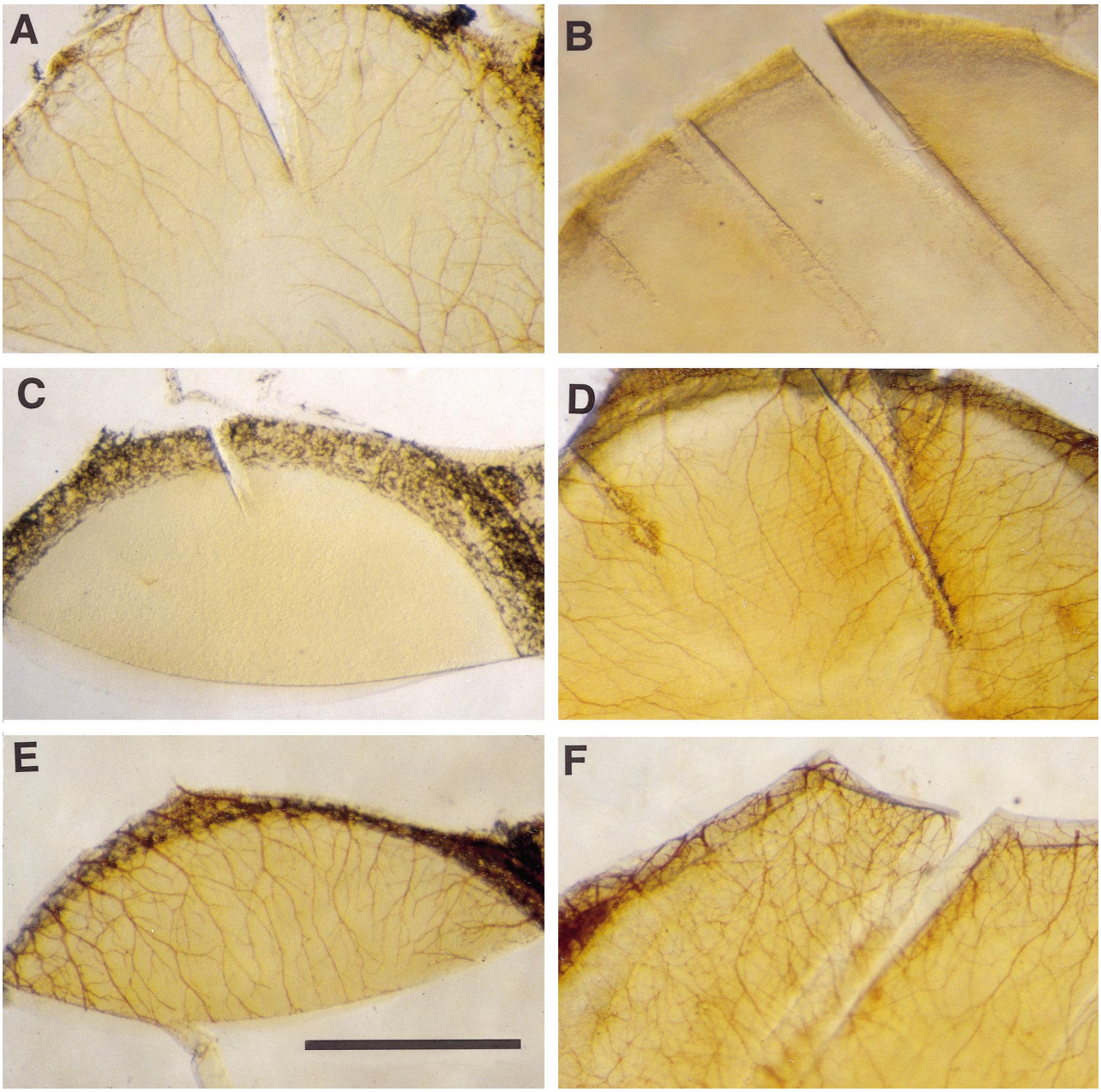

Fig. 1. Species- and nerve-specificity of QN, CN, and CQN MAbs illustrated by corneal nerve staining of Day 20 chick and Day 16 quail embryos. (A,C,E): quail corneas; (B,D,F): chick corneas. Pairs (A,B) were incubated with QN MAb; (C,D): CN MAb; (E,F): CQN MAb. Note species-specificity of QN for quail corneal nerves (A) and not for chick (B); species-specificity of CN for chick corneal nerves (D) and not for quail (C); nerve-specificity of CQN for both chick (F) and quail (E) corneal nerves. Scale bar = 1000 μm.

at room temperature. Corneal and DRG nerves then were revealed by final incubation with DAB (0.24 mg/ml) and 0.03% H_2O_2 (20–60 min at room temperature in the dark). Thereafter, tissues were rinsed in Saline G + 0.2% TX-100 and then in distilled H_2O. Stained tissues were stored in 100% glycerol at room temperature after being allowed to equilibrate on a rotator for 1 day.

A Wild M5 microscope with an automated photographic exposure system was used to record the nerve patterns as 35 mm color transparencies on Kodak and Fuji color T64 film.

3. Results

3.1. Species-specificity of QN, CN, and CQN MAbs—corneal and DRG nerve staining

The MAbs were species-specific in their staining of

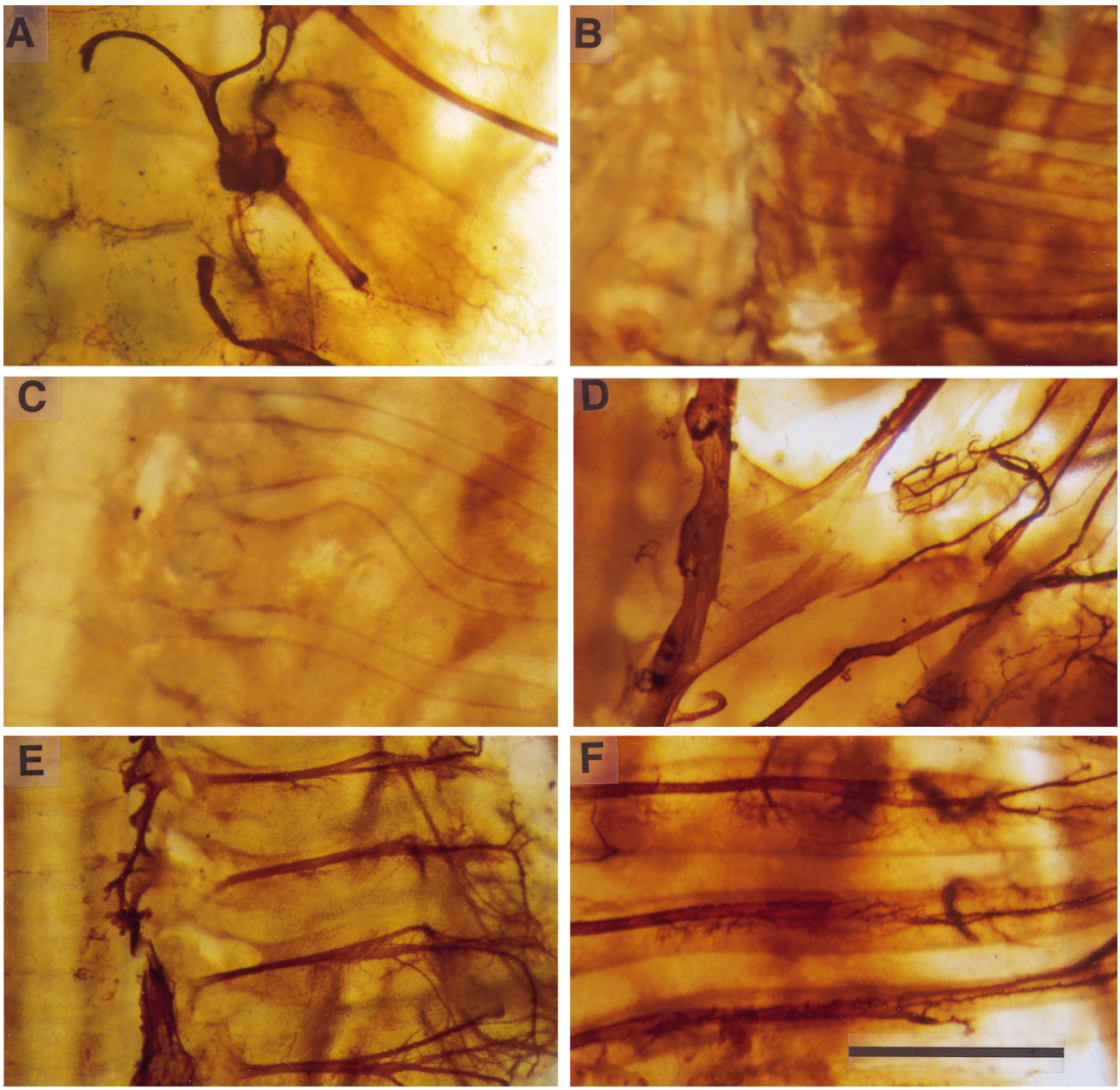

Fig. 2. Species-specificity of QN, CN, CQN MAbs illustrated by DRG nerve staining of Day 9 chick and Day 7 quail embryos. (A,C,E) represent quail DRGs; (B,D,F): chick DRGs. Pairs (A,B) were incubated with QN MAb; (C,D): CN MAb; (E,F): CQN MAb. Note species-specificity of QN for quail DRG nerves (A) and not for chick (B); species-specificity of CN for chick DRG nerves (D) and not for quail (C); nerve-specificity of CQN for both chick (F) and quail (E) DRG nerves. Scale bar = 1000 μm.

chick and quail corneal nerves (Fig. 1) and DRG nerves (Fig. 2), the latter confirming the conclusions of Tanaka et al. (1990).

Antibody QN bound to quail corneal nerves (Fig. 1A), but not to chick corneal nerves (Fig. 1B). Antibody CN bound to chick corneal nerves (Fig. 1D), but not to quail corneal nerves (Fig. 1C). Antibody CQN bound to both quail corneal nerves (Fig. 1E) and chick corneal nerves (Fig. 1F).

Antibody QN bound to quail DRG nerves (Fig. 2A), but not to chick DRG nerves (Fig. 2B). Antibody CN bound to chick DRG nerves (Fig. 2D), but not to quail DRG nerves (Fig. 2C). Antibody CQN bound to both quail DRG nerves (Fig. 2E) and chick DRG nerves (Fig. 2F).

3.2. Effects of tissue trimming on MAb penetration and nerve staining in chick and quail corneas using CQN MAb

Increasing the exposure area of the edge of the corneal stroma, by trimming and radial cuts, greatly

improved the whole-mount staining of the chick corneal nerves. A pattern of enhanced staining after trimming and slitting was observed in the nerve staining of the quail corneas if the incubation time with primary antibodies was shortened, indicating that the same phenomenon, in principle, occurs in the corneas of both animal species.

After overnight incubation with primary antibody, whole-mount staining of chick corneal nerves suggested a gradient in antibody diffusion. Non-trimmed chick corneas showed only very faint staining, if any, along the edges of the cornea (Fig. 3A). Nerves were especially visible along the circumferential cuts (Fig. 3B). However, except for the localized zones bordering radial cuts, staining appeared to diminish sharply towards the center of the halved cornea (Fig. 3C,D), suggesting that MAbs were penetrating the stromal matrix around the nerves mainly by diffusing inward from the cut edges of the stroma. Thus, even after tissue extraction with 0.2% TX-100, MAbs are not able to cross the chick basal laminae of the corneal epithelium and corneal endothelium (Descemet's membrane).

In contrast, after overnight incubation with primary antibody, whole-mount staining of quail corneal nerves occurred equally well in trimmed corneas (Fig. 3F) and in non-trimmed corneas (Fig. 3E), which were still surrounded by scleral tissues and limbal rim, suggesting fewer barriers to diffusion than in chick corneas. However, significantly decreasing the primary antibody (CQN) incubation time of quail corneas from overnight (Fig. 3E,F) to 30 min (Fig. 3G,H), although still producing acceptable staining, revealed enhanced staining of nerves in trimmed and radially slit corneas (Fig. 3H) compared with non-trimmed corneas (Fig. 3G), suggesting that cutting the edge of the corneal stroma does increase antibody access to the nerves in a lateral manner even in quail corneas.

3.3. Effects of pre-treatment techniques on nerve staining in corneas and DRGs

Classically, two types of tissue pre-treatment techniques have been used to inactivate endogenous peroxidases in preparation for immunohistological staining in which peroxidase conjugated to a secondary antibody is to be used to visualize sites of antibody binding: high concentration of H_2O_2 (0.3%) (Shimizu et al., 1992; Yoshinaga et al., 1992) or 1% PA (Ishikawa et al., 1986; Kuratani and Tanaka, 1990a,b; Kuratani et al., 1991; Kuratani and Aizawa, 1995). However, in using the three MAbs in the present experiments, these two techniques produced very different results when applied to corneas and DRGs. For the epitopes on both chick and quail corneal nerves, only H_2O_2 pre-treatment allowed subsequent immunostaining: corneal nerves were stained reproducibly following pre-treatment with 0.3% H_2O_2 (Fig. 4A), whereas staining was prevented by pre-treatment with 1% PA (pH 1.85–1.95) (Fig. 4C). Like those on corneal nerves, the epitopes on DRG nerves tolerated pre-treatment with 0.3% H_2O_2 (Fig. 4B). However, their responses to pre-treatment with 1% PA depended on the antibody, a pattern therefore different from that for corneal nerves: CQN did not stain quail DRG nerves that had been treated with 1% PA (Fig. 4D) (a sensitivity therefore resembling that of corneal nerves). In contrast, CN stained DRG nerves after such treatment, an indication of resistance to PA action not displayed by corneal nerves (data not shown); MAb QN gave equivocal results: two sets of experiments gave DRG nerve staining after PA pre-treatment and two sets gave no staining after such pre-treatment.

The striking effects of 1% PA in preventing corneal nerve staining with MAb CQN suggested that those epitopes were sensitive to either the oxidative effects of the periodate in the PA solution, or to its very low pH (pH 1.85–1.95), or, cumulatively, to both. Therefore, the effects of periodate and pH were examined separately. Oxidative effects of 10, 20, and 40 mM sodium *meta*-periodate in 0.05 M sodium acetate solution (pH 4.5) revealed steadily weaker corneal nerve staining, with barely any staining occurring after pre-treatment at 40 mM (Fig. 4E).

Parallel experiments were performed on DRG preparations to determine if the effects of 1% PA on such nerves arose from oxidation, very low pH, or both. In contrast to corneal nerves, DRG nerves revealed relatively the same intensity of immunostaining with CQN MAb, following pre-treatment with 10, 20, and 40 mM sodium *meta*-periodate (Fig. 4F), as with 0.3% H_2O_2 pre-treatment (Fig. 4B). Thus, corneal nerves and DRG nerves appeared to differ in their response to pre-treatment with periodate. Conversely, HCl pre-treatment of tissues with solutions of the same pH as that of 1% PA (pH 1.80), but without the presence of the oxidant, allowed subsequent immunostaining of quail corneal nerves (Fig. 4G), but not of quail DRG nerves, with the CQN MAb (Fig. 4H). Thus, corneal nerves and DRG nerves also appeared to differ in their response to pre-treatment with HCl.

4. Discussion

Results presented here indicate the species-specificity of MAbs QN and CN for both corneal and DRG nerves, and confirm the neural specificity of MAb CQN. In addition, antibody diffusion into the extracellular matrix of the corneal stroma, particularly of chicks, occurs predominantly from the exposed, cut edges of the stroma, rather than across the basement membranes that cover both surfaces of the stroma. Finally, the data indicate that comparison of two commonly used alternative methods for inactivating endogenous peroxidase activities can allow important discrimination between the epitopes of distinct nerve populations.

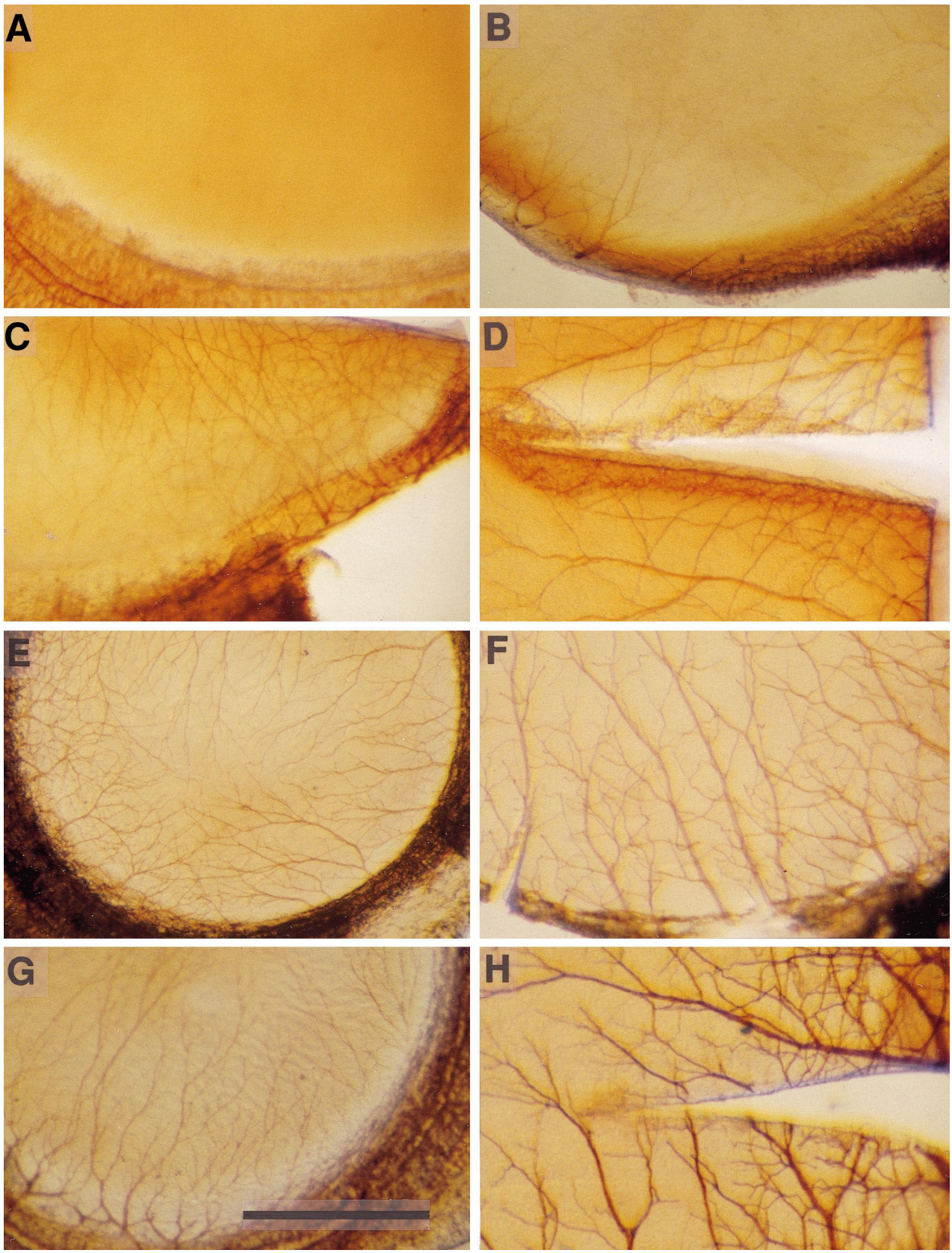

Fig. 3. Effect of trimming on nerve staining in Day 20 chick and Day 16 quail embryos suggesting lateral diffusion of MAb (CQN). (A,B,C,D) represent chick corneas incubated overnight with CQN. Note (A) non-trimmed chick cornea: no staining; (B,C) increasingly trimmed cornea: staining occurs only at cut edges of the tissue; (D) trimmed and radially slit cornea: staining of nerves at the edge, as well as nerves near cornea center. (E,F,G,H) quail corneas; (E,F) incubated overnight with CQN; (G,H) incubated 30 min with CQN; (E) non-trimmed quail corneal nerves stained as well as in trimmed, slit cornea (F); (G) non-trimmed quail corneal nerves stained, but not as intensely as in trimmed and slit corneas (H). For D, F, and H, scale bar = 400 μm; for all other photomicrographs, scale bar = 1000 μm.

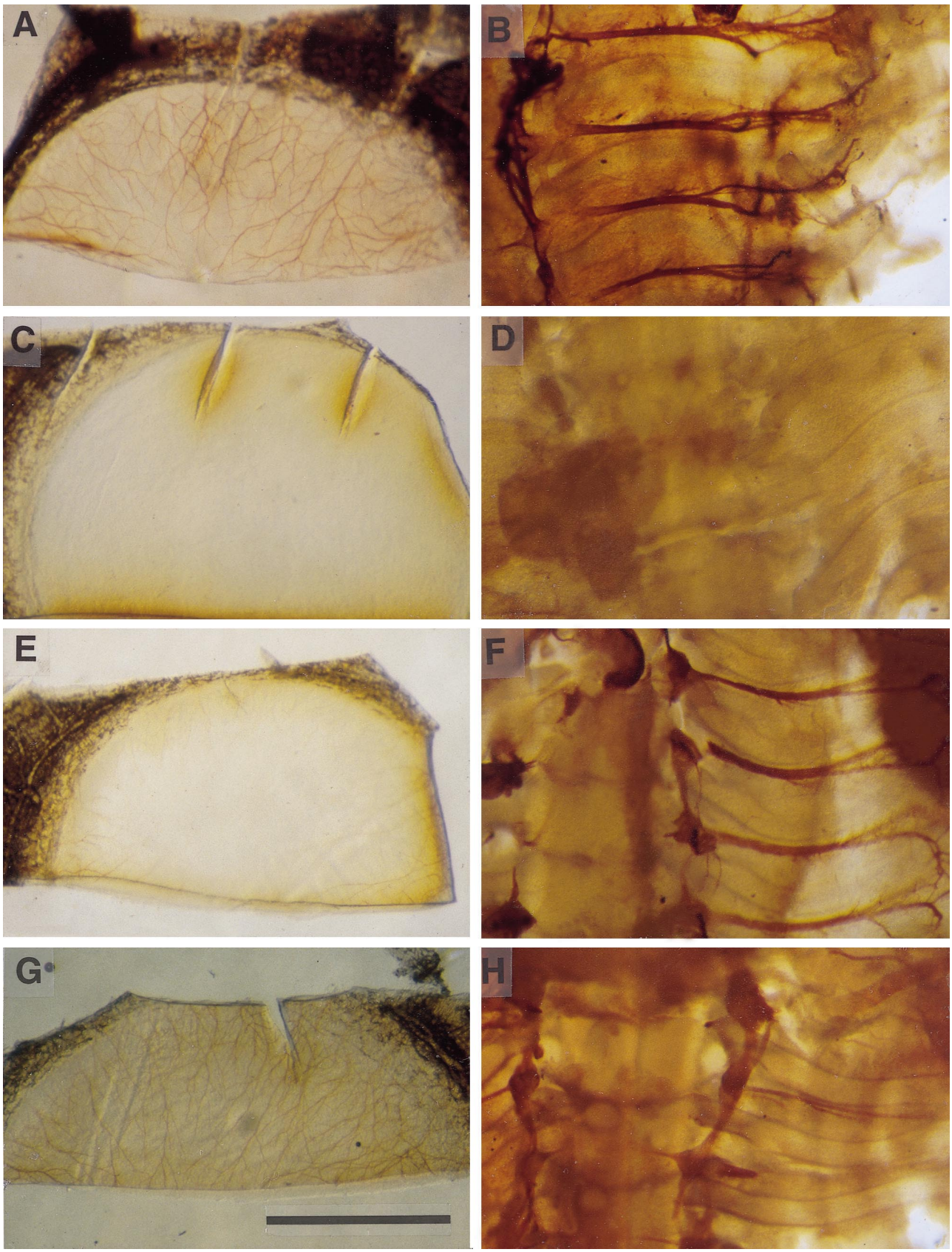

Fig. 4. Effects of pre-treatment techniques on nerve staining with MAb CQN in corneas of Day 16 quail and DRGs of Day 9 quail embryos. (A,C,E,G) represent quail corneas; (B,D,F,H): quail DRGs. Pairs (A,B) were pre-treated with 0.3% H_2O_2; (C,D): pre-treated with 1% PA; (E,F): pre-treated with 40 mM sodium periodate at pH 4.5; (G,H): pre-treated with HCl at pH 1.8. Following the pre-treatments described, together with rinsing, corneas and DRGs were incubated with CQN MAb, followed by secondary antiserum. Scale bar = 1000 μm.

The improvement in nerve staining that arises from trimming chick corneas demonstrates that, even after extensive incubation in 0.2% TX-100, the MAbs are able to penetrate the chick corneal stromal matrix only in the plane of the cornea, entering only via the narrow, cut edge of the stromal matrix exposed by the trimming. Quail corneal nerve staining also demonstrated this phenomenon when incubation with primary antibody was performed for a significantly shorter period of time. Therefore, in addition to entering by way of stromal cut edges, antibodies also penetrate quail corneas across the extracted cell layers and basement membranes lining the apposed stromal surfaces. Previous studies have examined diffusion of antibodies from the limbus into the stroma of corneas in vivo (Allansmith et al., 1979; Verhagen et al., 1990).

Our results also indicate that, whereas pre-treatment with 0.3% H_2O_2 allows staining of both corneal nerves and DRG nerves, PA pre-treatment preferentially prevents corneal nerve staining with all three antibodies. Following PA pre-treatment, DRG nerve staining similarly fails to occur in the case of one MAb (CQN), but still occurs in the case of another MAb (CN), and occurs inconsistently with a third MAb (QN). The effect of PA upon corneal nerves appears to be derived mainly from the oxidative effects of PA rather than from its very low pH, whereas the effect of PA on DRG nerve staining appears to arise from its acidic pH. Thus, the carbohydrate epitopes (Wolfe and Hage, 1995) that characterize the plasma membranes of corneal nerves appear to be distinct from those of DRG nerves, as assessed by the three MAbs. Differential destruction of epitopes by pre-treatment of tissue with PA has been described previously (Margolis et al., 1992; Shimizu et al., 1992).

The epitopes recognized by all three antibodies on both corneal nerves and DRG nerves tolerated the relatively gentle oxidation conditions of 0.3% H_2O_2. However, sensitivity of corneal nerve epitopes for all three MAbs to 1% PA pre-treatment suggests either that they were degraded by the periodate ion in the PA solution, or by the very low pH of the PA solution, or by both. Because corneal nerve staining, as visualized by CQN MAb, progressively disappeared with increasing concentrations of periodate (10–40 mM), we conclude that these epitopes are periodate-sensitive. This range represents the middle of conventionally utilized concentrations of periodate (Wolfe and Hage, 1995). In contrast, the epitope recognized by the CQN MAb on DRG nerves does not appear to be sensitive to periodate pre-treatment. This observation suggests that there may be major differences in the types of carbohydrate moieties attached to the cell surface glycoproteins of corneal nerves and DRG nerves.

A difference between corneal nerves and DRG nerves is also revealed by their response to pre-treatment with HCl: corneal nerve sensitivity to PA pre-treatment is due, largely, to its oxidative properties, and not to its low pH, because reactivity of corneal nerves is not significantly diminished by the tissue pre-treatment with HCl alone. In contrast, HCl pre-treatment does eliminate most DRG nerve staining, again suggesting that there are major differences in the carbohydrate epitopes of DRG nerves compared with those of corneal nerves. Although many studies already cited have used H_2O_2 pre-treatment to inactivate endogenous tissue peroxidases, an equally significant number have, alternatively, used PA for tissue pre-treatment (Ishikawa et al., 1986; Kuratani et al., 1988; Kuratani and Tanaka, 1990a,b; Kuratani et al., 1991; Kuratani, 1991; Kuratani and Wall, 1992; Kuratani and Kirby, 1992; Kuratani and Eichele, 1993; Kuratani and Aizawa, 1995).

These differences in responses to various tissue pre-treatment conditions may be due to modifications on a glycoprotein of the nerve cell surface, specifically on the carbohydrate epitopes. Periodic acid, an acidic, oxidizing agent, modifies polysaccharide chains by cleaving carbon–carbon bonds between vicinal hydroxyl groups. A concentration of 1% PA is equivalent to $\sim$44 mM sodium periodate. Sialic acid, a sugar normally present at the terminal ends of many of the oligosaccharide chains that are attached to cell surface glycoproteins, has been shown to be sensitive to exposure to PA (Påhlsson et al., 1994). Generically, sialic acids are acid-labile and have been shown to be cleaved from carbohydrate chains when exposed to PA at concentrations, temperatures, and times used in the present study (Reuter and Schauer, 1994; Kotani and Takasaki, 1997). At least some of the MAbs used here may recognize the sialic acid groups on these nerve-specific glycoproteins, or on glycoproteins associated with Schwann cells, types of sialic acids selectively releasable with periodate (Collins et al., 1997a,b). Corneal and DRG differences in sensitivity of epitopes to periodate and acid pre-treatment could conceivably arise if these two nerve populations expressed the same nerve-specific cell surface glycoprotein, but then added slightly different tissue-specific glycosylation moieties, such as different types of sialic acids that might differ in their sensitivities to periodate or acidic solutions. In addition, we recognize that recent preliminary data suggest that periodate oxidation may cause cleavage of specific peptide bonds, in addition to its expected release of sialic acids (Kimura and Stadtman, 1995). Elucidating the epitopes recognized by these three Mabs, perhaps by preparing independent batches of antisera to the 116 kDa glycoprotein(s), promises to provide useful information for understanding the exact location and biological role of a widely-distributed, nerve-associated molecule (or family of molecules) that appears to carry both species-common and species-specific epitopes.

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