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Protein G-horseradish peroxidase based method for light-microscope immunocytochemistry. Application to the pituitary gland of the killifish, *Fundulus heteroclitus*

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SUMMARY

Horseradish peroxidase-protein G conjugate was used to localize anti-human luteinizing hormone and anti-human chorionic gonadotropin primary antibodies bound to gonadotropins in paraffin and Historesin embedded sections of *Fundulus heteroclitus* pituitaries. The sensitivity and specificity of this method and those obtained after the avidin-biotin-peroxidase complex, the streptavidin-biotin-peroxidase complex and the peroxidase-antiperoxidase procedures were compared. The protein G-horseradish peroxidase method gave a clear immunostaining of gonadotrophic cells with virtually no background. Detection efficiency was reduced in comparison with the other techniques. As for the embedding medium, hydrophilic resin Historesin considerably enhanced the structural detail and the resolution of the immunostaining at light-microscope level.

INTRODUCTION

In light and electron microscopy, peroxidase enzymes coupled with different molecules have been widely used to detect antibodies specifically bound to their antigens (Kawarai and Nakane, 1970; Hanker *et al.*, 1977). Immunoperoxidase methods use antibodies conjugated to peroxidase (Nakane and Kawaoi, 1974), peroxidase-antiperoxidase complex (Sternberger *et al.*, 1970; Burns, 1975), protein A conjugated to peroxidase (Dubois-Dalcq *et al.*, 1977; Celio *et al.*, 1979), streptavidin-biotin-peroxidase complex (Larsson, 1989), or avidin-biotin-peroxidase complex (Hsu *et al.*, 1981a, b). All these methods have been shown to be useful for tissues embedded in paraffin, epoxy resins, and methacrylates (Kawarai and Nakane, 1970; Hogan and Smith, 1982; Rodríguez *et al.*, 1984; Larsson, 1989).

Protein A and protein G are cell surface bacterial proteins that bind to the Fc region of IgG molecules from many different species, without interfering with the antigen-antibody reac-

tion (Kronvall and Frommel, 1970; Björck and Kronvall, 1984). When conjugated to enzymes, they provide a useful method for detecting primary antibodies from a variety of origins, thus avoiding the preparation and use of secondary antibodies. Protein G binds to IgG molecules from a wider number of species (rat and mouse included), to more IgG subclasses, and with higher affinity, than Protein A, and it has been successfully utilized for the purification and detection of monoclonal and polyclonal antibodies in Western blots (Björck and Kronvall, 1984; Åkerström *et al.*, 1985); coupled to colloidal gold, it has been used in immunocytochemistry at electron-microscope level (Bendayan and Garzon, 1988; Bendayan, 1989).

In this study, we show the utility of protein G conjugated to horseradish peroxidase (PG-HRP) as a substitute for labelled or unlabelled secondary antibodies in light-microscope immunocytochemistry applied to paraffin-embedded sections of *Fundulus heteroclitus* pituitaries, and we compare this technique with other more conventional ones like peroxidase-antiperoxidase complex (PAP), avidin-biotin-peroxidase complex (ABC), or streptavidin-biotin-peroxidase complex (SBC) methods. We also present the application of this technique to tissue sections of *Fundulus heteroclitus* pituitaries embedded in the hydrophilic resin Historesin (hydroxy-ethyl-methacrylate), without deplastification and preserving a high resolution and immunostaining.

MATERIALS AND METHODS

Chemicals

All chemicals were analytical grade or better, purchased from Sigma Chemical (St. Louis, MO), Merck (Darmstadt, FRG), and Boerhinger (Mannheim, FRG).

Formulation of buffers

TBS (Tris Buffer Saline): 20 mM Tris-HCl, pH 7.5, 0.5 M NaCl; TTBS (Tween 20 Tris Buffer Saline): 0.05% Tween-20 in TBS; TCBS (Tween 20 Citrate Buffer Saline): 20 mM Na-citrate, pH 5.5, 0.5 M NaCl, 0.05% Tween-20.

Antibodies and immunological reagents

Polyclonal antibodies raised in the rabbit against the complete human luteinizing hormone (anti-hLH) and human chorionic gonadotropin (anti-hCG) were purchased from Fitzgerald (Chelm-sford, MA). Historesin embedding kit was from Reichert-Jung (Heidelberg, FRG) and Historesin mold tray was from LKB (Bromma, SW); Eukitt mounting medium was from Kindler GmbH (Freiburg, FRG); ABC and biotin blocking kit were both from Vector Laboratories (Burlingame, CA), and PG-HRP was from Bio-Rad (Richmond, CA). Goat antiserum to rabbit immunoglobulin G (anti-rIgG), biotinylated anti-rIgG, rabbit PAP, and streptavidin-peroxidase complex were from Sigma Chemical (St. Louis, MO).

Animals and tissue fixation

Fish (*Fundulus heteroclitus*) were collected in the salt-marshes surrounding the Bay of Cadiz (SW Spain) and kept in the laboratory in running sea-water. Animals were stunned in ice-water, killed by decapitation and the head fixed in phosphate buffer 0.1 M, pH 7.2, 4% formaldehyde, for two hours at room temperature. Brains with the pituitary attached were then carefully removed and further fixed overnight under the same conditions.

Embedding

After fixation, tissues were washed for one hour in running tap-water and embedded in paraffin or in the hydrophilic resin Historesin. For paraffin embedding, following dehydration in graded alcohol series, samples were transferred through graded acetone series to benzol and finally embedded in paraffin. Parasagittal sections (5-6 μm) were mounted on gelatin-coated glass-slides and deparaffinized through xylene. For Historesin embedding, tissues were dehydrated in graded alcohol series, preinfiltrated with 1:1 (v/v) absolute ethanol: infiltration solution (50 ml basic resin, hydroxyethylmethacrylate + 0.5 g activator, benzol peroxide) for two hours at room temperature to ensure even penetration, and then transferred to pure infiltration solution for 24 hours at 4°C. Infiltration was considered completed when the samples became translucent and sank. Polymerization

was carried in a Historesin mold tray filled with embedding medium (15 ml infiltration solution + 1 ml hardener, dimethyl sulfoxide) for 1-2 hours at room temperature. Sagittal sections (0.5-2 µm) of brain with the pituitary attached were mounted on gelatinized glass-slides. Sections embedded in Historesin were immunostained without plastic removal. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide in methanol for 30 min. Before immunostaining, sections were washed with distilled water, transferred for 1-2 hours to 3% bovine serum albumin (BSA) in TBS and washed (2 x 5 min) in TTBS. For sections processed according to the ABC and SBC methods, endogenous biotin was blocked with a biotin blocking kit.

Immunostaining techniques

Sections were incubated overnight in a moist chamber at 4°C with primary antibodies (anti-hCG or anti-hLH) diluted 1:500 to 1:5,000 in TBS containing 1% BSA; after rinsing in TTBS, we proceeded according to the following four immunoperoxidase methods:

Protein G-Horseradish Peroxidase (PG-HRP)

(a) 2 x 5 min wash in TCBS; (b) remove the excess of TCBS and incubate for 1-2 hours at room temperature with PG-HRP solution diluted 1:100 (v/v) in TCBS containing 1% BSA ; (c) 2 x 5 min wash in TTBS and 1 x 5 min wash in TBS; (d) incubate for 10 min at room temperature in the dark with 50 mM Tris-HCl, pH 7.6, 0.05% (w/v) 3-3'-diaminobenzidine tetrahydroclhoride, 0.01% (v/v) hydrogen peroxide; (e) 2 x 10 min wash in distilled water; (f) dehydrate in graded alcohol and clear in xylene, for paraffin sections, or dry on a hot plate for methacrylate sections; and (g) mount the sections in Eukitt.

Peroxidase-antiperoxidase (PAP)

(a) 2 x 5 min wash in TBS ; (b) incubate for 30 min with anti-rIgG diluted 1:25 in TBS containing 1% BSA; (c) 2 x 5 min wash in TBS; (d) incubate for 30 min at room temperature with PAP diluted 1:100 in TBS with 1% BSA ; (e) 2 x 5 min wash in TBS. Then, repeat steps (d), (e), (f) and (g) as in the PG-HRP technique.

Avidin-biotin-peroxidase complex (ABC)

All steps as above except for (b) incubated for 30 min at room temperature with biotinylated anti-rIgG diluted 1:50 in TBS with 1% BSA, and (d) incubate for 30 min at room temperature with ABC prepared before use according to the manufacturer's instructions.

Streptavidin-biotin-peroxidase complex (SBC)

All steps as in the ABC technique, except for (d) incubated for 30 min at room temperature with streptavidin-peroxidase complex diluted 1:100 in TBS with 1% BSA.

Controls

Control incubations conducted routinely to test for the specificity of the detection methods included substitution of the first, the second, or both antibodies, either by normal rabbit serum or by buffer. PG-HRP, PAP, ABC and streptavidin-peroxidase complexes were also replaced by normal rabbit serum or buffer.

RESULTS

Immunostaining reactions and the spatial distribution of gonadotrophic cells in the *F. heteroclitus* pituitary are similar for both antibodies we tested after all four immunocytochemical techniques. Nonspecific staining of cells and background are absent on control paraffin and historesin sections (Fig. 1).

Table 1 shows the staining reactions on paraffin embedded sections of pituitaries proccessed according to the four immunocytochemical techniques described in Material and Methods. At low dilutions of primary antisera (1:500 and 1:1,000), all four methods give an intense immunostaining of gonadotrophic cells in the ventral proximal pars distalis (Fig. 2). Background staining is evident in ABC, SBC and PAP methods (Figs. 2a, 2b and 2c, respectively). However, with the PG-HRP method, immunoreactive cells are clearly identified with no background (Fig. 2d). At higher dilutions of the primary antisera, both immunostaining and background are reduced for the ABC, SBC and PAP methods. No immunoreactivity is observed with the PG-HRP method at a 1:5,000

Table 1
Summary of immunocytochemical methods and staining reactions in paraffin sections of *Fundulus heteroclitus* pituitary

	Intensity of specific staining				Background staining			
Working dilutions	1:500	1:1,000	1:2,000	1:5,000	1:500	1:1,000	1:2,000	1:5,000
Immunocytochemical Methods								
ABC	+++	+++	++	+/++	+++	++	+	-
SBC	+++	++/+++	+/++	+	+++	+/++	+	-
PAP	+++	++	+/++	+	++	+	-	-
PG-HRP	+++	++	+	-	-	-	-	-

Results were similar with both anti-human LH and anti-human CG. The intensity of immunostaining was rated as follows: +++, strong staining; ++, moderate staining; +, weak staining; -, no staining. ABC: Avidin-biotin-peroxidase complex method; SBC: Streptavidin-biotin-peroxidase complex method; PAP: Peroxidase-antiperoxidase method; PG-HRP: Protein G-horseradish peroxidase method.

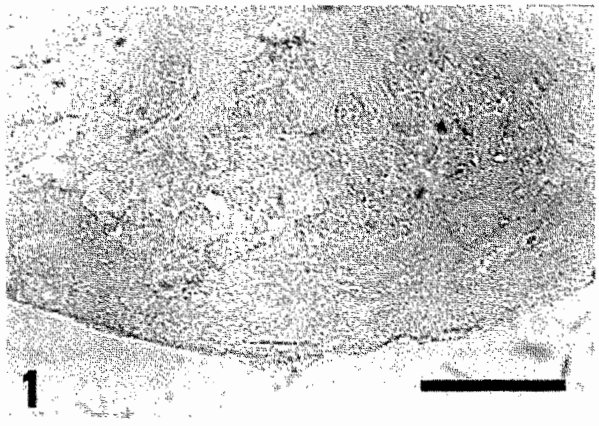

Fig. 1 - Light micrograph of proximal pars distalis in a control mid-sagittal paraffin embedded section of *F. heteroclitus* pituitary. First antibody was omitted. ABC method. Original magnification x 205. Bar = 100 µm.

dilution of the primary antisera. The ABC method provides the strongest specific staining at high dilutions of the primary antibodies.

For Historesin embedded sections, and considering the four methods as a whole, the immunostaining is weaker but similar in distribution to that obtained for paraffin embedded sections; however, cellular localization of immunostaining is more precise. Also, the ABC method gives the strongest staining, and the PG-HRP method gives no staining or very weak staining at a 1:5,000 dilution of the primary antisera. For the ABC, SBC and PAP methods, background is reduced or absent at low dilutions of the primary antibody, and for the PG-HRP method, background is absent irrespective of the primary antibody dilution. The results obtained with the PG-HRP technique are shown in Figure 3. The general morphology of gonadotrophic cells is well discerned (Fig. 3a); furthermore, the structural resolution of immunostaining is greatly enhanced, the immunoreactivity being restricted to rounded cytoplasmic secretory structures ranging from 0.9 to 1.8 µm in diameter (Fig. 3b).

DISCUSSION

In this study we show that the PG-HRP can be used in fish tissues as a substitute for secondary antibodies in indirect immunoperoxidase techniques, giving a clear immunocytochemical signal with reduced background. The

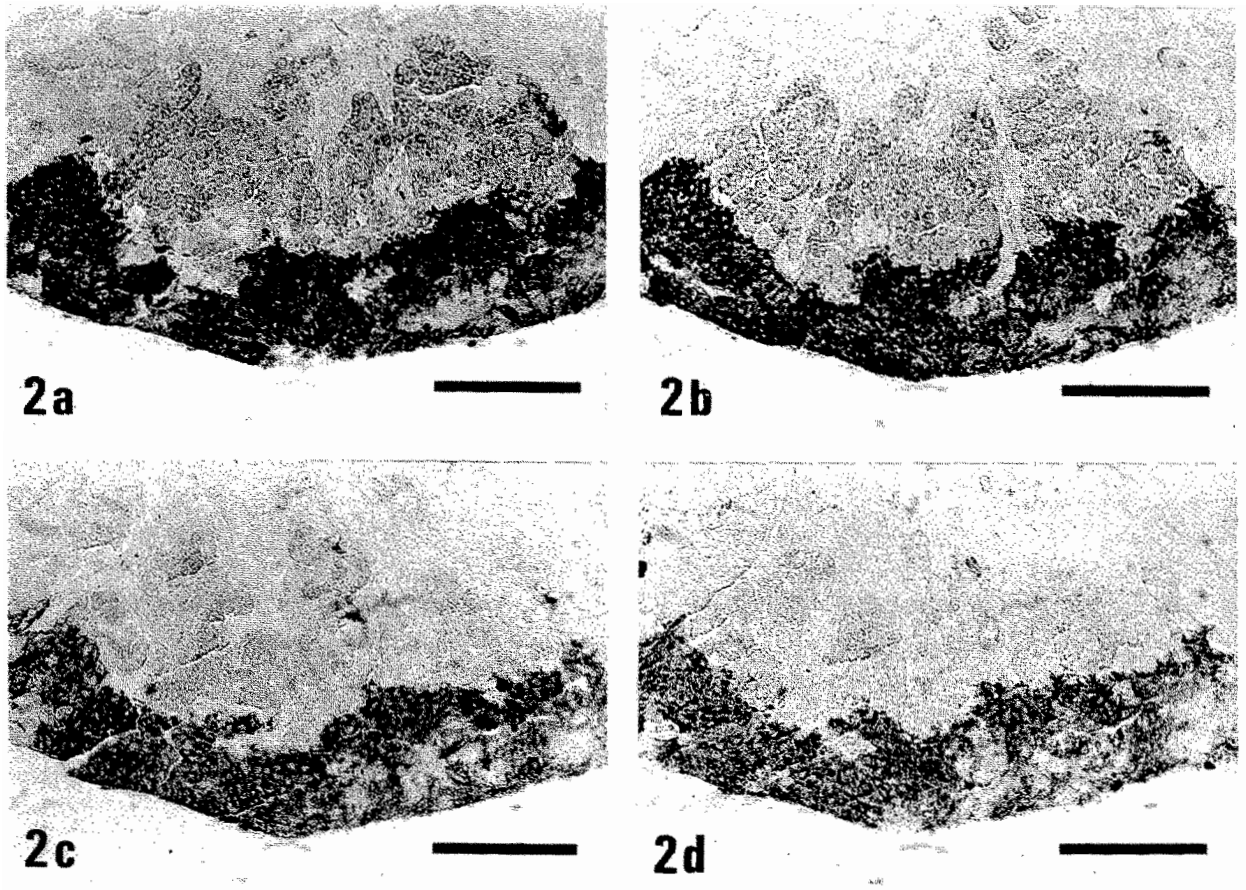

Fig. 2 - Light micrographs of proximal pars distalis in serial mid-sagittal paraffin embedded sections of *F. heteroclitus* pituitaries, immunostained with anti-human CG diluted 1:,000, and processed according to the ABC (a), SBC (b), PAP (c) and PG-HRP (d) methods. Note the differences in specific and background stainings. Original magnification x 205. Bars = 100 µm.

use of this IgG-binding protein avoids the constrain of raising the secondary antibody on a different species than that of the primary antibody (ABC and SBC methods) or, as in the PAP method, introducing a link-antibody directed against the IgG of the species from which the PAP complex was obtained.

Several studies compare the sensitivity and efficiency of different immunocytochemical methods (Burns, 1975; Celio *et al.*, 1979; Hsu *et al.*, 1981a, b; Scopsi and Larsson, 1986; Larsson, 1989). Celio *et al.* (1979), after comparing four immunoperoxidase techniques, conclude that the protein A-peroxidase gives more contrast and less background than PAP at low dilutions of the primary antibody, PAP being more sensitive at higher dilutions. In this study, we find a similar result when comparing the PG-HRP and PAP methods.

According to Hsu *et al.* (1981a), the ABC technique gives an immunostaining 20 to 40 times stronger than the PAP method. In our hands, too, the ABC method is the most sensitive and gives stronger immunostaining; but quantitatively our result is closer to that reported by Larsson (1989), i.e., 4 to 5 times stronger than PAP or other indirect immunostaining techniques.

On the other hand, the ABC method also exhibits the strongest background and nonspecific staining. Some of the nonspecific staining could be accounted for by a cross-reaction of

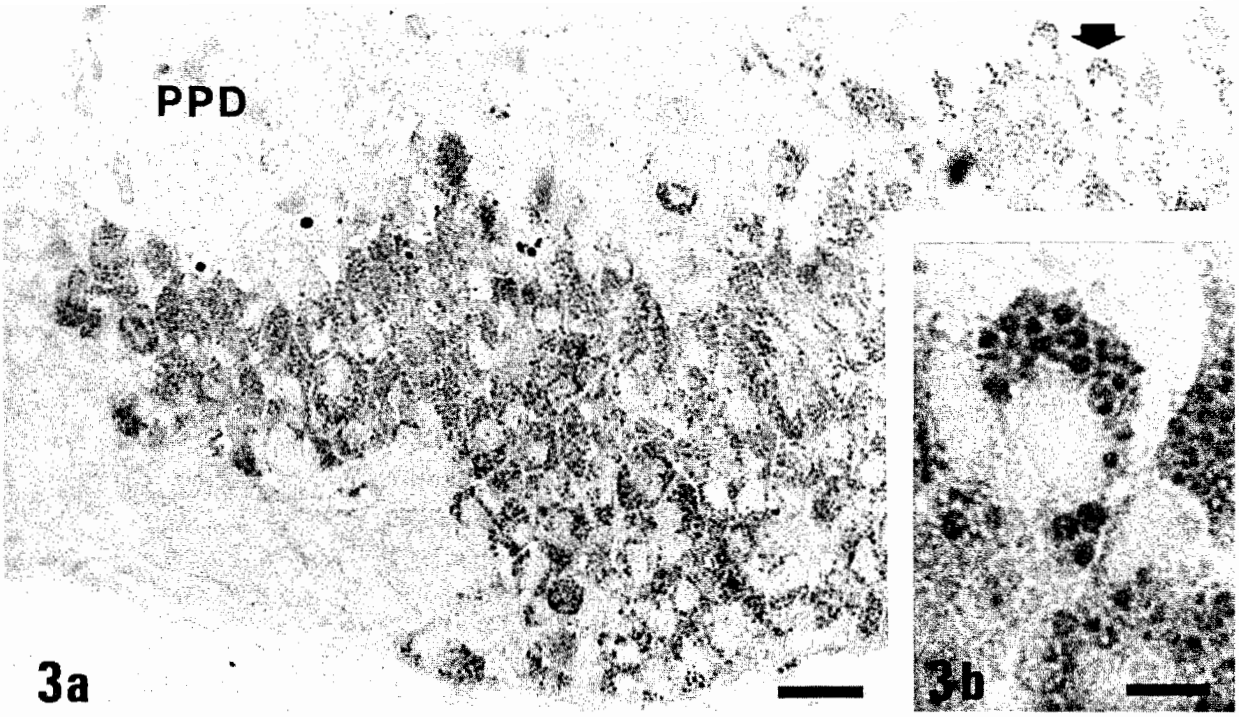

Fig. 3 - (a) Proximal pars distalis (PPD) in a sagittal Historesin embedded section of *F. heteroclitus* pituitary immunostained with anti-human CG at a 1:500 dilution and PG-HRP method. Original magnification x 550. Bar = 20 μm. Arrow marks the cell enlarged in (b). Immunoreactive secretory structures range from 0.9 to 1.8 μm in diameter. Original magnification x 2200. Bar = 5 μm.

the anti-hCG antibody with TSH in thyrotrophic cells, because the α-subunits of both gonadotrophic and thyrotrophic hormones present a variable degree of homology within Vertebrates (Stockell Hartree and Renwick, 1992). Nonspecific staining by the ABC technique may also be related to the pH (7.6) of the buffer system, considering that a decrease in the nonspecific staining of mast cells was found using a pH 9.4 buffer system (Bussolati and Gugliotta, 1983). Additionally, a high ABC concentration can cause nonspecific staining (Hsu *et al.*, 1981b).

The PG-HRP method gives less background than the ABC and SBC methods at dilutions of the primary antibody between 1:500 and 1:2,000. This may be due to the reduction of binding reactions which diminish nonspecific adsorption; as a consequence, less controls for specificity are needed. Nevertheless, like other indirect immunoperoxidase procedures, the PG-HRP technique shows less detection efficiency in terms of primary antibody dilution, compared to the ABC, SBC and PAP methods. Therefore, this technique is not recommended when small quantities of the antigen are present and minimal amounts of the primary antibody are available.

The results shown here demonstrate that the PG-HRP method, as well as the other procedures, can be applied effectively to tissue sections embedded in hydrophilic resin Historesin. Plastic embedded sections, by being much thinner (0.5-2 μm) than paraffin embedded sections (5-6 μm), contain a lower amount of antigen and thus show a weaker immunocytochemical staining; this constraint could be overcome by using methods such as diaminobenzidine intensification (Rodríguez *et al.*, 1984; Scopsi and Larsson, 1989). Nevertheless, the enhanced

structural resolution, the higher specific immunoreactivity and reduced background so attained, in comparison with paraffin embedding, allow the morphology of gonadotrophic cells, as well as the localization of immunoreactivity within rounded secretory structures ranging from 0.9 to 1.8 μm in diameter to be clearly discerned. These immunoreactive structures may represent an accumulation of secretory granules (0.1-0.5 μm diameter in fish). However, they could also represent the large secretory globules (0.5 to 2 μm in diameter) described in fish gonadotrophic cells (Van Oordt and Peute, 1983). At least in salmonids, granular and globular gonadotrophs seem to represent different stages of maturation. Although quantitative methods applied to immunoperoxidase techniques remain controversial, differences in number, size and intensity of immunostaining of secretory globules could be related with differences in the amount of antigen present and maturation stage. Hydrophilic resins achieve a more homogeneous tissue infiltration, polymerize at low temperature and do not require deplastification because they facilitate the interaction between aqueous reagents and substrates (Casanova *et al.*, 1983; Scala *et al.*, 1992). As a consequence, protein denaturization is minimized and antigenicity is better preserved (Carlemalm and Villiger, 1989). On the other hand, thin sections allow different antigens in the same cell to be compared by contrasting serial cuts.

To our knowledge, this is the first report studying the immunocytochemical application of PG-HRP in fishes. In addition to the pituitary, this method is also useful for other organs and tissues of fish processed with a variety of human and piscine primary antibodies (anti-human trypsin, anti-human gastrin, anti-gilthead sea bream growth hormone, anti-carp gonadotropin) (in preparation), opening the possibility for wider applications.

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REFERENCES

Åkerström B., Brodin T., Reis K., and Björck L.: Protein G: a powerful tool for binding and detection of monoclonal and polyclonal antibodies. J. Immunol. *135*, 2589-2592, 1985.

Bendayan M.: Protein A-gold and protein G-gold postembedding immunoelectron microscopy. In Hayat M.A. (ed) Colloidal Gold: Principles, Methods and Applications, vol. *1*. Academic Press, San Diego, pp. 33-94, 1989.

Bendayan M. and Garzon S.: Protein G-gold complex: comparative evaluation with the protein A-gold for high resolution immunocytochemistry. J. Histochem. Cytochem. *36*, 597-607, 1988.

Björck L. and Kronvall G.: Purification and some properties of streptococcal protein G, a novel IgG-binding reagent. J. Immunol. *133*, 969-974, 1984.

Burns J.: Background staining and sensitivity of the unlabelled antibody-enzyme (PAP) method. Comparison with the peroxidase labelled antibody sandwich method using formalin fixed paraffin embedded material. Histochemistry *43*, 291-294, 1975.

Bussolati G. and Gugliotta P.: Nonspecific staining of mast cells by avidin-biotin-peroxidase complexes (ABC). J. Histochem. Cytochem. *31*, 1419-1426, 1983.

Carlemalm E. and Villiger W.: Low temperature embedding. In Bullock G.R. and Petrusz P. (eds.) Techniques in Immunocytochemistry, vol. *4*. Academic Press, London, pp. 29-45, 1989.

Casanova S., Donini U., Zini N., Morelli R., and Zucchelli P.: Immunohistochemical staining on hydroxyethyl-methacrylate-embedded tissues. J. Histochem. Cytochem. *30*, 1000-1004, 1983.

Celio M.R., Lutz H., Binz H., and Fey H.: Protein A in immunoperoxidase techniques. J. Histochem. Cytochem. *27*, 691-693, 1979.

Dubois-Dalcq M., McFarland H., and McFarlin D.: Protein A-peroxidase: a valuable tool for the localization of antigens. J. Histochem. Cytochem. *25*, 1201-1206, 1977.

Hanker J.S., Yates P.E., Metz C.B., and Rustioni A.: A new specific, sensitive and non-carcinogenic reagent for the demonstration of horseradish peroxidase. Histochem. J. *9*, 789-792, 1977.

Hogan D.L. and Smith G.H.: Unconventional application of standard light and electron immunocytochemical analysis to aldehyde-fixed, araldite-embedded tissues. J. Histochem. Cytochem. *30*, 1301-1306, 1982.

Hsu S.-M., Raine L., and Fanger H.: A comparative study of the peroxidase-antiperoxidase method and an avidin-biotin complex method for studying polypeptide hormones with radioimmunoassay antibodies. Am. J. Clin. Pathol. *75*, 734-738, 1981 a.

Hsu S.-M., Raine L., and Fanger H.: Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. J. Histochem. Cytochem. *29*, 577-580, 1981 b.

Kawarai Y. and Nakane P.K.: Localization of tissue antigens on the ultrathin sections with peroxidase-labeled antibody method. J. Histochem. Cytochem. *18*, 161-166, 1970.

Kronvall G. and Frommel D.: Definition of staphylococcal protein A reactivity for human immunoglobulin G fragments. Immunochemistry *7*, 124-127, 1970.

Larsson L.-I.: Immunocytochemistry: Theory and Practice. 2nd ed. CRC Press, Boca Raton. 1989.

Nakane P.K. and Kawaoi A.: Peroxidase-labeled antibody. A new method of conjugation. J. Histochem. Cytochem. *22*, 1084-1091, 1974

Rodríguez E.M., Yulis R., Peruzzo B., Alvial G., and Andrade R.: Standarization of various applications of methacrylate embedding and silver methenamine for light and electron microscopy immunocytochemistry. Histochemistry *81*, 253-263, 1984.

Scala C., Cenacchi G., Ferrari C., Pasquinelli G., Preda P., and Manara G.C.: A new acrylic resin formulation: a useful tool for histological, ultrastructural, and immunocytochemical investigations. J. Histochem. Cytochem. *40*, 1799-1804, 1992.

Scopsi L. and Larsson L.-I.: Increased sensitivity in peroxidase immunocytochemistry. A comparative study of a number of peroxidase visualizing methods employing a model system. Histochemistry *84*, 221-230, 1986.

Sternberger L.A., Hardy P.H., Jr Cuculis J.J., and Meyer H.G.: The unlabeled antibody enzyme method of immunohistochemistry: preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. J. Histochem. Cytochem. *18*, 315-333, 1970.

Stockell Hartree A. and Renwick A.G.C.: Molecular structures of glycoprotein hormones and functions of their carbohydrate components. Biochem. J. *287*, 665-679, 1992.

Van Oordt P.G.W.J. and Peute J.: The cellular origin of pituitary gonadotropins in teleost. In: Hoar W.S., Randall D.J., and Donaldson E.M. (eds) Fish Physiology, vol. *IXA*. Academic Press, New York, pp. 137-186, 1983.