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Increased sensitivity in immunocytochemistry

Effects of double application of antibodies and of silver intensification on immunogold and peroxidase-antiperoxidase staining techniques

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Summary. Sensitivity and detection efficiency of immunocytochemical methods were tested on cytochemical models and tissue material, respectively. Use of silver intensification procedures revealed that staining with immunogold reagents could be rendered equally or even more sensitive than the standard peroxidase-antiperoxidase (PAP) method. Further increases in sensitivity with both methods could be obtained by double application of the primary antiserum. Combined use of the immunogold techniques and the PAP method with development in diaminobenzidine and subsequent silver intensification resulted in the most sensitive procedures. The procedures were applied to a wide variety of tissue preparations, including whole mount preparations of the external longitudinal muscle layer of the gut wall and were found not to produce any unspecific staining in any tissue tested. Use of immunogold-silver and, particularly of the combined immunogold-silver-PAP methods may be valuable for analyzing tissues and tumours containing small amounts of antigen, for testing the quality of immunogold staining procedures intended for ultrastructural studies and for electroblotting techniques.

Introduction

Colloidal gold constitutes an excellent probe for immunocytochemical and lectin staining techniques at the transmission and scanning electron microscopical level (De Mey et al. 1981; Geoghegan and Ackermann 1977; Geuze et al. 1981; Handley and Chien 1983; Horisberger and Rosset 1977; Horisberger 1979, 1981; Larsson 1979, 1981b; Roth 1982, 1983; Slot and Geuze 1981; Van den Pol 1984). One of the advantages of the probe is that it is highly electron-dense and thus easily recognised also in optimally contrasted transmission electron microscopic preparations. Colloidal gold particles may, hence, easily be quantitated by simple counting procedures. Moreover, they can be fabricated in different sizes, providing opportunities for double staining at the ultrastructural level.

Although colloidal gold particles, particularly those of larger diameters, confer a red stain to immunopositive elements at the light microscopical (LM) level (Gu et al. 1981), the detection efficiency of the gold as an LM marker is no better and probably poorer than that of preexisting methods and the probe has, thus, not enjoyed wide-spread use at this level. Use of polarized epiillumination may result in a higher detection efficiency of the gold probe but requires specialized microscopical equipment (De Mey et al. 1981). Access to a convenient all-purpose LM and EM level method is desirable, not least since this would enable investigators to test staining parameters at the LM level before advancing to ultrastructural studies.

Very recently, reports have appeared showing that metallic silver, through a process of physical development, can be deposited around colloidal gold particles (Danscher and Nørgaard 1983; Holgate et al. 1983a, b). As the length and intensity of the physical development is easily controlled, the amount of stain deposited (i.e. metallic silver) can also be controlled. Consequently, this new immunogold-silver staining method has been successfully exploited at the LM level and suggested to be more "sensitive" than the PAP method as expressed in terms of dilution of the primary antibody (Holgate et al. 1983a).

Stimulated by these reports, we have now evaluated the applicability of the immunogold-silver staining method for the detection of neurohormonal peptides in immunocytochemical models and in a wide variety of light microscopical tissue preparations. Moreover, we have also increased the detection efficiency and sensitivity of the PAP and immunogold-silver methods by combining them with each other and by employing the recently suggested double application immunocytochemical method of Gu et al. (1983). For comparisons between the immunogold-silver method and the PAP method we have also applied the silver intensification procedure of Gallyas et al. (1982) to the latter.

Material and methods

Tissue material: Adult albino rats (body-weight 150 to 200 g) were anaesthesized with diethyl ether and either decapitated or perfused via the heart with 20 ml saline followed by 60–80 ml 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. Antropyloric mucosa, duodenal lobe and tail of pancreas from non-perfused rats were immersionfixed over-night in Bouin's solution and routinely embedded in paraffin. Antropyloric and oxyntic mucosa, duodenum, duodenal lobe and tail of pancreas from the formaldehyde-perfused rats were postfixed in 4% paraformaldehyde at 4° C overnight and then either routinely embedded in paraffin or soaked in 20% sucrose in 0.1 M sodium phosphate buffer, pH 7.4, and frozen in melting Freon-13 for cryostat sectioning (Larsson 1981b). Jejunum and ileum of the formaldehyde-perfused rats were, after

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postfixation in formaldehyde over-night dehydrated in ascending ethanols, cleared in xylene and then rehydrated in descending ethanols (permeabilization procedure, cf. Larsson and Stengaard-Pedersen 1982), transferred to 0.05 M Tris buffer, pH 7.4, containing 0.15 M NaCl and 1% triton X-100 (TBS-triton), whereafter the longitudinal muscle with the adhering myenteric plexus was separated from the rest of the gut wall with a pair of jewellers forceps. In addition, specimens from the antropyloric mucosa and pancreas of mice and rats were rapidly dissected out from diethyl ether-anaesthesized animals, snap-frozen in melting Freon-22, freeze-dried, exposed to paraformaldehyde gas for 1 h at 80° C and embedded in paraffin in vacuo (for details see Björklund et al. 1972). Green monkeys were anaesthesized with Nembutal and pieces of the antropyloric mucosa and the duodenal lobe of the pancreas rapidly dissected out and immersionfixed in a 3% paraformaldehyde: 2% glutaraldehyde mixture in 0.1 M sodium phosphate buffer pH 7.3 for 15 min at 4° C. After fixation the specimens were rinsed for 3 h in 0.1 M sodium cacodylate buffer pH 7.3, dehydrated in graded ethanols and embedded in Epon 812 over propylene oxide (polymerization at 45° C for 3 days).

Immunocytochemical models: Strips of Whatman No. 1 filter paper and of nitrocellulose filter (0.45 μm pore size; Millipore HAWP 000 10) were used. Droplets of 2 μl of pentagastrin solutions (Peptavlon, ICI) were applied so that initially a concentration range of peptide from 1 μg to 10 pg was covered. In most subsequent experiments the range was narrowed so that the total amounts of peptide added in the 2 μl volume were 40, 120, 360, 1000, 3,000, and 10,000 pg. The paper or nitrocellulose filters were dried with a gentle stream of air from a hairdryer and were then exposed to paraformaldehyde vapours at 80° C for 1 h as described (Larsson 1981a). After this treatment the strips with the immobilized peptides were submitted to immunocytochemical staining exactly as described for the tissue material below. Antiserum No. 2717, which recognizes the C-terminal tetrapeptide amide of gastrin/CCK, contained within the sequence of pentagastrin, was used for staining the models.

Antisera: Antisera raised in rabbits against synthetic human gastrin I (Ab. 2717, 4562), synthetic met-enkephalin (Ab. KA_3), synthetic somatostatin-14 (Ab. R37/3), synthetic somatostatin-28[1–14], serotonin, glucagon (Ab. 4304) and human pancreatic polypeptide (HPP), and in guinea pigs against porcine insulin, previously characterized and described in detail, were employed at dilutions varying between 1:800 and 1:2 048 000 (Larsson 1981a and b; Larsson et al. 1976, 1979; Larsson and Moody 1980; Larsson and Rehfeld 1979; Larsson and Stengaard-Pedersen 1982).

Immunocytochemical staining: Cryostat sections (7 μm) and immunocytochemical models were soaked in TBS-triton, paraffin sections (5 μm) were deparaffinized and carried down to TBS-triton through descending ethanols and Epon sections (0.5–1 μm) were deplastisized in saturated KOH in ethanol, and rinsed in TBS-triton (Larsson 1981b). Subsequently, all specimens were incubated with 1% human serum albumin (HSA) in TBS for 1 h at room temperature, briefly rinsed in TBS-triton and exposed to the various antisera diluted in TBS containing 0.25% bovine serum albumin for 20 h at 4° C followed by 2 h of reequilibration at room temperature. All specimens were then rinsed for 3 × 10 min in TBS-triton, whereafter some were reincubated for a second time (20 + 2 h as above) with the primary antiserum and others were carried directly to the subsequent procedure. Occasionally, some specimens were continuously incubated for 42 h at 4° C plus a subsequent 2 h at room temperature and then submitted to the subsequent procedure.

The site of antigen-antibody reaction was revealed by *1*) the peroxidase-antiperoxidase (PAP) procedure of Sternberger (1979) using unlabelled antirabbit IgG (SBL, Stockholm, Sweden), diluted 1:30 for 30 min at room temperature in case of primary rabbit antibodies and unlabelled antiguinea pig IgG cross-reacting with rabbit IgG (Cappel Labs.), diluted 1:20 for 1 h at room temperature in the case of primary guinea pig antibodies and a commercial PAP complex (Dakopatts, Copenhagen, Denmark), diluted 1:75 for 30 min at room temperature. The reaction product was revealed by the diaminobenzidine (DAB) procedure of Graham and Karnovsky (1966) without postosmification of the reaction product; *2*) colloidal gold particles (5, 12 or 18 nm in diameter) coated with antirabbit or antiguinea pig IgG. The 5 nm gold particles were coated with affinity-purified antirabbit or antiguinea pig IgG and obtained from Janssen Pharmaceutica (Beerse, Belgium). The 12 nm gold particles were fabricated by the sodium ascorbate method (Horisberger 1979) and the 18 nm particles by the sodium citrate method of Frens (1973). These particles were coated with unlabelled antirabbit IgG from sheep (SBL, Stockholm, Sweden) according to Geoghegan and Ackermann (1977). All types of gold particles were diluted between 1:5 and 1:30 in gold buffer (0.05 M or 0.5 M Trisbuffer pH 7.4 containing 0.15 M NaCl, 1% BSA and 0.05% sodium azide) with or without 0.3% triton X-100 added. Diluted gold particles were applied to tissue sections and models for 30 min or 1 h at room temperature and to whole-mount preparations for 20 h at 4° C and for a subsequent 2 h at room temperature. Subsequently, specimens were washed for 3 × 10 min in TBS-triton and whole-mounts washed for 3–24 h in TBS-triton. The sections were first observed directly in the light microscope and then submitted to silver intensification.

Silver intensification: Sections, immunocytochemical models and whole-mounts stained by either the PAP method and DAB-development or by the colloidal gold method were submitted to one of three silver intensification methods:

1) The silver nitrate/hydroquinone method of Timm as modified by Danscher and Schrøder (1979). The specimens coming from TBS-triton were rinsed for 10 min in TBS without triton and then rinsed for 10 min in at least 3 changes of redistilled water. Subsequently, the specimens were immersed in the silver solution consisting of 60 volumes gum arabic stock solution + 10 volumes citrate buffer + 30 volumes hydroquinone solution + 0.5 volumes silver nitrate solution. The silver nitrate solution was added to the mixture only just before development commenced. The gum arabic stock solution was prepared by dissolving 1 kg gum arabic (Merck) in 2 l distilled water under vigorous stirring for 3–4 h. The solution was allowed to stand for 4 days at room temperature and was then filtered through multiple layers of gauze and stored in aliquots at − 20° C. This stock solution was used either undiluted or diluted 1 + 1, 1 + 3 or 1 + 9 with redistilled water. Occasionally, the gum arabic component was substituted for redistilled water. The citrate buffer was prepared by dissolving 25.5 g citric acid and 23.5 g trisodium citrate dihydrate in 100 ml redistilled water and, if necessary, adjusting the pH to 3.5 with citric acid. The hydroquinone solution was composed of 0.85 g hydroquinone (Fotopur, Merck) in 15 ml redistilled water and the silver nitrate solution of 85 mg silver nitrate in a final volume of 0.5 ml redistilled water. The hydroquinone solution has to be carefully dissolved under stirring. If necessary, dissolution can be increased by gentle warming. Both the silver nitrate and the hydroquinone solutions should be protected from light. All solutions except the gum arabic stock should be freshly prepared.

The specimens were left in this solution protected from light for 5 min to 2 h at room temperature or at 26° C. Following development the specimens were rinsed in running tap water of 37–40° C for at least 40 min. Subsequently, the sections were either mounted in glycerine-TBS (4:1) or dehydrated, cleared in xylene and mounted in Canada balsam.

2) The silver lactate/hydroquinone method of Danscher (1981). The specimens were washed as described for the above silver method and immersed in 60 volumes of gum arabic stock solution + 10 volumes citrate buffer + 15 volumes of hydroquinone solution + 15 volumes of silver lactate solution. The gum arabic stock solution, the citrate buffer and the hydroquinone solution were prepared as in the preceeding method. The silver lactate solution was freshly

prepared by dissolving 0.11 g silver lactate (purum, Fluka) in 15 ml redistilled water while protected from light. It was added to the developing solution just prior to use.

Specimens were developed protected from light for 5 min to 2 h at room temperature or at 26° C. They were then rinsed and mounted as in the preceeding method.

3) The silver intensification method of Gallyas et al. (1982). Sections were rinsed in TBS and then exposed to a solution consisting of 20 ml conc. thioglycolic acid, 5 ml 37% HCl and 75 ml distilled water at room temperature for 4 h. After washing in three changes of redistilled water (5 min each) the specimens were immersed in the physical developer made up freshly by slowly adding 10 ml of stock solution B (2.0 g ammonium nitrate, 2.0 g silver nitrate, 10 g tungstosilicic acid and 5 ml 37% formaldehyde added in the order given to 1,000 ml redistilled water) to 10 ml of stock solution A (50 g anhydrous sodium carbonate dissolved in 1,000 ml redistilled water) under vigorous stirring. The sections were developed until the desired degree of intensification was reached (about 5–10 min) and were then washed in 3 changes of 1% acetic acid (10 min each) and in 3 changes of redistilled water (10 min each).

Controls: Conventional staining controls as recommended (Sternberger 1979; Larsson 1981b), including deletion of the various antibody layers in each procedure and substitution of primary antisera with normal rabbit serum or with unrelated hyperimmune sera were carried out. All antisera used have been extensively characterized before with respect to their region specificity for the different antigens and absorption controls have been performed on the tissues described (Larsson 1981a and b; Larsson et al. 1976, 1979; Larsson and Moody 1980; Larsson and Rehfeld 1979; Larsson and Stengaard-Pedersen 1982). All controls were negative and, with the exception of the Gallyas method, as described in the text, the silver intensification procedures did not induce unspecific staining in the tissue. Thus, in agreement with the findings of Danscher (1981), the silver methods employed do not seem to detect endogenous heavy metals in the tissue unless specific pretreatment and fixation precautions are undertaken.

Results

Initial screening of the various methods employed was carried out with gastrin antiserum 2717 on Bouin-fixed, paraffin-embedded sections of rat antropyloric mucosa.

Double application of this antibody resulted in an increase in the working dilution with all methods tested (PAP method: single application 1:128,000; double application: 1:512,000). This was observed also with the other antibodies tested (Abs. 4562, KA_3, R37/3, anti-somatostatin-28[1–14], anti-serotonin, 4304, anti-HPP and anti-insulin) although the magnitude of increase in dilution varied from antibody to antibody. Simple prolongation of the single incubation from 20+2 h to 42+2 h also resulted in an increase in the working dilution. Extensive comparisons between long continous single incubations and double incubations of equivalent lengths of time were not carried out. However, clear-cut differences between the two modes of application were not observed on sections (cf. results on immunocytochemical models later).

Comparisons between the different methods on Bouin-fixed paraffin sections of rat antropyloric mucosa using either single or double application of Ab. 2717 revealed that the highest end-point dilution was obtained with the immunogold-silver methods and that more concentrated antibody solutions had to be used with the PAP method (Fig. 1). In terms of end-point dilution the silver nitrate and silver lactate intensification methods (methods Nos. 1 and 2) proved equally effective in enhancing immunogold staining. The Gallyas method (No. 3), however, was less efficient for immunogold intensification and was less easy to handle because of the very short development time necessary for specimens that had not been pretreated with the thioglycolic acid solution (times over 2–3 min produced excessive background). In contrast, in specimens stained with the PAP method, the Gallyas method (No. 3) was preferred and produced excellent results in tissues pretreated with thioglycolic acid (Fig. 1). The background problem of the Gallyas method could thus be handled by pretreating the sections for at least 4 h with the thioglycolic acid solution, which reduces general tissue argyrophilia. The two other silver developers were preferred for immunogold intensification, as these produced no unspecific staining/argyrophilia in any of the tissues studied (as listed under Material and methods) and as they consequently did not require at 4-h thioglycolic acid pretreatment. Development times for both the silver nitrate/hydroquinone and silver lactate/hydroquinone methods were similar and averaged 50 min if the concentrated gum arabic stock solution was used. With decreasing concentration of gum arabic the development time also decreased (without gum arabic: about 10 min, with the stock solution diluted 1+9: about 20 min and with 1+3: 30 min). Otherwise no major differences in tissue staining results with various concentrations of gum arabic were noted. One particular advantage of a longer development time is that the quality of the staining can be observed in the microscope and the development prolonged if deemed necessary.

Further comparisons between the two immunogold-silver methods (No. 1 and 2) and the PAP method (without silver intensification) were carried out with other antisera and other tissue preparations including cryostat sections, whole mount preparations of the intestinal wall and semithin, non-osmicated Epon sections. Organs tested included the gastrointestinal tract and the pancreas and sera used included somatostatin, met-enkephalin, gastrin, insulin, glucagon, HPP and serotonin antisera (for a complete listing see Material and methods). All antisera worked well with all methods in all tissues and tissue preparations used. Invariably, the immunogold-silver methods produced clear staining results with, at optimal antisera dilutions, minimal background and permitted use of antisera at higher dilutions than the PAP method did (Figs. 1 and 2). With the whole-mount preparations from the intestinal wall good results were invariably obtained with the PAP method. With the immunogold-silver methods results were at first disappointing when 12 nm gold particles and ordinary staining buffers without triton X-100 were used. Attempts to solve the problem aimed at increasing the penetration of the gold particles into the tissue by using smaller gold particles, and by varying the molarity and content of triton X-100 in the gold buffer. Use of 5 nm gold particles diluted 1:10 in 0.5 M gold buffer containing 0.3% triton X-100 provided a good localization of enkephalin immunoreactive nerve terminals in whole-mount preparations of the myenteric plexus (Fig. 2). Small gold particles (5 nm) also produced better results on tissue sections than did larger gold particles (12 and 18 nm). Addition of 0.3% triton X-100 to the gold buffer used for diluting the gold particles also resulted in a lower background, whereas the molarity of

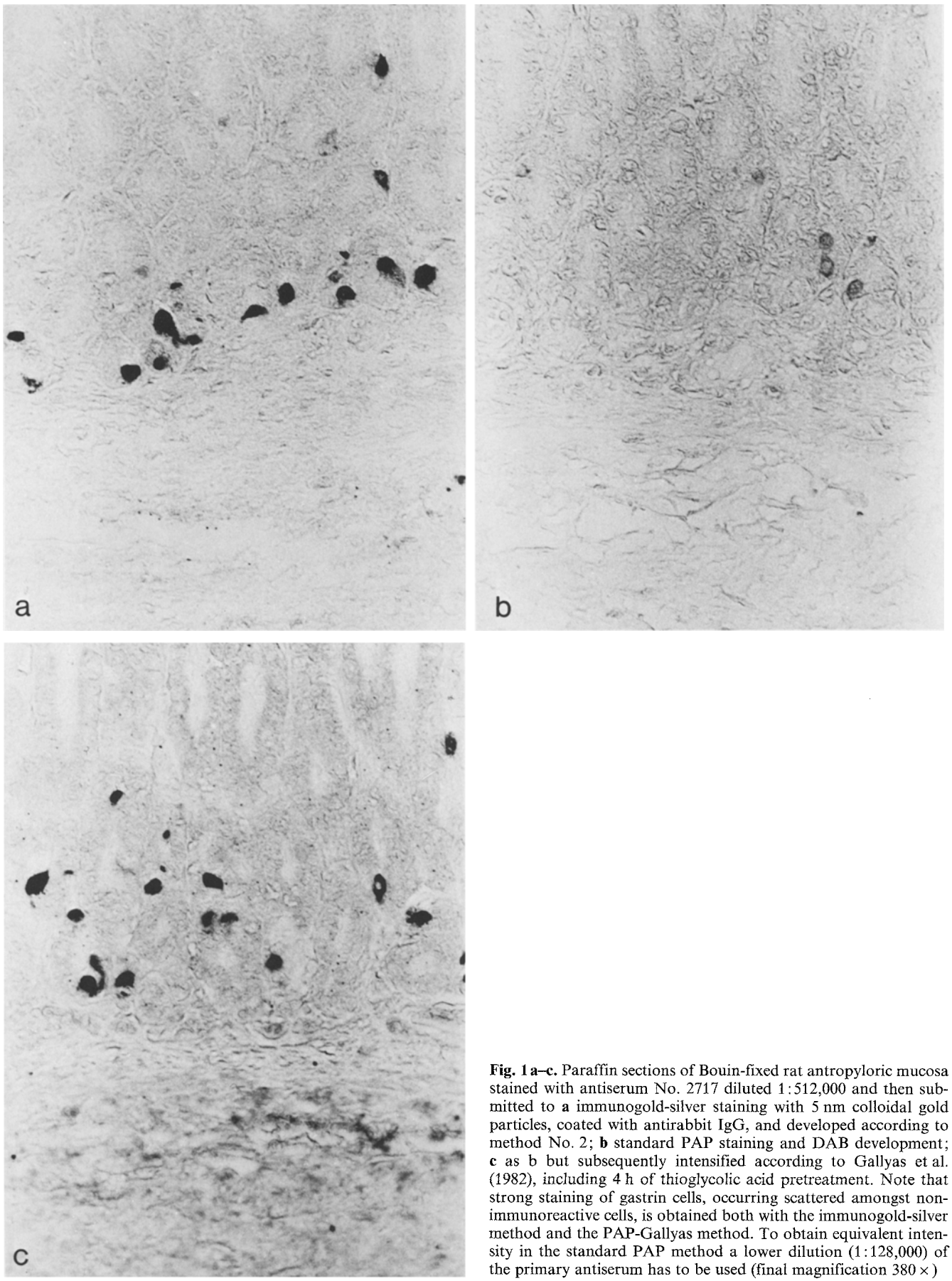

Fig. 1 a–c. Paraffin sections of Bouin-fixed rat antropyloric mucosa stained with antiserum No. 2717 diluted 1:512,000 and then submitted to **a** immunogold-silver staining with 5 nm colloidal gold particles, coated with antirabbit IgG, and developed according to method No. 2; **b** standard PAP staining and DAB development; **c** as b but subsequently intensified according to Gallyas et al. (1982), including 4 h of thioglycolic acid pretreatment. Note that strong staining of gastrin cells, occurring scattered amongst non-immunoreactive cells, is obtained both with the immunogold-silver method and the PAP-Gallyas method. To obtain equivalent intensity in the standard PAP method a lower dilution (1:128,000) of the primary antiserum has to be used (final magnification 380 ×)

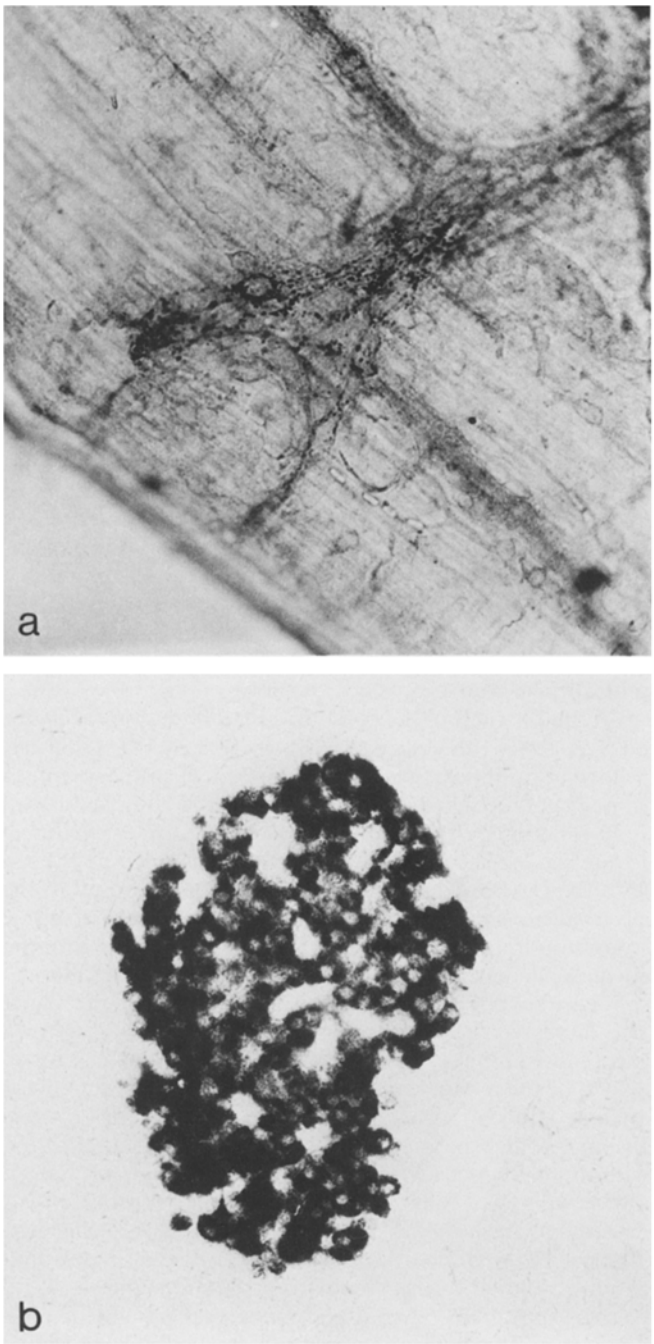

Fig. 2a, b. In **a** is shown a whole-mount preparation of rat ileal longitudinal muscle wall with its adherent myenteric plexus. The specimen was stained for demonstration of enkephalin immunoreactive nerve fibers using antiserum KA_3, 5 nm antirabbit IgG-gold particles and silver development according to method No. 2. Enkephalin nerves appear as dark varicose fibers in ganglia and interganglionic strands of the myenteric plexus (final magnification 304 ×). In **b** is shown a rat pancreatic islet stained with a guinea pig anti-insulin serum, 5 nm antiguinea pig IgG-gold particles and silver development according to method No. 2. Insulin cells are heavily stained against an unstained background (final magnification 304 ×)

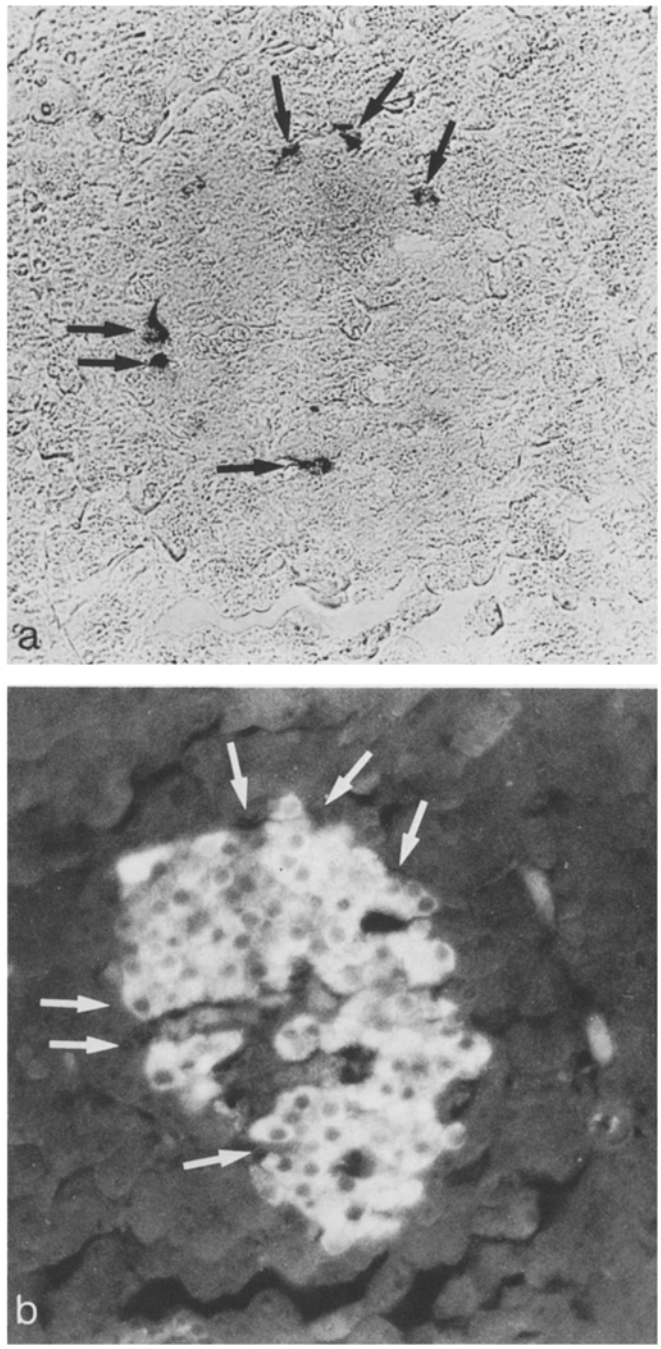

Fig. 3a, b. Rat pancreatic islet first stained with anti-somatostatin-28[1–14] and the immunogold silver procedure (**a**). Subsequently, the section was stained for insulin using indirect immunofluorescence (**b**). Note that different cells stain for somatostatin (*arrows*) and for insulin and that the preceeding silver stain does not interfere with the subsequent insulin immunofluorescence. The high contrast in the immunogold-silver method picture (**a**) is due to the fact that the specimen is mounted in glycerin-TBS for the immunofluorescence. (final magnification 380 ×)

the buffer (0.5 versus 0.05 M) did not seem to have a major impact on our staining results with tissue sections.

In order to test whether the immunogold-silver staining adversely affected the antigenicity of other peptides a set of double-staining experiments was performed. Sections of pancreas were first stained with anti-insulin, anti-somatostatin-14 (R37/3), anti-somatostatin-28[1–14], anti-HPP or anti-glucagon (4304) and developed with the immunogold-silver method (No. 2). Subsequently the stained sections were restained for another hormone using indirect immunofluorescence (the same antisera as those above) (Fig. 3).

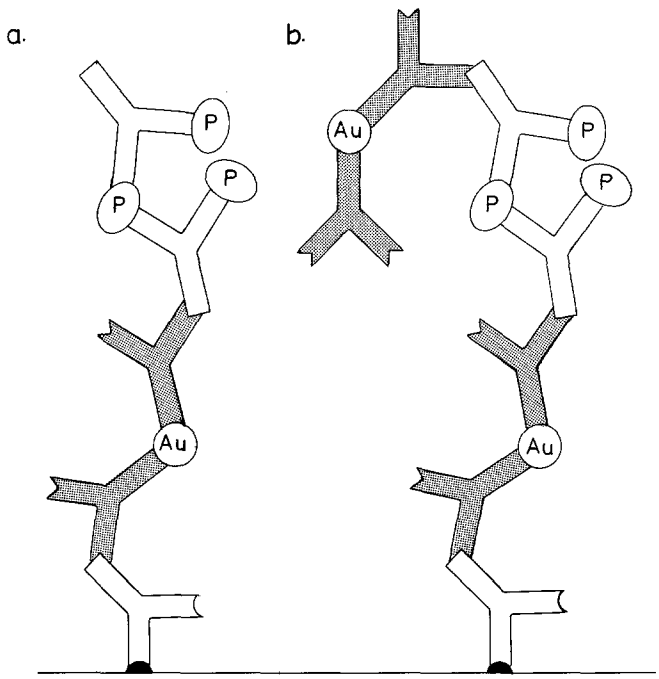

Fig. 4a, b. Schematic drawing illustrating the principles of the three-(**a**) and four-(**b**)-layer combined immunogold-silver-PAP methods. The three-layer method depends upon the use of antirabbit IgG-coated colloidal gold particles (*Au*) as a link between the primary, tissue-bound rabbit antibody and the rabbit PAP complex (*P* = peroxidase molecules). In the four-layer method (**b**), antirabbit IgG-coated colloidal gold is applied also after the PAP complex (antirabbit IgG molecules are hatched). Free antigenic epitopes on the rabbit PAP complex will, hence, react with the antirabbit IgG-coated colloidal gold and thereby affix more gold particles per tissue antigen. For simplicity only one site of reaction between the latter and the PAP complex has been illustrated. In reality, multiple points of reaction exist and thereby the number of gold particles and peroxidase molecules per molecule of antigen is greatly amplified

In all combinations tested previous immunogold-silver staining of one antigen was found not to destroy the reactivity of other antigens present in the same section.

In one set of experiments we ventured to combine the immunogold method and the PAP method. Thus, after incubation with the primary antiserum (2717), sections were exposed to gold-labelled antirabbit IgG (5 nm, diluted 1:10–1:30 for 30 min–1 h at room temperature) and then to the PAP complex (diluted 1:75 for 30 min at room temperature) (Fig. 4). Subsequently, sections were developed with diaminobenzidine for a maximum of 10 min and were thereafter either observed directly or were exposed to the silver developer (method No. 1 or 2) for an average of 1–2 min. Visual inspection of the sections revealed that a higher staining intensity of immunoreactive antropyloric gastrin cells was achieved by this combination than with immunogold-silver staining or PAP staining alone.

In order to define our results in terms of absolute sensitivity (i.e. minimum amount of antigen that can be distinguished from zero) rather than in terms of detection efficiency (i.e. dilutions), immunocytochemical model staining experiments were carried out on nitrocellulose or filter paper models (Fig. 5). Synthetic pentagastrin was used as antigen and antibody No. 2717 as primary antiserum. Models containing various concentrations of pentagastrin revealed that with the standard PAP method the smallest amount detectable was 1,000 pg, whereas with subsequent intensification according to Gallyas et al. (1982) 360 pg could be detected (the background of nitrocellulose presenting a problem). With immunogold-silver staining and development according to method No. 2 (using undiluted gum arabic stock solution) 360 pg were barely detectable whereas with method No. 1 (undiluted gum arabic stock solution) 120 pg were barely detectable. With immunogold-silver staining and development according to method No. 3 1,000 pg were barely detectable. With immunogold-silver staining and development according to method No. 2 but with varying concentrations of gum arabic the sensitivity increased somewhat as the concentration of gum arabic was lowered. Best result was obtained with the gum arabic stock solution diluted 1+9 when 120 pg became detectable. Development according to this method without gum arabic did not produce a further increase in sensitivity. Combined staining with the immunogold-PAP techniques as described above for sections, followed by DAB development and by silver intensification according to method No. 2 (undiluted gum arabic stock solution) did result in the 40 pg spot becoming barely detectable. Somewhat stronger staining of the spots with this method was obtained by reducing the gum arabic concentration as above.

A final variant of the combined PAP immunogold method was tested on nitrocellulose. In this modification the following antibody layers were applied *1*) primary antiserum; *2*) gold-labelled sheep antirabbit-gold complex (5 nm) 1:30 30 min; *3*) PAP complex 1:75 30 min; *4*) identical to the second layer. Thereafter, the strips were developed with the DAB method (5–10 min) and then exposed to the silver lactate solution (method No. 2; undiluted gum arabic stock solution). In this variant the spots stained even more strongly than in the PAP-immunogold combination described above. However, as the background of the paper also increased the minimum detectable quantity of antigen was higher (120 pg yielding a well-stained spot) (Fig. 5).

All of the model experiments referred to above involved a single application of the primary antiserum for 20+2 h. In one set of experiments we compared single application with double application (2 × 20+2 h) and single prolonged application (42+2 h). The antigen-antibody reaction was detected by the immunogold-silver method No. 2 using gum arabic solution diluted 1+3. With this method single application for 20+2 h (using equivalent dilutions of Ab. 2717) resulted in the 360 pg spot becoming weakly stained. With 42+2 h of incubation no more spots became detectable but the staining of the 360 pg spot was slightly intensified. With double application (2 × 20+2 h) the 120 pg spot became weakly stained.

Comparisons were carried out using both nitrocellulose and filter paper as support. With the filter paper used (Whatman No. 1) the 2 µl droplets spread over a larger area than on the nitrocellulose. However, the droplets seemed to spread also on the nitrocellulose but only on the back, producing a target-like area. The results, as expressed in minimum detectable amounts were the same for paper and nitrocellulose.

Discussion

In order to define sensitivity at the immunocytochemical level use of model experiments is necessary. The term sensi-

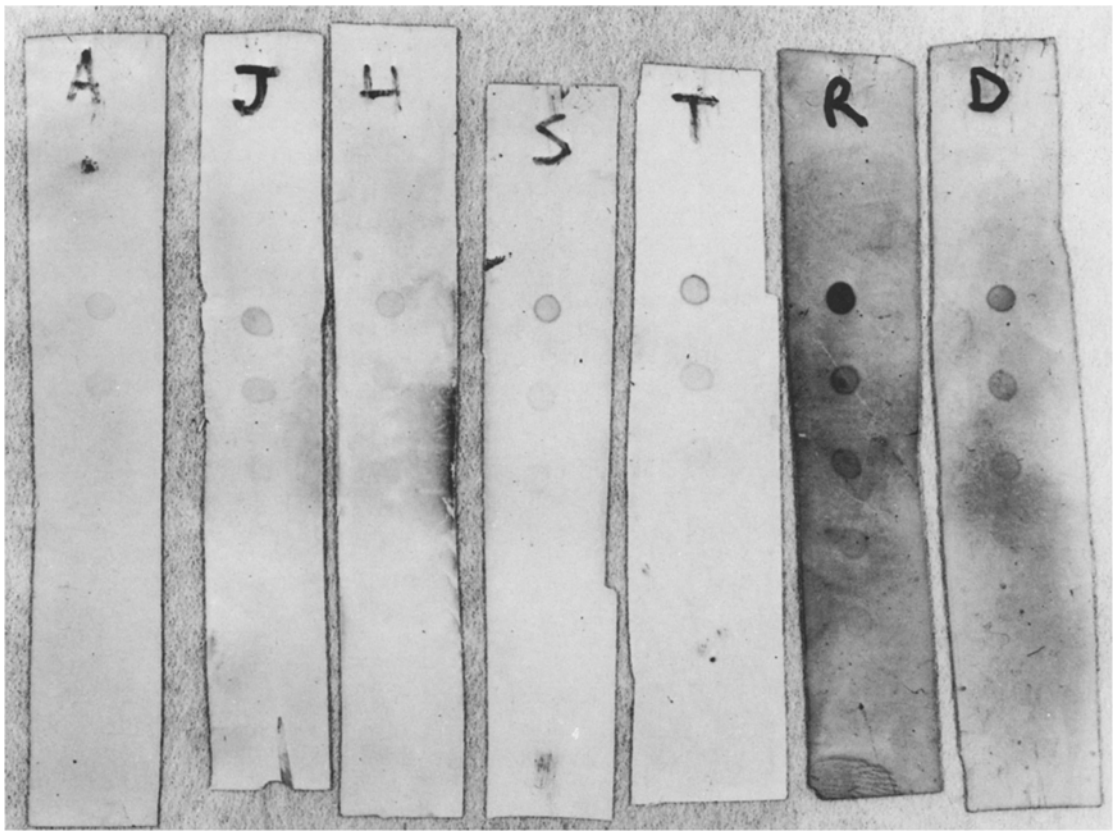

Fig. 5. Nitrocellulose immunocytochemical models containing 2 µl droplets of pentagastrin (final content per spot: 1,000 pg, 360 pg, 120 pg, 40 pg and 12 pg in descending order from top to bottom on each strip). All strips were stained with Ab. 2717 diluted 1:16,000 for 20+2 h (*A, J, H, R* and *D*), for 40+2 h (*S*) or 2×20+2 h (*T*). The site of antigen-antibody reaction in the strips was then revealed either by the standard PAP technique and DAB-development (*A*), by the immunogold silver method and development according to method No. 2 with gum arabic stock solution diluted 1+3 (*J* and *S*), similar to *J* and *S* but using method No. 1 and gum arabic stock solution diluted 1+3 (*H* and *T*), by the combined four-layer immunogold-silver-PAP method and development in DAB for 15 min and then in silver method No. 2 using gum arabic stock solution diluted 1+3 for 15 min (*R*), and by the three-layer combined immunogold-silver-PAP method and development as *R* (*D*). Although some of the contrast is lost in the photographic reproduction it is obvious that the standard PAP method is the least sensitive and the combined immunogold-silver-PAP methods the most sensitive (cf. text)

tivity is often confused with "detection efficiency" as defined by Petrusz and co-workers (1975, 1980). The effective working dilution (or end-point dilution) is often cited as an argument for the "sensitivity" of a method (cf. Holgate et al. 1983a). However, when only this parameter is used we agree with Petrusz et al. (1975, 1980) that the term sensitivity should be replaced by "detection efficiency".

Studies on cytochemical paper or nitrocellulose models allows us to define a minimum detectable amount of antigen and hence to use the term sensitivity in its original biochemical meaning (Larsson 1981a).

Our model experiments clearly demonstrate that the combined immunogold-silver-PAP methods are the most sensitive so far tested by us. Simple immunogold-silver staining is somewhat less sensitive and the concentration of gum arabic as well as the nature of the silver salt used (silver lactate or silver nitrate) influences the sensitivity. Thus, with the immunogold-silver methods use of low concentrations of gum arabic along with silver nitrate (method No. 1) produces the most sensitive staining. By comparison, the standard PAP method is much less sensitive. In tissue material, intensification of the PAP method by the procedure of Gallyas et al. (1982) produces a tremendous increase in detection efficiency. Unfortunately, as this method (both with and without thioglycolic acid pretreatment) produced an excessive background on paper and nitrocellulose models we could not compare its sensitivity with the other methods.

Another major factor that influenced the sensitivity in our model experiments was the mode of application of the primary antiserum. Prolongation of the incubation time or double application of the primary antiserum for an equivalent length of time both produced important increases in sensitivity. As double-application was slightly more sensitive than single, prolonged incubation this method may be preferred. Gu et al. (1983) have discussed the possibility that the beneficial effect of double application is due to the fact that polyclonal antisera, such as those used in their and in our study, are heterogenous with respect to the binding avidity of their component antibodies. Thus, upon washing, part of the antibodies with weak avidity are dislodged from their binding sites in the tissue and do not contribute to subsequent immunocytochemical staining. By introducing an intermediate step of washing during the incubation, antigenic epitopes may hence become free of poorly bound antibodies and may bind to more avid antibodies in the subsequent incubation. Our quantitative model staining experiments concur with this tentative explana-

tion since lower concentrations of antigens can be detected upon double, as opposed to prolonged, single, application of the primary antiserum.

The results obtained from the model experiments agree closely with our tissue staining experiments although strict quantitative data are not possible to obtain in the latter. Thus, using rat antropyloric gastrin cells as substrate the same staining parameters as those tested with the cytochemical models were investigated. In the case of the gastrin cells no difference between single, prolonged incubation and double incubation with the primary antiserum was found. This may probably be related to the fact that normal gastrin cells contain quite high concentrations of the hormone and it is likely that a difference may be observed in cells that contain lower and more variable quantities of antigen (e.g. tumour cells). Therefore, for the time being, we advocate the use of double incubation also in tissue staining experiments.

As found with the models, immunogold-silver staining and, particularly, immunogold-silver-PAP staining possessed a detection efficiency that was much higher than that obtained with the standard PAP method. However, as mentioned above, intensification of the standard PAP method by the procedure of Gallyas et al. (1982) brought this method to a level of detection efficiency comparable to that seen with immunogold-silver staining. It should be emphasized that the 4-h pretreatment with thioglycolic acid solution was mandatory for obtaining useful results with the PAP-Gallyas method.

As regards the size of the colloidal gold particles, tissue staining experiments clearly demonstrated that small (5 nm) gold particles were to be preferred. Such small gold particles were essential for staining whole mount preparations of the gut wall indicating that penetration of the marker was important. A second factor of importance may be an increased density of small, as opposed to large, gold particles over antigen-containing areas (unpublished electron microscopical observations).

For general purposes the immunogold-silver and, particularly, the immunogold-silver-PAP methods appear to be very useful. Thus, these techniques are more sensitive than methods previously employed, they incorporate use of the colloidal gold probe which can now be employed for both light and electron microscopical studies. Moreover, these methods (as opposed to the PAP-Gallyas method) work well on paper and nitrocellulose models and may therefore also be of value in electroblotting and related chromatographical techniques. Notably, in these latter techniques, sensitivity is of prime importance. Highly sensitive techniques are also badly needed in conventional immunocytochemistry including studies on enzymes with rapid turnover rates and analysis of tumor antigens. Use of the immunogold-silver and immunogold-silver-PAP methods on light microscopical or model preparations also enables the investigator to quickly assess the quality of home-made colloidal gold probes for use in conventional immunocytochemistry, in the gold-labelled antigen detection (GLAD) technique (Larsson 1979) and in other applications. A modification of the GLAD method, the clono-GLAD technique (Scopsi, Bock and Larsson: in preparation) has also been developed for use at the light microscopical level.

In the most sensitive techniques tested, the immunogold-silver-PAP methods, the gold particles coated with anti-rabbit IgG serve as link-antibodies between the primary (tissue-bound) rabbit antiserum and the subsequently applied rabbit PAP complex. In the variant described last in the results section a fourth layer of anti-rabbit-IgG-coated gold particles were applied after the PAP complex in order to increase the amount of gold particles per tissue-bound antigen. Both variants worked well both in models and on tissue sections but, as pointed out above, the four-layer method produced an increased background. Development with these techniques depend both upon detection of peroxidase activity with the standard DAB reagent as well as upon silver intensification of the gold particles. The silver developers (Nos. 1 and 2) employed for this purpose also increase the intensity of the peroxidase-DAB reaction product. Thus, these methods develop the reaction at three different levels which probably contribute to their high sensitivity. It should be emphasized, however, that, with tissue material, short development times with DAB (not exceeding 10 min) and with the silver intensifier (1–2 min) have to be employed in order to avoid excessive background. With cytochemical models slightly longer development times are permissible (cf. Fig. 5). One factor which, moreover, could contribute to the increased sensitivity would be if the gold particles were coated with more than one anti-rabbit IgG molecule per particle, thus making them a more polyvalent link than free anti-rabbit IgG.

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References

Björklund A, Falck B, Owman Ch (1972) Fluorescence microscopic and microspectrofluorometric techniques for the cellular localization and characterization of biogenic monoamines. In: Berson SA (ed) Methods of investigative and diagnostic endocrinology. Vol. I. Rall JE, Kopin TJ (eds) The thyroid and biogenic amines. North-Holland, Amsterdam, pp 318–368

Danscher G (1981) Histochemical demonstration of heavy metals. Histochemistry 71:1–16

Danscher G, Nørgaard JOR (1983) Light microscopic visualization of colloidal gold on resin-embedded tissue. J Histochem Cytochem 31:1394–1398

Danscher G, Schrøder HD (1979) Histochemical demonstration of mercury induced changes in rat neurons. Histochemistry 60:1–7

De Mey J, Moeremans M, Geuens G, Nuydens R, De Brabander M (1981) High resolution light and electron microscopic localization of tubulin with the IGS (Immuno Gold Staining) method. Cell Biol Int Rep 5:889–899

Frens G (1973) Controlled nucleation for the regulation of the particle size in monodisperse gold suspensions. Nature (Phys Sci) 241:20–22

Gallyas F, Görsc T, Merchenthaler I (1982) High-grade intensification of the end-product of the diaminobenzidine reaction for peroxidase histochemistry. J Histochem Cytochem 30:183–184

Geoghegan WD, Ackermann GA (1977) Absorption of horseradish peroxidase, ovomucoid and antiimunoglobulin to colloidal gold for the indirect detection of concanavalin A, wheat germ agglutinin and goat antihuman immunoglobulin G on cell surfaces

at the electron-microscopic level: A new method, theory and application. J Histochem Cytochem 25:1187–1200

Geuze HJ, Slot JW, Van der Ley PA, Scheffer RCT (1981) Use of colloidal gold particles in double-labeling immunoelectron microscopy of ultrathin frozen tissue sections. J Cell Biol 89:653–665

Graham RC, Karnowsky MJ (1966) The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney. Ultrastructural cytochemistry by a new technique. J Histochem Cytochem 14:291–302

Gu J, De Mey J, Moeremans M, Polak JM (1981) Sequential use of the PAP and immunogold staining methods for the light microscopical double staining of tissue antigens. Its applications to the study of regulatory peptides in the gut. Regul Pept 1:365–374

Gu J, Islam KN, Polak JM (1983) Repeated application of first-layer antiserum improves immunofluorescence staining. A modification of the indirect immunofluorescence staining procedure. Histochem J 15:475–482

Handley DA, Chien S (1983) Colloidal gold: a pluripotent receptor probe. Proc Soc Exp Biol Med 174:1–11

Holgate CS, Jackson P, Cowen PN, Bird CC (1983a) Immunogold-silver staining: New method of immunostaining with enhanced sensitivity. J Histochem Cytochem 31:938–944

Holgate CS, Jackson P, Lauder I, Cowen PN, Bird CC (1983b) Surface membrane staining of immunoglobulins in paraffin sections of non-Hodgkin's lymphomas using immunogold-silver staining technique. J Clin Pathol 36:742–746

Horisberger M (1979) Evaluation of colloidal gold as a cytochemical marker for transmission and scanning electron microscopy. Biol Cell (Paris) 36:253–258

Horisberger M (1981) Colloidal gold: a cytochemical marker for light and fluorescent microscopy and for transmission and scanning electron microscopy. In: Jahari O (ed) Scanning electron microscopy. Vol. II. SEM Inc O'Hare, pp 9–31

Horisberger M, Rosset J (1977) Colloidal gold, a useful marker for transmission and scanning electron microscopy. J Histochem Cytochem 25:295–305

Larsson LI (1979) Simultaneous ultrastructural demonstration of multiple peptides in endocrine cells by a novel immunocytochemical method. Nature 282:743–746

Larsson LI (1981a) A novel immunocytochemical model system for specificity and sensitivity screening of antisera against multiple antigens. J Histochem Cytochem 29:408–410

Larsson LI (1981b) Peptide immunocytochemistry. Prog Histochem Cytochem 13:1–85

Larsson LI, Moody AJ (1980) Glicentin and gastric inhibitory polypeptide immunoreactivity in endocrine cells of the gut and pancreas. J Histochem Cytochem 29:925–933

Larsson LI, Rehfeld JF (1979) A peptide resembling COOH-terminal tetrapeptide amide of gastrin from a new gastrointestinal endocrine cell type. Nature 277:575–578

Larsson LI, Stengaard-Pederson K (1982) Immunocytochemical and ultrastructural differentiation between met-enkephalin, leu-enkephalin and met/leu-enkephalin immunoreactive neurons of feline gut. J Neurosci 2:861–878

Larsson LI, Sundler F, Håkanson R (1976) Pancreatic polypeptide – a postulated new hormone: Identification of its cellular storage site by light and electron microscopic immunocytochemistry. Diabetologia 12:211–226

Larsson LI, Goltermann NR, de Magistris L, Rehfeld JF, Schwartz TW (1979) Somatostatin cell processes as pathways for paracrine secretion. Science 205:1393–1395

Petrusz P, DiMeo P, Ordronneau P, Weaver C, Keefer DA (1975) Improved immunoglobulin-enzyme bridge method for light microscopic demonstration of hormone-containing cells of the rat adenohypophysis. Histochemistry 46:9–26

Petrusz P, Ordronneau P, Finley JCW (1980) Criteria of reliability for light microscopic immunocytochemical staining. Histochem J 12:333–348

Roth J (1982) The preparation of protein A-gold complexes with 3 nm and 15 nm gold particles and their use in labelling multiple antigens on ultra-thin sections. Histochem J 14:791–801

Roth J (1983) The colloidal gold marker system for light and electron microscopic cytochemistry. In: Bullock GR, Petrusz P (eds) Techniques in immunocytochemistry, vol 2. Academic Press, London, pp 217–284

Slot WJ, Geuze HJ (1981) Sizing of protein A-colloidal gold probes for immunoelectron microscopy. J Cell Biol 90:533–536

Sternberger LA (1979) Immunocytochemistry. 2nd edn. John Wiley and Sons, New York

Van Den Pol AN (1984) Colloidal gold and biotin-avidin conjugates as ultrastructural markers for neural antigens. Q J Exp Physiol 69:1–33