Application of Protein A Gold for Light Microscopic Immunohistochemistry in Paraffin Sections

(Protein A Gold-Silver Staining Method)

SUMMARY

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Since the advent of colloidal gold as a marker particle for immunoelectron microscopy, a series of studies using the gold marker has successfully been made for solving various problems in electron microscopic histo- and cytochemistry. As a result of these studies, it has been revealed that colloidal gold particles can be conjugated to a variety of macromolecules such as immunoglobulins, enzymes, lectins, various proteins and polysaccharides to form stable complexes, and that those substances reacting with such complexes can be visualized by virtue of the presence of gold. The immunohistochemical methods employing gold-labeled immunogloblins are, however, subjected to the serious limitation that immunoglobulins of all animal species will not necessarily bind firmly to colloidal gold. To circumvent such a limitation, an indirect immunohistochemical method named the protein A gold technique has been developed, in which a protein A-colloidal gold complex was used as the second step reagent. Protein A, a major cell wall component of Staphylococcus aureus, shows a strong affinity for the Fc fragment of immunoglobulin G among different animal species. Most of the immunohistochemical techniques employing protein A gold have been developed for electron microscopy, and Roth was the first to use protein A gold for light microscopy and obtained pale reddish reaction products in pancreatic islets of the rat. The light microscopic protein A gold technique developed by Roth involved, however, at

least a couple of difficulties ; in his technique, higher concentrations of primary antibodies and protein A gold were needed and the final reaction products obtained were not contrastive enough to be clearly distinguished from the background. To overcome these problems, an attempt has been made to photochemically intensify the reaction products obtained by the protein A gold technique using antibody against insulin in rat insular tissues and a protein A gold-silver method was successfully developed in which a refined procedure of physical development was performed. In this protein A gold-silver technique of Fujimori and Nakamura, however, high concentrations of primary antibody and protein A gold were employed. To establish a more sensitive and efficient protein A gold-silver staining method, in the present study, the availability of the staining technique was estimated in the course of the immuno-histochemical demonstration of insular hormones in rat, dog, monkey and human pancreas.

Pieces of tissues from the splenic portion of rat, dog, monkey and human pancreas were fixed in Bouin's fixative at 4°C for 18 hours, dehydrated in a graded ethanol series, cleared in benzene and embedded in paraffin wax. Sections were cut at a thickness of 4 μm and mounted on uncoated glass slides.

Guinea pig antiserum against porcine insulin and rabbit antisera against porcine glucagon, somatostatin and against human

pancreatic polypeptide were employed as the primary antibody. For the second step reagents of protein A gold-silver staining, protein A gold with particle sizes of both 5 nm and 10 nm were used. As reagents for the physical development combined with the immunohistochemical procedures, gum arabic, silver nitrate, citric acid and bromohydroquinone were employed.

In the protein A gold-silver staining procedure previously developed by the author and co-worker, primary antibody against insulin and protein A gold employed were diluted 1:400 and 1:40 respectively and a gold particle size of 10 nm was specified. Based upon the results obtained by the previous procedure, attempts have been made to elevate sensitivity of the previous procedure by diluting primary antibody against insulin up to 1:10,000 and protein A gold up to 1:640. For further elevation of sensitivity of the staining procedure, protein A gold of a smaller particle size (5 nm) was used instead of 10 nm particles.

To substantiate the wide applicability of the protein A gold-silver procedure to a variety of tissue antigens in light microscopy, the procedure was likewise employed for the immunohistochemical detection of three other endocrine pancreatic hormones, glucagon, somatostatin and pancreatic polypeptide (PP). In the course of this, the primary antibody against each hormone and protein A gold were investigated in terms of concentrations, and the gold particle sizes of both 5 and 10 nm were tested, as

in the case with insulin. For substantiating the applicability of the staining procedure, in addition, double immunostaining has been examined to combine the protein A gold-silver staining method for glucagon with the immunoperoxidase technique for insulin.

The formula of the developing solution employed is as follows: Solution A: 20% gum arabic aqueous solution, 45 ml; 10% silver nitrate aqueous solution, 1 ml. The gum arabic solution was prepared by centrifugation at 18,000 rpm for 30 min at 0°C and resultant supernatant fluid was separated for use. Solution B: distilled water, 15 ml; bromohydroquinone, 200 mg; citric acid, 300 mg. The working developing solution was prepared by mixing solutions A and B immediately prior to use. The results obtained by the procedure of physical development may immediately be checked microscopically either in an open laboratory or in a dark room under a photographic safelight illumination.

To compare the potency of this developer with that of different developers described previously, the former was replaced by the latter in the protein A gold-silver staining method. The developers compared were those of Danscher and of Moeremans et al. which contained silver lactate and hydroquinoe as developing reagents.

To test the specificity of the staining reactions, four types of control procedures were performed : (1) incubation with the antigen-preabsorbed antibodies at concentration of 1 mg (insulin,

glucagon and somatostatin) or 1 μg (PP) antigen per 100 μl (anti-insulin, -glucagon and -somatostatin) or 10 μl (anti-PP) of undiluted antibody, followed by treatment with protein A gold solution and physical development; (2) incubation with the specific antibodies, followed by 1 hour incubation with unlabeled protein A (1 mg/1 ml), then with protein A gold solution and finally with physical developer; (3) omission of incubation with both antibodies and protein A gold solutions followed by physical development and (4) omission of physical development only.

As compared with the previous technique, the protein A gold-silver staining procedure developed in the present study was found to show a significantly higher sensitivity. In view of the final staining results obtained, the dilutions of 1:2,000 for primary antibody against insulin and of 1:80 for protein A gold were found to be most appropriate in the staining with protein A gold of 10 nm particle size. In the protein A gold-silver procedure employing 5 nm particle size, the most optimal dilutions of primary antibody against insulin and of protein A gold were shown to be 1:10,000 and 1:320 respectively. Further, the most appropriate concentrations determined similarly for the primary antibodies against three other insular hormones and protein A gold in each case are shown as follows.

ANTI-SERUM / PAG	INSULIN	GLUCAGON	SOMATOSTATIN	PANCREATIC POLYPEPTIDE
P A G ϕ 10 nm	2000 / 80	2000 / 40	2000 / 40	5000 / 40
P A G ϕ 5 nm	10000 / 320	4000 / 160	8000 / 160	20000 / 160

In the present study, the following staining protocols were established as a standard technique.

(1) Deparaffinize sections and hydrate them in an ethanol series of descending concentrations.

(2) Rinse for 15 min. in 3 changes of phosphate buffered saline (PBS : 0.01 M, pH 7.4).

(3) Immerse for 30 min. in 5 % ovalbumin in PBS.

(4) Incubate for 60-90 min. with a primary antibody diluted in PBS containing 1% BSA (BSA-PBA).

(5) Rinse for 30 min.in 4 changes of PBS.

(6) Immerse for 15 min. in 5 % ovalbumin in PBS.

(7) Incubate for 60 min. with protein A gold diluted in BSA-PBS.

(8) Rinse for 30 min. in 4 changes of phosphate buffer (0.01 M, pH 7.4).

(9) Place in the physical developer (Fujimori and Nakamura 1985) for 60-80 min. at 20°C either in a dark box or in a dark room.

(10) Wash for 5 min. in running tap water and then immerse for 1 min. in a photographic fixer diluted 1:4.

(11) Wash for 10 min. in running tap water.

(12) Counterstain with Kernechtrot.

(13) Rinse in water.

(14) Dehydrate in a graded ethanol series, clear in xylene and mount in permanent media such as Canada balsam.

In the endocrine pancreatic tissues from the four mammalian species immumostained for insulin, glucagon, somatostatin and pancreatic polypeptide by means of the present procedure, reaction products were visualized in black shades. Higher magnifications of the cytoplasm of each endocrine cell type revealed that these products consisted of distinct fine particles of different sizes. In the present procedure, the staining images at both low and high magnifications obtained with gold particle size of 5 nm were comparable in quality to those obtained by with gold particle size of 10 nm.

In the present double staining procedure, two types of endocrine cells secreting glucagon and insulin respectively have simultaneously been visualized by reaction products of different shades in an identical pancreatic islet, i.e. reaction products obtained by the protein A gold-silver technique were revealed in black shades, whereas those obtained by the immunoperoxidase method were visualized in brown colors.

In the protein A gold-silver staning procedure employing Danscher developer, weak reaction products were obtained as pale black shades. Contrary to this, the use of Moeremans et al. developer resulted in abolishment of all immunostaining.

Negative results were constantly obtained, whenever sections were subjected to the control staining procedures (1),(2),(3) and (4).

In the present protein A gold-silver staining procedure employing gold particle size of 10 nm, the higher dilutions of primary antibodies were found to result in a higher efficiency of the procedure. Moreover, the present staining procedure with smaller gold particle size of 5 nm revealed a higher sensitivity, as compared with that using 10 nm gold particle size, and this has made possible further dilution of primary antibodies and protein A gold.

In the present staining procedure employing 5 nm gold particles, a longer duration of development was needed so as to obtain satisfactory results, as compared with that using 10 nm gold particles. This appears to reflect that a longer time lapse is necessary for the visualization of silver precipitates in light microscopy. A higher dilution of primary antibodies and protein A gold and smaller particle size of protein A gold could induce time-consuming effects on the visualization of precipitates.

In double stainings of immunohistochemistry, it is generally

recognized that two antigenic sites are to be clearly distinguished without difficulty and unreliability. In the present double staining procedure, the black reaction products obtained by the protein A gold-silver staining method were highly contrastive to the brown ones obtained by the immunoperoxidase technique. In view of this, the present double staining procedure is believed to be superior to those previously developed such as immunoperoxidase and immunocolloid techniques, in terms of the sharp contrast between the colors exhibited by the two reaction products obtained,

In light microscopy, attempts have hitherto been made to photochemically intensify the reaction products obtained by techniques employing gold-labeled RN-ase for RNA, immunogold for antigens, protein A gold for antigens, lectin-gold for glycoproteins and biotinylated lectin-avidin gold for glycoproteins. In all these techniques, except for the protein A gold-silver staining procedure, Danscher's developer or its modification such as that of Moeremans et al. were employed and these developers involved silver lactate and hydroquinone as developing reagents. On the contrary, an improved physical developer was employed in the protein A gold-silver staining procedure, which contained silver nitrate and bromohydroquinone as developing substances and ultracentrifugated gum arabic solution as protective colloid. Silver nitrate is known to be more potent in terms of silver ion

supply, as compared with silver lactate. Bromohydroquinone exhibits a significantly higher developing capacity than hydroquinone. The purified gum arabic solution is effective for suppression of autocatlytic activity of the developer. These ingredients are thought to result in a potent developer, which can lead to the visualization of even a small amount of gold particles on antigenic sites in light microscopy.

In view of all the results obtained by the present experimental and control stainings, the protein A gold-silver staining procedure established here is believed to be not only high in specificity and efficiency but marked in intensity. In addition, the present double staining method in combination with the immunoperoxidase method was conceived to be a reliable and useful technique for the simultaneous detection of two tissue antigens in light microscopic immunohistochemistry.

As the results obtained indicate, the present staining procedure was found to be useful consistently for the detection of four pancreatic hormones from different mammalian species. Since protein A shows a strong affinity towards the Fc fragment of IgG from numerous animal species, furthermore, the present protein A gold-silver staining procedure can be widely applied to demonstrate a variety of tissue antigens. Protein A gold-silver techniuque is, therefore, a reliable method of choice in light microscopic immunohistochemistry.