PROTEIN A-GOLD POSTEMBEDDING IMMUNE ELECTRON MICROSCOPY

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SUMMARY : *Protein A-gold technique, applied in postembedding approach, reveals antigenic sites on tissue sections with very high resolution. Since it is a two step indirect technique, the results obtained depend on the specificity and affinity of the antibody used and on the* amount of antigenicity retained in the tissue. Since protein A interacts with IgGs from most mammalian species, with the polyclonal and monoclonal antibodies, it can be applied almost universally. This highly reactive probe can also be ap*plied for double labeling techniques.*

Furthermore since it is a postembedding approach, the technique is not confronted with problem of accessibility of the antigen and diffusion of the reagents and thus, does not require permeabilization of membranes. Since the marker is particulate, it allows for an easy identification of the labeled structure with high resolution and for quantitative evaluations, introducing a certain dynamic aspect to the approach. When compared to other techniques, it certainly appears as a simple, sensitive, and reliable approach, providing superior results. Because it gives far more information than what can be obtained from either morphological or biochemical means alone and because of the levels of sensitivity and resolution obtained with this approach, immunocytochemistry is having a great impact in cell biology and pathology and is creating great interest.

Key Words : *Protein A, Colloidal Gold, Postembedding.*

In recent years, several proteins having property of binding immunoglobulins have been identified and isolated from different strains of bacteria. Protein A from staphylococci (19), protein G from streptococci (13) and protein L from peptococcus (14). These proteins are located at the surface of bacteria and are thought to play a role in the pathogenesis of infectious agent and in the host-parasit relationship. Among these proteins, the most common and most completely studied is the protein A. Found in the cell wall of the staphylococcus aureus (19, 42), protein A is produced by most strains of staphylococcus and is either covalently attached to the cell wall of the bacteria or secreted (34). The most relevant property of protein A consists in its affinity tword immunoglobulins of type G through binding to the Fc fragments (19). An interaction of lower affinity has also been reported between protein A and Fab regions of the IgGs and IgEs (17, 29, 30, 43). Protein A interacts with immunoglobulins type G of several mammalian spieces, which represents additional advantages to this protein (33). It also displays moderate affinity to certain IgA, IgE

and IgM molecules (23, 24, 30).

According to Sjödahl (28), protein A displays four Fc-binding sites, starting from the N-terminal part of the molecule located outside the bacteria cell wall, in which the C-terminal part is anchored (32). However, once released from the bacteria wall, it appears to be functionally bivalent, able to bind only two immunoglobulins (32). Protein A purified most strains of staphylococcus aureus consist of a single elongated polypeptide chain having a molecular weight of 42.000 (15). The interaction that take place between protein A and the immunglobulins occurs through the Fc fragment on the CH_2 and CH_3 domains (16). Thus it is a pseudo-immune reaction that does not interfere with the binding of the immunoglobulin to its antigen. Protein A displays very high stability to change in temperatures, PH values and various denaturating agens (39). The affinity properties of the protein A toward the immunoglobulins have been studied extensively and are of great use in immunocytochemical techniques.

The high affinity of protein A for immunoglobulins has been extensively applied in immunocytochemical techniques which led to the introduction of these reagents in immunocytochemistry (36, 37). This molecule is of low molecular weight, cannot be visualized directly, and must be tagged to an electron-dense marker for its detection in microscopy. Among the different electron-dense markers, colloidal gold has many advantages when compered to others and has been extensively used in this field. Once associated with colloidal gold particles, the protein A form a complex, protein A-gold complex (36, 37) which can be applied in immunocytochemistry at light and electron microscope levels.

The first application of the protein A-gold complex was reported by Romano and Romano (36) for the preembedding labeling of surface antigens on red blood cells. Roth and his workers (37) adapted this approach for the postembedding detection of tissue and intracellular antigens and have since then contributed to major developments in immunocytochemistry.

COLLOIDAL GOLD MARKER

Since its introduction in immuno electron microscopy by Faulk and Taylor in 1971 (18), colloidal gold has proven to be one of the best electron-dense makers in cytochemistry, displaying several major advantages when compared to other markers such as ferritin and peroxidase. Because of its particulate nature, very accurate identification and delination of the labeled structure is possible without masking them. Being one of the smallest markers (down to 3 nm), it allows for the best resolution in cytochemistry. Furthermore, quantitative evaluations of the intensity as well as spatial distrubution of the labeling can performed. Since it can be easily prepared in different sizes from 3 to 100 nm (20), one can perform multiple labeling of various binding sites in the same section.

Colloidal gold is a negatively charged hydrophobic sol, formed by electron - dense metalic particles. Its binding to macromolecules seems to occur by noncovalent electrostatic, stable adsorption; it is a complex reaction that is still poorly understood (27). It is assumed to take place between the charged surface of the particles and those of the protein throughout electrostatic van der Waals forces. A number of physicochemical factors such as the pK_1 and the concentration of protein influence the adsorption process (21). The adsorption and the formation of the protein - gold complex do not affect the biological activity of tagged macromolecules (28). In contrast to ferritin, colloidal gold has no spontaneous affinity to the various resins used in electron microscopy, which results in negligible nonspecific adsorption to tissue sections and makes it a suitable marker for postembedding labeling.

TISSUE PROCESSING

The purpose of any cytochemical study consist in revealing specific components and assigning them to particular tissues or cell compartments. Thus the cytochemical technique used for such a purpose represents only part of the work : the preservation and good characterization of the structures is the other important aspect of the process. For optimal results, one has to combine the use of a cytochemical approach with a technique of tissue processing that enables fine structural identification. To achieve this in immunocytochemistry, one must work with two different parameters, which are usually not compatible with each other : the retention of the affinity properties (antigenicity) of the components, and the preservation of the fine structure. Indeed, preparation of tissue for microscopic observation requires procedures that alter the chemical properties of most components. Usually one has to compromise between both criteria and work

out the best approach.

The optimal condition can be defined as those that allow for retention of adequate biological activity to enable cytochemical reactions to take place in adequately preserved structure. All cytochemical techniques including those applying the colloidal gold marker are confronted with this prob'em. The structural preservation of a tissue depends on two different procedures, the fixation and embedding protocols. Fixation can be carried out with different chemicals at different concentrations (25, 26). Each class of antigen behaves differently to the fixation protocol (1, 2, 31). In general, the fixative concentration should be kept as low as possible, though many antigens can be revealed after routine fixation with high concentrations of glutaraldehyde (2 %) and postfixation with 1 % O_SO4. When postfixation with O_SO4 is performed, tissue sections should be treated with a strong oxidizing agent. Hydrogen peroxide and sodium metaperiodate have been used. The later has been fonud to give optimal results, since, hydrogen peroxide tends to strongly etch the surface of the sections (3). The solution of metaperiodate should be kept at room temperature instead of 4° C and is stable for several months. In the case of nonosmicated tissue, the treatment of sections with sodium metaperiodate many enhance somehow the intensity of the labeling, without altering the ultrastructural preservation. The method of fixation (perfusion versus immersion) solud also be taken into consideration since it may introduce artifactural modifications and displacement of certain antigens (2). The fixatives can introduce free aldehyde radicals into specimen, which should be quenched prior to exposing the tissue to the antibodies or any other probe. To do this, after fixation, tissue specimens should be soaked for 30-60 min in 0.15 M glycine in PBS or 0.5 MnH_4Cl solution and then processed for embedding protocol.

Various embedding procedure are available and can be applied for immunocytochemistry. Some appear to be less denaturating than others; but again for each class of antigen one has to determine the one yielding the best result (2). Five protocols commonly used for electron microscopic postembedding immunocyto-chemistry are 1. embedding in epoxy resins (mainly Epon 812, Araldite and Spurr); 2. embedding in glycol metacrylate resins (GMA and low-cid GMA); 3. embedding in lowicryl type resins (K4M and HM20); 4. embedding in LR type ins (LR white and LR gold); and no embedding, processing through ultramicrotomy, with labeling on ultrathin cryosections. Cryosectioning is particularly useful when embedding in resins drastically interferes with immunolabeling. Tissue thin sections of resin-embedded material can be mounted on Parlodion-carbon-coated nickel grids. These grids can be stored for several months without any problem; they yield labeling as good as that by freshly prepared sections. The use of a support film (Butvar, Collodion or Formvar) is not required to perform the labeling. However, a film provides a good support for the sections that might other wise be lost during incubation and washing procedures. The use of nickel grids (or gold grids) is strongly recommended for general use, and absolutely required when postfixation with O_SO4 is carried out. Indeed, oxidation of the copper grids during the various incubations leads to the formation of contaminants and dirt deposits on the tissue sections.

IMMUNOLABELING

Protein A-gold is an indirect two-step immunocytochemical technique; its principle is illustrated in Figure 1. The antigen present at the surface of the tissue section are first exposed to a specific antibody to form an antigen-antibody complex. In the second step, the tissue sections are exposed to pro-

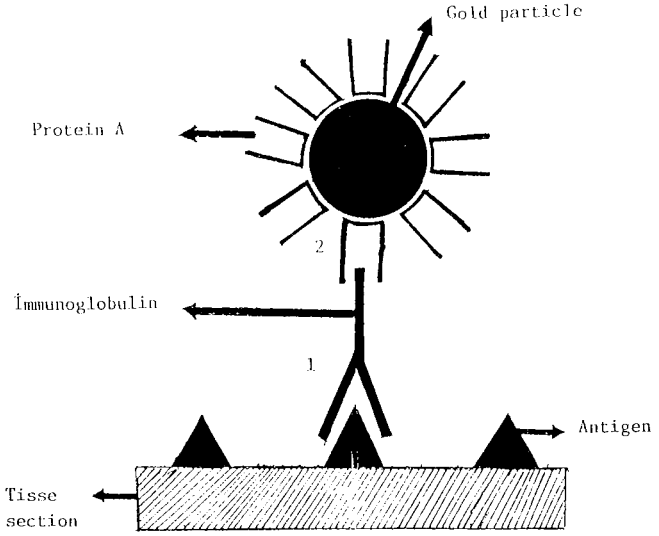

Fig - 1 : Diagram illustrating the principles of the protein A-gold approach. The labeling is carried out in two steps; in the first one, the immunoglobulin interacts specifically with the antigen present at the surface of the tissue sections; in the second step, the molecules of protein A surrounding the gold particle interact with the Fc fragment of the immunoglobulin and the gold particle allows for the indirect localization of the antigenic sites.

tein A-gold complex for interaction with the immunoglobulins retained at the surface of the sections through their previous binding with the antigen molecules. The gold particles will by superimposition, reveal the side of the protein A-IgG-antigen complex.

On the basis of the principle involved in this approach, it is clear that the specificity of the results will depend on the quality of the antibody used in the first step of the technique. The antibody should be a good specificity and high affinity. The specificity refers to the purity and uniqueness of its antigen, while the affinity corresponds to the recognition of binding sites on the antigen molecule. The antibody should be directed against a highly purified and well characterized antigen and the interaction should be assessed through various immunocytochemical techniques. Whole sera, IgG fractions or affinity-purified antibodies can be used with Protein A-gold technique. Monoclonal antibodies in either supernatant ascites fluid or purified form can also be used, although they are strongly recommended application of the protein G-gold complex rather than the protein A-gold (4). Fab fragments should be absolutely avoided since the major interaction between protein A and the immunoglobulins occurs through the Fc frogment. The antibodies should be of the IgG class rather than IgM or IgA.

The antibodies are used diluted in PBS, which may contain 0.1 % sodium azide and 1 % albumin. They should be stored frozen in buffer but always in small aliquots to avoid freeze-thaw damage. While performing a labeling study, the diluted antibody should be stored at 4°C. Dilution can vary from 1/10 to 1/1000 or higher, depending on the titer of the antibody. Several dilutions should be tried before finding the optimal one. This can be defined as giving the highest specific labeling with acceptable levels of background staining.

DOUBLE - LABELING TECHNIQUES

Gold particles are highly electron-dense, particulate in nature, and able to be prepared in different sizes. These characteristics allow for their use in combination with themselves or with other electron-tense tracer for the simultaneous detection of two or more tissue or cell components. There is considerable interest in cell biology in comparing locations of various components simultaneously in the same tissue. This can be done either using serial sections or more elegantly by double labeling on the same tissue section.

LABELING OF TWO ANTIGENS ON THE SAME SECTION

Using antibodies directed toward two different antigens (even if they are raised in the same animal species) and protein A-gold complexes prepared with gold particles of different sizes, two specific antigenic sites can be localized simultaneously on the same section (5, 22). However, since protein A is known to interact with the Fc fragments as well as to a lesser extent with the Fab fragments of the immunoglobulins G, it has been a technical approach that allows for the detection of two antigenic sites on the same section without artifactual interaction among the different reagents (5). The two surfaces of the section should be used, performing the labeling of each antigen on a separate face of the tissue. Since it has been demonstrated that labeling occurs only at the surface of the section without penetration, there is no interference among the labeling taking place on separate surfaces. Furthermore, this approach also avoids the steric hindrance phenomena that would occur where both antigens are present in the same site.

In this technique protein A-gold complexes are prepared using gold particles of different size. Thin sections are mounted on uncoated nickel grids, so that both faces of the section are exposed and avaible for labeling. In order to increase adhesion of the sections to the grid, it can be treated for a few seconds with 20 % solution of acetic acid, then with 90 % ethanol, and rinsed in distilled water. The labeling of one surface of the section (face A) is carried out as described previously using a specific antibody and a protein A-gold complex formed with large gold particles (e.g., 10nm). Care should be taken to perform the whole procedure by floating the grid on the different solutions and not contaminating face B of the section with any of the reagents.

After completing the first labeling, the grid is dried carefully and turned over for the second labeling. In this case, face B of the section is exposed to a second antibody and then protein A-gold is formed with small gold particles (e.g., 5nm). Again, care should be taken not to contaminate face A with these reagents. All procedures should be carried out by floating the grid on the different reagents. After drying the grids, they can be stained (on one side only) with uranyl acetate and lead citrate before examination in the microscope. The transparency

of the sections under the electron beam allows the simultaneous visualization of the gold particles present on both sides of the sections. Since each labeling is performed in an independent way, problems of interactions between reagents leading to artifactual colocalization, which can occur if both labelings are performed on the same side of the section, are avoided.

APPLICATION OF THE PROTEIN A-GOLD COMPLEX

Protein A-gold was introduced in 1977 by Romano and Romano (36) for the preembedding localization of membrane antigens at the electron microscopy level. Roth and his coworkers (37) extended the technique in 1978 for postembedding ultrastructural ocalization of antigens in cells and tissues. Since that first publication, a large number of applications have been published using the postembedding protein A-gold approach for high-resolution localization of intracellular as well as extracellular antigens. Various types of antigens have been succesfully revealed : secretory proteins and peptides in the rough endoplasmic reticulum, Golgi complex and secretory granules or vesicles, as well as in lysosomal structures of the corresponding secreting cells in various organs and tissues, mitochondrial, peroxisomal, nuclear, cytosolic and cytoskeletal proteins and various associated and integral membrane proteins in different types of cell. Other examples include several viral proteins present either in the viral particles or in infected cells.

In the extracellular space, the technique has been applied to the collagen family of proteins, proteins of enamel and dentin layers, and bone matrix, and to components of plant cells and bacteria and their corresponding walls. In addition, the protein A-gold technique has been adapted for the study of vascular permeability as well as for the dynamic passage or diffusion of various components through the interstitial space. All these applications have repeatedly demonstrated the high potential of the protein A-gold technique has been adapted for the study of vascular permeability as well as for the dynamic passage or diffusion of various components through the interstitial space. All these applications have repeatedly demonstrated the high potential of the protein A-gold approach, since specific results of high resolution were obtained.

STUDIES OF PROTEIN SECRETION

The immunocytochemical study of protein secretion from rat pancreatic acinar cells was the first application in which the protein A-gold postembedding approach was applied (7). The high resolution of labeling combined with the fine structural preservation of the cellular compartments and the quantitative evaluations have allowed for the demonstration of 10 different pancreatic secretory proteins and/or proproteins in the different cellular compartment and subcompartments involved in the processing of protein secretion in the acinar cells, namely the rough endoplasmic reticulum, the transitional elements of reticulum, all the cisternae of the Golgi complex, and the immature and mature secretory granules (6, 7). In the Golgi area, the smooth vesicles were specifically labeled while the coated ones were. The last trans-cisternae of the Golgi complex, the GER presented low levels of labelings. This was particularly well demonstrated by performing a double-labeling technique for the simultaneous localization of phosphatase and amylase (8). Application of double-labeling protein A-gold technique, with antibodies directed aginst various secretory proteins, has demonstrated that the diffrenet proteins coexist in the same compartments (5), supporting the proportion of paralel intracellular processing of different secretory proteins by the acinar cells (35). Şeftalioğlu (41) used protein A-gold technique on the pancreatic B-Cells of mouse, appliying Agar resin 100 embedding material (Figures 2, 3, 4). Furthermore, the double-labeling approach has also demonstrated that, although they share the same compartments, same classes of

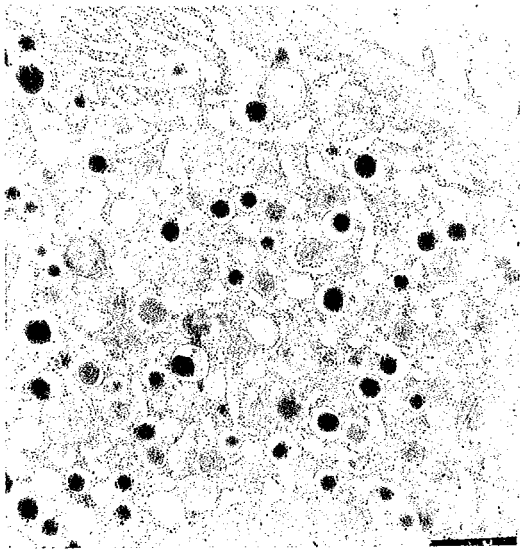

Fig - 2 : An electron micrograph of mouse pancreatic B-cells located around a capillary. They were incubated with anti-insulin antibody and protein A-gold complex. The gold particles are seen over the secretory granules X 28500.

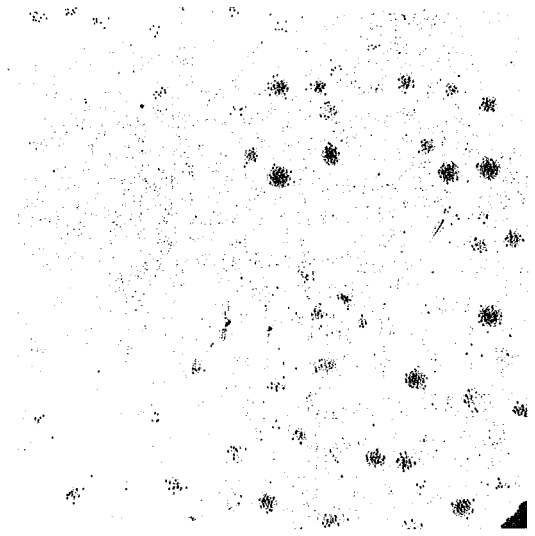

Fig - 3 : One of the cytoplasmic part of pancreatic B-cell. The gold particles are intensely observed over the dense core of the B-cell granules. X 28500.

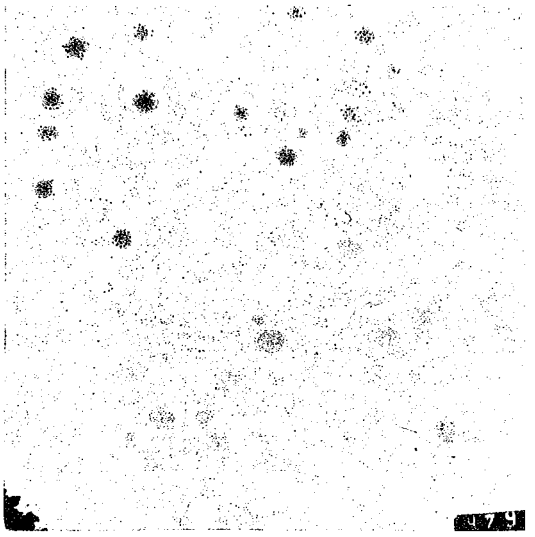

Fig - 4 : Pancreatic B-cells and centroacinar cell are seen side by side. The protein A-gold labeling is positive in the granules of pancreatic B-cell. X 28500.

proteins are concentrated in the same subregions of the secretory granules (9).

In a second series of studies, the location of cytoskeletal proteins was investigated in various types of cells, including those involved in protein secretion. Actin, myosin, keratin, and desmin were detected by the postembedding protein A-gold technique over corresponding thin, thick, and intermediate filaments in various contractile and epithelial cells with high resolution (10, 11, 14, 40). Actin, myosin, and keratin antigenicity was also found in close relationship whith the membranes of the trans-Golgi cisternae and secretory granules in pancreatic cells, suggesting an importent involvement of cytoskeletal elements in the process of secretion (10, 11).

APPLICATION TO PATHOLOGICAL SPECIMENS

Immunocytochemistry, particularly at the light microscope level, is one of the major techniques in diagnostic histopathology. It has been introduced in electron microscopy, although the length of time as well as the difficulties in processing the tissues makes it less practical. However, several immunogold studies have been performed on pathological specimens obtained mainly from tissue biopsies. It has been studied the localization of immunoglobulins (kappa light chains) in large deposits in the glomerular basal laminae of patients suffering of lupus erythematosus and the secretion of prolactin and growth hormones by endocrine cells of human pituitary adenomas (12).

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