

Immunolabelling of prolactin at ultrastructural level using the protein A-gold technique on Epon-embedded tissue

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Abstract

The use of the colloidal-gold technique in electron microscopy immunocytochemistry has provided important information on the *in situ* localisation of intracellular antigens. We have developed a post-embedding technique for prolactin localisation on resin-embedded human pituitary tissue sections by the use of the protein-A gold conjugate. Human pituitary tissue obtained at autopsy was processed for electron microscopical study without post-osmication and then embedded in Epon. The indirect immunoperoxidase method was used for light microscopical targetting of lactotroph cells for subsequent electron microscopical antigen localisation. Ultra-thin sections were labelled with human anti-human prolactin followed by protein-A gold conjugate. Specific labelling was observed over secretory granules with a density of 15-30 particles per granule, as determined by the Quantimet 570 image analysis system. This technique provides a means of studying the pathophysiology of hormonal secretion at ultrastructural level and can be a useful tool in diagnostic and research investigations.

Key words: Protein A-Gold, immunolabelling, prolactin, antigen localisation

INTRODUCTION

Immunocytochemistry has provided an important means for microscopical *in situ* localisation of intracellular antigens. In order to extend such visualisation to the ultrastructural level, different techniques for electron microscopical labelling have been developed, amongst them the protein A-gold conjugate method.

Protein A is a cell wall constituent produced by most strains of *Staphylococcus aureus*.¹ Its unique and high affinity binding with immunoglobulins, especially immunoglobulin G (IgG) is the basis of its application in immunocytochemistry.^{2,3,4} Moreover, being non-species specific, it is a useful reagent for binding immunoglobulins from several animal species. Protein A has been conjugated to ferritin⁵ and peroxidase⁶ for antigen localisation at light and electron microscopical level.

In 1971, Faulk and Taylor⁷ initiated the immunocolloid method for electron microscopy by using an antibody-colloidal gold complex for cell surface antigen localisation. Since then, gold particles have gained popularity as markers in light and electron microscopical immunocytochemistry.^{2,8,9,10} Soon after, Roth *et al*^{8,11} successfully applied the protein A-gold conjugate in a post-embedding staining technique.

Various attempts to refine the techniques above to improve specificity and resolution followed.^{3,4,10,12}

Osmication and Epon embedding techniques have been cited as unsuitable for immunolabelling due to the destruction of antigens during osmication and processing.^{2,3,4} Most of the literature on post-embedding techniques thus far report the use of araldite or low-temperature embedding media such as Lowicryl. However, in our laboratory, the Epon embedding technique has been the mainstay in routine work. As such, in this study, we endeavoured to develop an immunolabelling method that can be applied to unosmicated Epon-embedded tissue. The protein-A gold technique is used here because it is a relatively simple technique and precludes the use of various preparations of species specific immunoglobulins. In this study, we have chosen to apply this method to the detection of prolactin in human pituitary cells as an example of hormone detection at ultrastructural level. The ability to localise hormonal or other protein antigens at ultrastructural level is envisaged to have far-reaching applications in the study of cell physiology in health and disease. Unlike detection of antigens using immunohistochemistry at light microscopical level, ultrastructural studies can

relate protein localisation to various cytoplasmic organelles. Furthermore, the gold particles used also lend themselves more easily to some form of quantitation.

MATERIALS AND METHODS

Principles of protein A-gold technique

The principles of the protein A-gold technique as a two-step post-embedding labelling procedure carried out on thin tissue sections is described in Fig 1. In the first step, the specific immunoglobulin binds to the antigen exposed at the surface of the section. In the second step, molecules of protein A conjugated with gold particles interact with the Fc region of the immunoglobulin. The first antigen-antibody reaction is then revealed by the protein A-gold complex. In this indirect way, the location of the antigen is indicated by the gold particles.

Tissue preparation

Small cubes (about 1 mm³) of fresh human pituitary tissues obtained from 4 human subjects at autopsy were fixed in 4% glutaraldehyde for four hours at 4°C. They were then thoroughly washed with several changes of cacodylate buffer to remove unbound glutaraldehyde followed by washing briefly in double-distilled water and block stained in 4% uranyl acetate for 10 minutes at room temperature. The tissues were washed free of uranyl acetate in several changes of double-distilled water, transferred to 35% alcohol and dehydrated in a graded ascending series of alcohol up to absolute alcohol. After clearing in propylene oxide the tissues were infiltrated and embedded in Epon 812 (Bio-Rad).

Antisera

The primary antibody used was anti-human

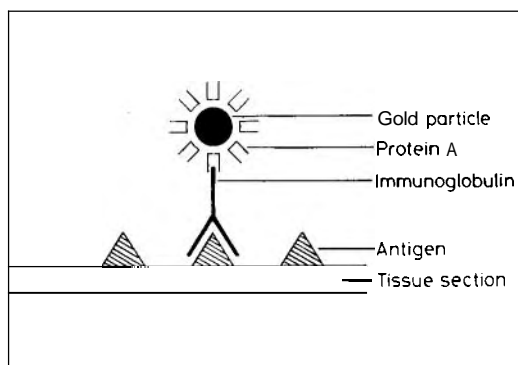


FIG. 1: Diagram illustrating the principles of the protein-A gold technique.

prolactin (Dakopatts). The gold label used was protein-A gold conjugate (10 nm, Sigma). Ovalbumin (Grade VI, 99%, Sigma) was the blocking agent used to reduce background staining.

Light microscopical localisation of prolactin-producing cells

One micron thick Epon sections mounted on glass slides were stained by the indirect immunoperoxidase method to determine the location of prolactin-producing cells. After these were identified, the blocks were trimmed down in size to target these cells prior to ultra-thin sectioning for immunogold labelling.

Electron microscopy (Immunogold procedure)

Ultrathin sections of 60 nm were obtained using a Sorvall MT-5 ultramicrotome and subsequently collected on Nickel grids (300 Mesh, Bio-Rad).

The following steps were used for immunogold labelling:

1. Etch Epon section with 10% aqueous hydrogen peroxide for 10 minutes.
2. Wash with several changes of double-distilled water.
3. Rinse with phosphate-buffered saline (PBS).
4. Incubate section on grid in 1% ovalbumin in PBS for 30 minutes.
5. Blot dry to remove excess ovalbumin.
6. Incubate in primary antibody (anti-human prolactin, diluted 1:100 in PBS) overnight at 4°C.
7. Wash with several changes of PBS and then blot dry excess buffer.
8. Incubate in Protein-A gold in PBS (1:20) for 60 minutes.
9. Wash with several changes of PBS and double-distilled water.
10. Counterstain with uranyl acetate and lead citrate.

RESULTS

At light microscopical level, prolactin-producing cells were stained brown with the immunoperoxidase method for human prolactin (Fig 2). Electron microscopy revealed that colloidal gold particles were concentrated over secretory granules of these cells (Figs 3a & b). The background was relatively clean. While the gold particles were easily identified, it was observed that ultrastructural preservation of organelles was not optimal. This was felt to be due to the omission of osmium tetroxide post-fixation.

Quantitation of the spatial distribution of gold particles over the granules was carried out using the Quantimet 570 (Leica Cambridge, U.K.) image analysis system. The **number** of gold particles per granule or per unit area of the granule were analysed automatically from the micrographs and **was** found to range from 15 to 30 particles per granule.

DISCUSSION

Previous attempts at immunogold labelling in our laboratory using osmicated tissue, even though subjected to sodium metaperiodate etching, had proved unsuccessful. This **was** most likely due to the loss of antigenicity during the osmication process. However, omission of osmication, as in the present work, resulted in cell organelles being more vulnerable to distortion/disruption during dehydration and embedding due to the lipophilic nature of Epon. This artifact was observed in the micrographs (Figs 3a & b). Notwithstanding the problem of optimal morphological preservation, we found that the technique described is an acceptable and practical compromise for immunoelectron microscopical investigations.

Other limitations of this technique are relatively minor. Being a post-embedding technique, only antigens on the exposed cut surface of the sections can be labelled. Another limitation concerns the affinity of protein A to IgG molecules.

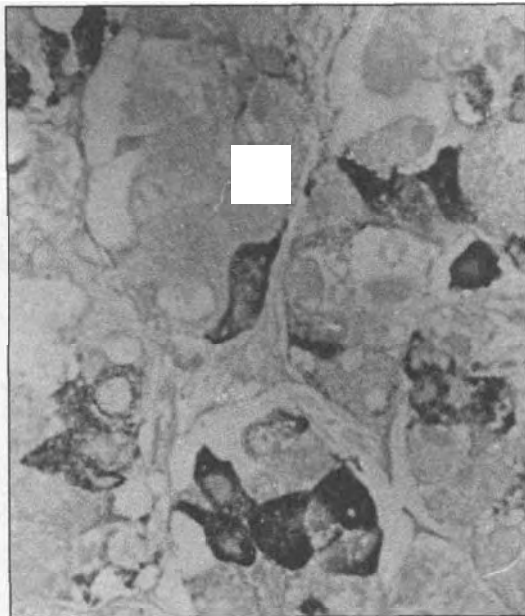


FIG. 2: Light micrograph of pituitary gland stained with the indirect immunoperoxidase technique for human prolactin. Cells expressing prolactin are stained dark brown.

This varies from one animal species to another. It has been found that antisera raised in rabbits and guinea pigs are preferred and yield better results over those from goat or sheep." Furthermore, Epon embedding suffers from the disadvantage that Epon is hydrophobic and therefore liable to cause high background staining due to its strong attraction for hydrophilic immunoreagents.

The combined desirability of strong, specific antigen binding together with optimal morphological preservation remains the main problem, as is also the case with all other immunocytochemistry techniques. However, this can be circumvented by varying the conditions for fixation and embedding. The use of newly developed resins such as LR White and Lowicryl or of modified embedding procedures for processing the tissues at very low temperatures improve antigenic preservation and offer promising results.¹⁴ However, no single procedure can be recommended as the best for immunoelectron microscopy as optimal labelling has to be worked out for each type of antigen." From our study, we observed that Epon embedded tissue can be used for immunolabelling even though it is at the expense of ideal morphological preservation. This compromise allows satisfactory detection of antigenic sites and provides a valuable means to study the pathophysiology of enzyme activities at ultrastructural level in a routine electron microscopy set-up.

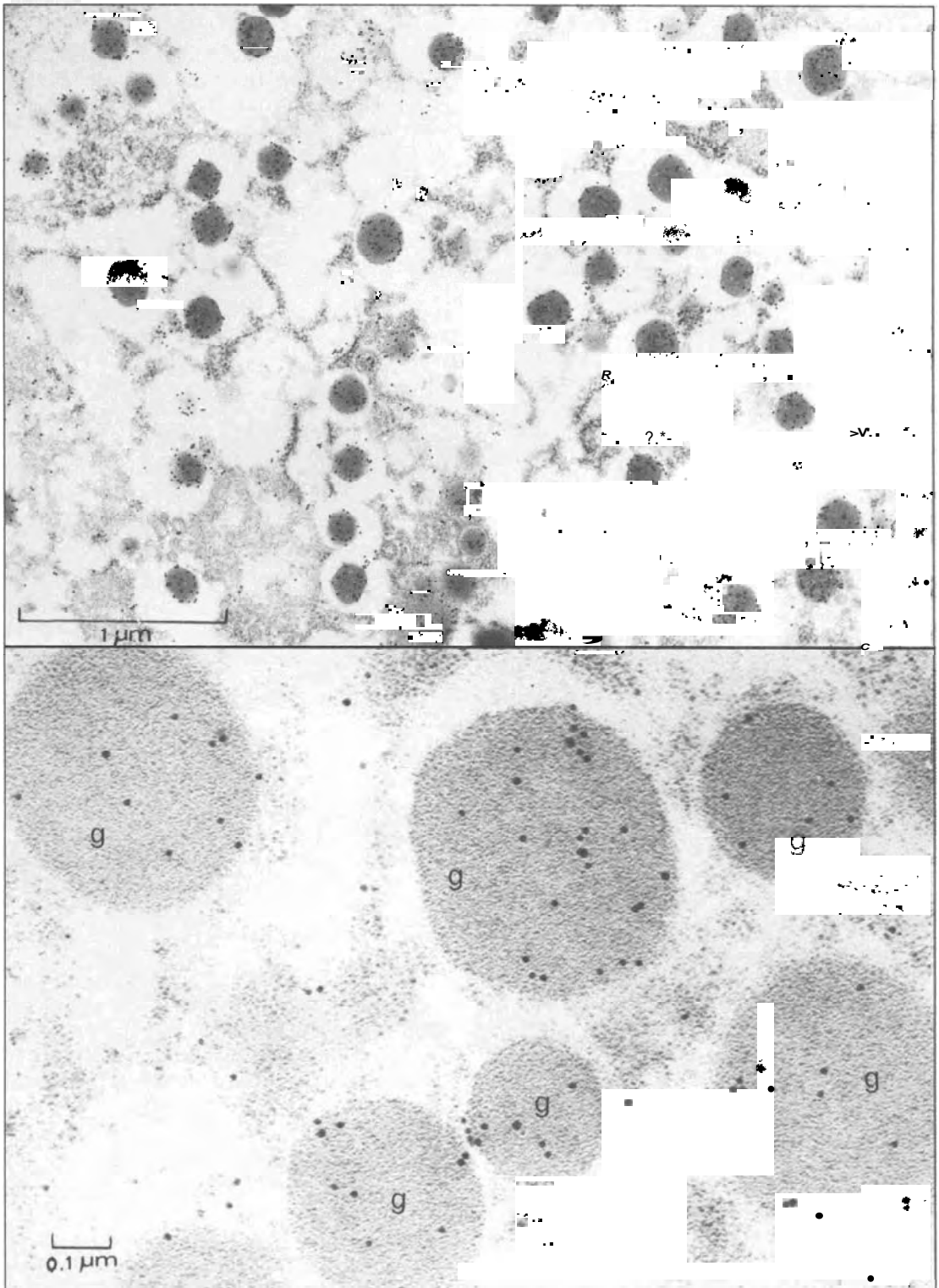
We also suggest the use of a computerised image analyser (such as the Quantimet 570 in this study) as an objective evaluation of the specificity of labelling. It also serves to ensure quality control of the immunogold labelling technique.

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FIGS. 3a, b: Localisation of prolactin in human pituitary cells. Gold labelling is observed over secretory granules (g).

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