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Immunohistochemistry for detection of Aujeszky's disease virus antigens: Protein A-gold labeling of ultrathin sections for electron microscopy

Soon-bok Kim

College of Veterinary Medicine, Gyeongsang National University

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오제스키병 바이러스 항원검출을 위한 면역조직화학적 연구 : 전자현미경적 관찰을 위한 초박절편내 protein A-gold labeling

김 순 복

경상대학교 수의과대학

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초록 : 오제스키병 바이러스를 배양세포에다 감염시켜, 냉동 및 araldite포매 초박절편에서 protein A-gold labeling을 통해 바이러스항원 검출을 시도하였다. 오제스키병 바이러스항원은 10nm gold probes로 표지되었으며, 전자밀도가 높은 gold 입자들은 바이러스의 nucleocapsid와 envelope에서 주로 관찰되었고, 초냉동박절표본에서의 immunogold labeling은 조직구조물들과 극히 미미한 비특이결합만을 보였다.

초냉동박절표본에서의 immunogold labeling은 오제스키병 바이러스항원을 검출하는데 있어 효과적이었으며, 이는 또한 여러가지 바이러스들과 숙주세포들간의 상호작용에 관한 면역세포화학적 연구에 크게 이용될 수 있을 것으로 생각된다.

Key words: protein A-gold labeling, Aujeszky's disease virus antigen, ultrathin cryosections

Introduction

Gold particles are easily prepared and bound to lectins[1,2] and proteins.[3-5] Gold markers also can be prepared in a variety of sizes.[6]

The cell wall of *Staphylococcus aureus* contains a covalently bound protein which is called protein A.[7] Because of its characteristic ability to interact with the Fc fragment of IgG molecules from several species[8,9] protein A has become of special interest in immunology.

Recently protein A-gold has been introduced for the localization of different antigens at the light[10,11] and electron microscope[12-15] levels. In 1981 the application of an immunogold method for the ultrastructural labeling of viruses replicating in cell cultures was introduced by Weiland.[16]

Immunocytochemistry using frozen thin sections has become an important technique in cell biology during the last ten years following the pioneering work of Tokuyasu.[17,18] The technique has, however, not yet gained a widespread usage, notably because of difficulties encountered in structural preservation.

The purpose of the present study is to demonstrate Aujeszky's disease virus(ADV) antigens in ultrathin frozen and araldite-embedded sections of cell cultures, as well as its morphogenesis, and to provide a better understanding of ultrathin cryosection in order that greater control of its critical steps is made possible.

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Materials and Methods

Materials: Fibroblastic cells originated from pig brain and BHK cells were grown and confluent monolayers were infected with ADV-Phylaxia strain, as described earlier.[11]

Ultracryosection: Monolayers were fixed with 2% paraformaldehyde and 0.4% glutaraldehyde in PBS (sodium phosphate buffer, pH 7.6) containing 5% sucrose for 1 hour, followed by rinsing in PBS containing 5% sucrose, 3 times for 5 minutes. Cells were removed from the monolayer using rubber policeman, centrifuged for 30 minutes at 2,400 rpm, resuspended in PBS containing 10% sucrose, and repelleted in a conical plastic vial for 10 minutes at 13,000 rpm. For crysectioning, small pieces of the pellets were processed through 25%, 50% and 2.2M sucrose-PBS solutions for 60 minutes respectively, and then infused with 2.2M sucrose overnight at 4°C, and mounted on specimen holders, freezed in liquid nitrogen. The blocks were cryosectioned to an estimated thickness of approximately 70nm at −106°C to −108°C in a Reichert-Jung ultramicrotome equipped with a FC4 cryochamber. Thin sections were cut with glass knives and picked up on 100 mesh, pioloform-coated hexagonal copper grids.

Araldite-embedded section: Monolayer were prefixed for 50 minutes in 2.5% glutaraldehyde, followed by rinsing in cacodylate buffer 3 times for 30 minutes, and cells were removed from the monolayer, pelleted for 25 minutes at 6,000 rpm. The pellets were postfixed for 1 hour in 1% osmium tetroxide, and embedded in araldite by standard techniques. Sections were cut with an LKB ultramicrotome and stained with uranyl acetate prior to viewing on an Carl Zeiss EM 109 electron microscope.

Immunolabeling of protein A-gold: All grids were passed onto drops of the following solutions at room temperature for immunolabeling.

—stored on 10% sucrose-PBS drops on ice until enough are collected
—1.5% bovine serum albumin(BSA)-PBS, 10 minutes
—0.02M glycine-PBS, 10 minutes
—rabbit hyperimmune serum to ADV in 1% BSA-PBS, 60 minutes
—4×1 minute in 1% BSA-PBS
—protein A-gold(10nm probe, Janssen) diluted 1:50 just before use with 1% BSA-PBS, 30 minutes
—4×5 minutes in PBS
—3×3 minutes in destilled water
—2% uranyl acetate in 0.15M oxalic acid, pH 7.0, 8 minutes
—3× a few seconds in destilled water
—a fresh mixture of 0.2% uranyl acetate in 0.5% tylose on ice, 8 minutes; 1% tylose solution was prepared by mixing 25 centipoise MH300 methyl cellulose in destilled water at 90°C, followed by stirring for 24 hours at 4°C, and then centrifuged for 30 minutes at 13,000 rpm. 1% tylose solution was mixed with 0.4% uranyl acetate just before use.
—removed excess fluid by touching the surface of filter paper; The final thickness of the tylose was controlled by the amount of excess fluid removed.

Results

In the ultrathin both frozen and araldite sections of cell cultures immunomarked with hyperimmune serum, ADV antigens were labeled with 10nm electron-dense round gold particles. Gold particles were frequently seen on the envelope and on the capsid membrane(Fig 1, 2). Distribution of the gold particles was similar to that of virus particles observed in the araldite sections(Fig 3, 4).

In the ultracryosections protein A-gold showed a very low degree of interaction with tissue structures and the embedding materials. A faint nonspecific staining was observeр in the background and in the control sections. Viral structure and cell organelles in the cryosections, however, were not so well preserved and contrasted(Fig 5, 6) as those observed in the araldite sections. In the araldite secions immunogold labeling was often not specific, and nonspecific bindings to cell structures and embedding materials were found.

In the araldite sections ultrastructural morphologic details of cells and virus particles were well preserved and had a resonable contrast. The detailed

morphology of internal components of the virus appeared as a small round electron dense structure (Fig 4, 7). Envelopment of internal component occurred by budding at cytoplasmic membranes(Fig 8) and, more frequently, at the inner nuclear membrane gave rise to complete particles between the two membranes and these were released into the cytoplasm(Fig 4). Enveloped virus particles were frequently seen in the perinuclear space. Virions were also present in the cytoplasm of infected cells as enveloped particles within vesicles or as capsids budding into membraned vesicles(Fig 4). Enveloped mature virions were frequently observed in the extracellular space(Fig 7). Infected nuclei showed margination of chromatin, and small groups of capsids within such nuclei were often found in a dense granular matrix(Fig 5). The majority of virus particles observed appeared to invade the cell only after the envelope had been partially digested and fused with the plasma membrane(Fig 3).

The ultrathin cutting were carried out very slowly, 0.3mm/sec, and using an eyelash probe the sections were moved away carefully from the knife edge and grouped together for picking up. For ultracryosectioning optimal cutting temperatures of specimen holder, knife, and in chamber were $-107\pm1°C$, $-117°C$, and $-140\pm5°C$ respectively. Sections were retrieved with platinum loop containing 2.2M sucrose in PBS. It is important that the sections touch the sucrose drop after the latter has cooled but before it freezes completely. Optimal freezing time of sucrose in the cooling chamber was 1~2 seconds for ultrathin sections, and 13 seconds for semithin sections. Optimal knife angle was 6° for both sections. Optimal thickness of methyl cellulose films had white to silver interference colors, and as the film got thicker film structure preservation improved at the expenses of contrast.

Discussion

At the ultrastructural level, colloidal gold used as a tracer for viral particles has been reported by Weiland.[16] He used a colloidal gold-protein A probe for tagging viral particles in the ultrathin araldite sections. Immunogold labeling of ADV antigens at the light microscopic level is superior in sensitivity and intensity of staining to previous immunostaining methods.[10,11] An advantage of the immunogold-silver method is the absence of perceptible background staining. In the ultrathin cryosections of cell cultures immunomarked with hyperimmune serum, ADV antigens, both intracellular and extracellular, were labeled with gold probes. Electron-dense gold particles were mainly present on the envelopes and the capsids. Distribution of the gold particles in the present study was comparable with the previous observation.[16] Presense of the gold particles on the virus particles and absence of this phenomenon in the control sections illustrated the specifity of the immunologic reaction. A faint nonspecific staining was observed in the background and in the control sections. These observations may be attributed in part to nonspecific adsorption of labeled protein or free gold particles that were not washed out completely during the rinsing procedures. The results indicated that tagging of ADV antigens in the ultrathin cryosections provides a useful tool for the detection of viral antigens in tissues and the study of virus-host cell interactions in vivo.

In the araldite sections of this study, immunolabeling of the gold particles was often not specific, and nonspecific bindings were not only observed in the cells but also in the intercellular background. These nonspecific reactions to embedding materials differed from the results observed by Ducatelle and co-workers.[19]

The direct observation of frozen hydrated sections in the cytoelectron microscope has recently become a practical method.[13,14,20~23] In this way one overcomes the usual preparation artifacts except those due to the sectioning process itself, and improves antigenicity preservation. The ultrathin cutting were carried out very slowly, 0.3mm/sec, in the cryochamber. It is important that the sections touch the 2.2M sucrose drop after the latter has cooled but before it freezes completely. The introduction of methyl cellulose to protect thawed frozen sections against surface tension damage caused by air-drying was an essential step in the development of the technique[17,18] The final thickness of the methyl

cellulose is critical with respect to both contrast and fine structure preservation, and this thickness is assessed by the interference colors on the film, after drying. As the film got thicker fine structure preservation improved at the expenses of contrast. Optimal films had white to silver interference colors, and the result differed somewhat from that observed by others.[6,15,18]

In general the replication and the morphogenesis of ADV in cells are similar to those of other herpes virus.[24,25] In the infected cells of this study all the main stages of virus development were observed, i.e., capsid formation, budding of virus particles, the appreance of enveloped virus particles within the cytoplasmic vesicle and their release. The majority of capsids acquired their envelope by budding from the inner nuclear membrane into the perinuclear space. However, many capsids also acquired an envelope by budding into cytoplasmic membraned structures, and this cytoplasmic envelope was mainly derived from thin-walled vesicles.

Summary

The present study was carried out to determine viral antigens and its morphogenesis in the ultrathin frozen and araldite sections of cell cultures infected with ADV by protein A-gold labeling.

ADV antigens were labeled with 10nm gold probes, and electron-dense gold particles were mainly present on viral nucleocapsids and viral envelopes. Immunogold labeling in the ultracryosections showed a very low degree of interaction with tissue structures.

Immunogold labeling in the ultrathin cryosections can be useful tool for the detection of ADV antigens, and the technique also may provide its great potential for immunocytochemical studies on various virus-host cell Interactions.

Legends for figures

Fig 1. Labeling of gold probes on virus particles in ultracryosection. 20,000×

Fig 2. Immunogold labeling on viral particles in ultracryosection. 30,000×

Fig 3. Virus particles in process of entering cell. 20,000×

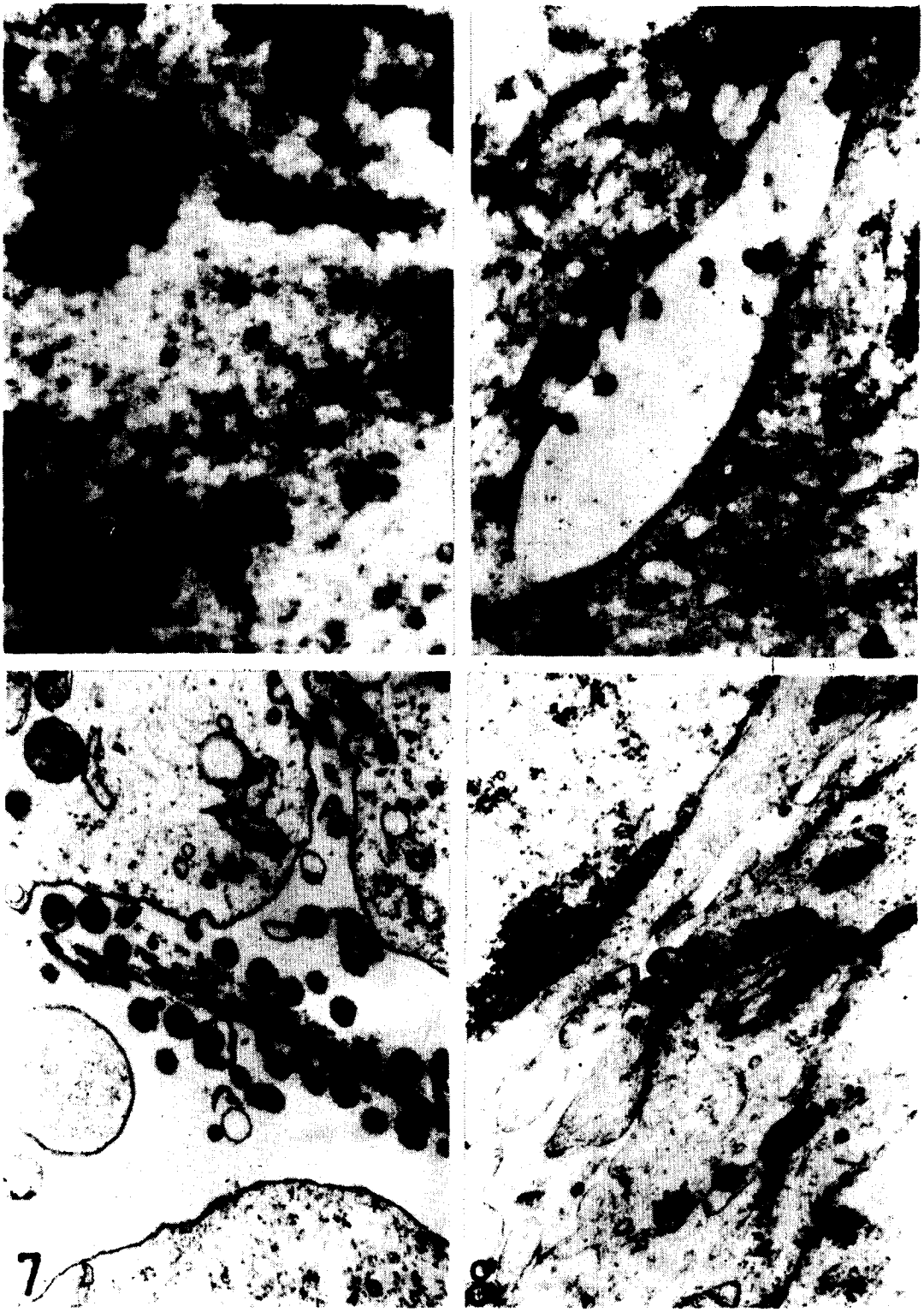
Fig 4. Many virus particles in the cytoplasm, and capsids budding at folded nuclear membranes. 20,000

Fig 5. Clusters of virus particles in the nucleus of ultracryosection. 20,000

Fig 6. Immunogold labeling on virus particles in ultracryosection. 12,000

Fig 7. Virus particles of varying morphology in extracellular space. 20,000×

Fig 8. Nucleocapsids budding through cytoplasmic membrane. 20,000×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