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Immunohistochemistry for detection of Aujeszky's disease virus antigens: Immunogold-silver method in tissue sections

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오제스키병 바이러스 항원검출을 위한 면역조직화학적 연구 —조직절편내 immunogold-silver 법—

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초 록: 본 연구는 감염돼지와 조직배양세포의 동결 및 파라핀 절편에서 immunogold-silver(IGS)법을 이용하여 오제스키병 바이러스를 검출코자 하였으며, 이를 위해 자돈 5두와 돼지뇌유래 섬유아세포 및 BHK 세포를 공시하였고, IGS법과 immunoperoxidase법의 1차 항원으로는 2종의 단크론성 항체와 고도면역혈청을 사용하였다.

IGS법은 양성부위에서 흑색의 미세한 과립의 침착을 볼 수 있었고, immunoperoxidase법에 비해 탁월한 효과를 얻었으며, IGS법은 조직내에 존재하는 각종 바이러스 항원을 검출하는데 이용할 수 있을 것으로 생각된다.

Key words: immunogold-silver method, Aujeszky's disease virus antigens

Introduction

Immunoglobulin adsorbed to colloidal gold, the so-called immunogold reagent, has been used previously for the demonstration of antigens by electron microscopy,[1] and during last twenty years or so it has become increasingly popular in cytochemistry.

The gold particles seen under the electron microscope are too small for demonstration at the light microscope level, although using highly concentrated immunogold.[2] In 1983 a new immunohistochemical technique, the immunogold-silver(IGS) method, was introduced by Holgate and co-workers.[3,4] In this method dilute immunogold is employed and the gold particles that are localized at antigenic sites are subsequently revealed under direct light microscope by a silver precipitation reaction.[2] As they have claimed that the method has greater sensitivity than the immunoperoxidase method,[6~8] the present experiments were tried to investigate its potential for the immunostaining of Aujeszky's disease virus(ADV) antigens in fixed, paraffin embedded tissue or frozen sections.

Materials and Methods

Specimens: Three tested young Landrace pigs were inoculated intranasally with 1.0ml of infected culture medium containing10^{-4} $TCID_{50}$/ml of the ADV-phylaxia strain and were anesthetized at thr 6th or 7th day after virus inoculation. A variety of tissues

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from 5 pigs involving 2 controls were dissected and fixed in Bouin's fluid for 4 hours or 10% neutral formalin solution overnight at 4°C, and both were processed through graded alcohols and xylene to paraffin wax. Another tissue specimens were freezed in liquid nitrogen for frozen section. Fibroblastic cells originated from the pig brain and BHK cells were grown and confluent monolayers were infected with ADV. Cells were removed from monolayers, pelleted in PBS(sodium phosphate buffer, pH 7.6) for 25 minutes at 6,000 rpm, 4°C, and embedded in PBS containing 3% Bactoagar at 42°C, followed by cooling and freezing in liguid nitrogen.

IGS method: All procedures were performed at room temperature and immunostaining was done as follows.

--sections were washed 2×5 minutes in PBS
—pretreated with 2% normal goat serum in PBS, 10 minutes
—covered with primary antibodies respectively; rabbit antiserum to ADV for 1 hour, or monoclonal antibodies for 4 hours or more
--washed 6×3 minutes in PBS
—incubated with goat anti rabbit IgG immunogold conjugate(Auro Probe™ LM, Janssen), 60 minutes
—washed 3×5 minutes in PBS, and then 3×3 minutes in distilled water
—covered with fresh silver enhancement mixture (IntenSE™ Ⅱ, Janssen), 100 minutes
—washed quickly in distilled water
--processed sequentially in graded alcohols and xylene
—mounted with balsam

When appropriate, two adjacent sections were cut and one was stained with immunoperoxidase, another with hematoxylin and eosin.

Indirect immunoperoxidase method[9,10]: Rabbit hyperimmune serum to ADV and goat anti rabbit IgG (F(ab')2) peroxidase of comercial origin(Sigma) were used as the source of primary antibody and conjugate. The staining solution contained 0.01% hydrogen peroxide and 0.1% 3.3'-diaminobenzidine in 0.1M tris-HCl-buffer, pH 7.6. Incubations with the sera were carried out in a moist chamber at 23~25°C.

Results

The IGS method was found to give very intense staining and positive cells were easily identified at low magnification. Positive regions of the pellet sections of cell cultures labelled with the IGS conjugate appeared clearly as areas of fine granular black reaction product and background colouring was weak and absent(Fig 1). Plaques of infected cells exhibited labelling of both nuclei and cytoplasms, and isolated nuclei, often more highly coloured, were seen in cells at an earlier stage of virus replication.

The pattern of staining was similar to that demonstrated by the immunoperoxidase method using the same primary antiserum at a concentration of 1 : 100, however, intensity and specificity of the IGS method were superior to the immunoperoxidase for all antibodies tested. In cases where staining was acceptable when using immunoperoxidase, the IGS method produced a much more intense reaction in the adjacent section. In many cases where very little or no immunoreaction was obtained with immunoperoxidase, positive staining was often obtained using the IGS method.

In paraffin sections positive reactions from both the IGS and Immunoperoxidase methods were usually weaker and background colourings were darker than those in frozen sections.

Uninfected control was completely negative and the cells were difficult to see under the bright field illumination.

Application of the immunoperoxidase procedure to the frozen and paraffin sections yielded a dark brown fine granular reaction in the positive areas of all the organs(Fig 2) and cell pellets examined. Nonspecific background staining and endogenous peroxidase activity were low and did not interfere with the interpretation of the specific stain. Also the pseudoperoxidase activity of the red blood cells could be distinguished from the specific stain. In the negative control sections, this faint nonspecific background staining and endogenous peroxidase and pseudoperoxidase activities could also be seen. The negative control sections never contained specific stain.

The immunoreaction product was mostly confined

to the small necrotic foci. Infected cells with homogeneous amphophilic nuclei were often seen inside and at the margin of the necrotic areas. Granular eosinophilic intranuclear inclusions were rare even in the ADV-infected cell cultures(Fig 3), and when present, were seen in the cells around the necrotic foci. Focal necrosis in association with immunostain was observed in 3 tonsils(Fig 4), 2 spleens, 2 lymph nodes, and 1 liver. In the tonsils, apart from infected foci in the lymphoid tissues, groups of infected epithelial cells were also abserved. These were present both in the tonsillar crypts and in the surface epithelium.

Discussion

This study was carried out to examine an application of the IGS method for the localization of ADV antigens in tissue sections. The sections first were incubated with primary antibody and secondary antibody conjugate coupled to gold particles, and then emersed in a physical developer. The principle of physical development of the IGS method as first used by Roberts[11] is based on creation of a shell of silver around the gold particle. In principle, the same events take place by chemical or normal development of a photographic plate. The only difference between the two methods is the source of silver ions. A physical developer contains both silver ions originate from dissolution of the silver bromide crystals in the photographic emulsion.

I have found that in all cases tested, the IGS method was more intense than that obtained using the immunoperoxidase method. In some cases, staining was obtained with the IGS when no immunoreactivity was visible with immunoperoxidase. With optimal staining conditions, the labelling was seen as an accumulation of fine dark granules on the positive cells. It seems reasonable to assume that the presence of ADV antigens as demonstrated with the IGS method, in direct association with degeneration and necrosis of the affected cells, represented active multiplication and production of viral particles.

Eosinophilic intranuclear material, designated Cowdry type A inclusion has been described in Aujeszky's disease,[12~14] and the lesion seems to be rare.

In conclusion, the IGS method is a simple technique that is easy to perform, and is a valuable alternative to immunoperoxidase and immunofluorescent methods used for this kind of purpose. The thchnique thus has great potential in the field of diagnostic pathology, and might be a reliable tool for the detection of various viral antigens in tissue sections.

Summary

The present study was done to demonstrate ADV antigens in frozen and paraffin sections from ADV-infected pigs and cell cultures by using of the IGS method.

Tissue specimens from 3 young pigs infected with ADV-phylaxia strain and of 2 healthy pigs were used. Fibroblastic cells originated from pig brain and BHK cells were grown and confluent monolayers were infected with the virus. Two monoclonal antibodies and a specific hyperimmune serum to ADV were used as the source of primary antibodies for both the IGS and immunoperoxidase methods.

Application of the IGS method yielded a black fine granular reaction in positive areas, and the results were superior to those obtained using the immunoperoxidase technique for all cases tested. The IGS method might be useful in the detection of various viral antigens in tissue sections.

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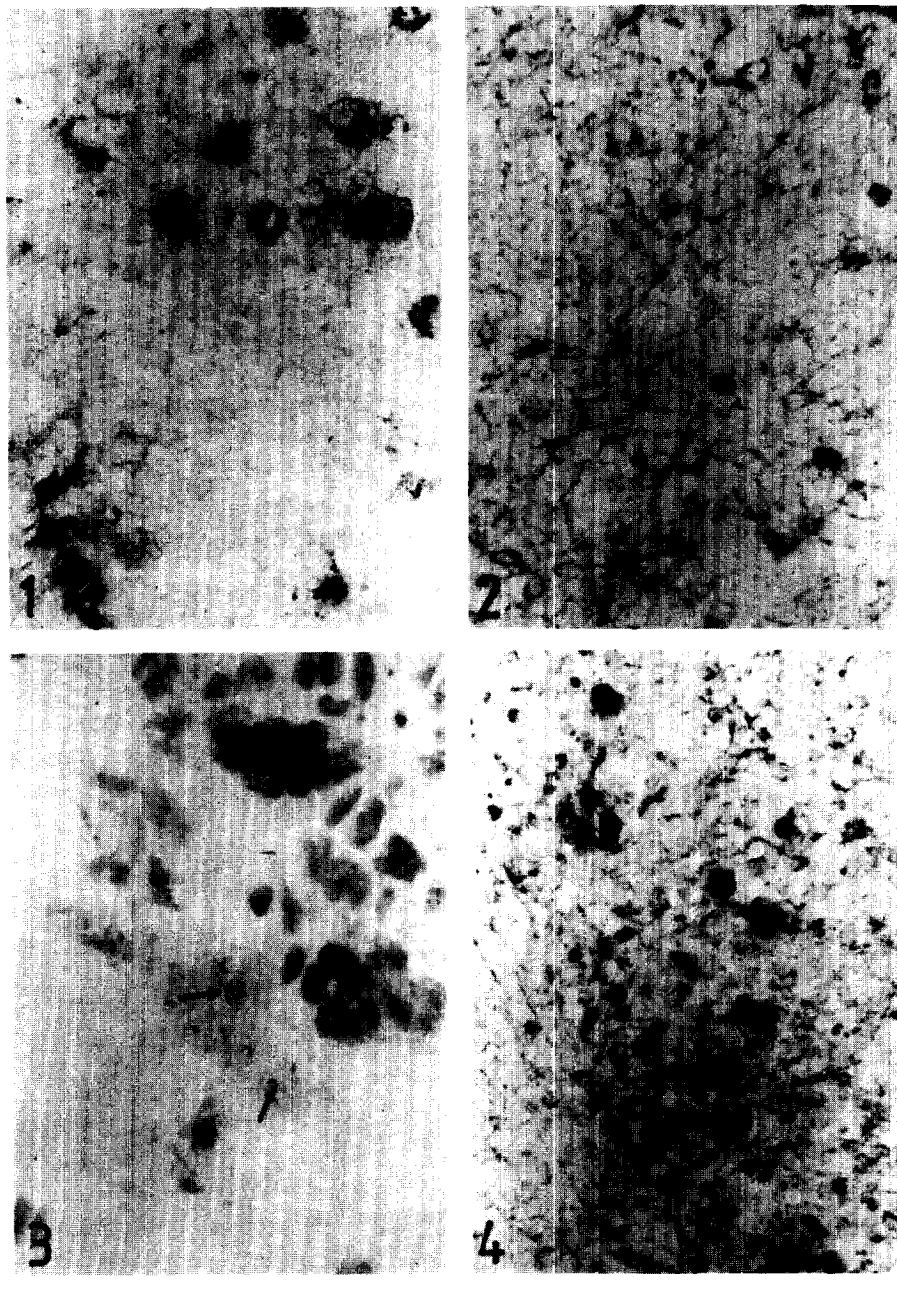
Legends for figures

Fig 1. Positive cells showed an accumulation of dark fine granules. Usually both the cytoplasm and the nucleus are positive for viral antigen. ADV-infected culture cells. IGS method. 125 X.

Fig 2. Positive cells were scattered in the white pulp of the spleen. Immunoperoxidase method. 50 X.

Fig 3. Eosinophilic intranuclear inclusions(arrows) of the infected culture cells. Hematoxylin-eosin stain. 200 X.

Fig 4. IGS positive cells in the infected tonsil. 125 X.



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