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Indirect Immunoperoxidase Staining for Newcastlle Disease Virus (NDV)

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Introduction

Newcastle disease (ND) is the most economically important disease of poultry in many parts of the world, causing serious mortality which may reach 100% in some flocks.

Although vaccination is extensively used for the control of ND in commercial flocks, the village unit of production with only a few birds is seldom vaccinated. Therefore Newcastle disease continues to be a major constraint to increased poultry production in Indonesia.

There is no single gross lesion which is pathognomonic for ND and lesions of avian influenza, infectious laryngotracheitis, infectious bronchitis and other diseases may resemble Newcastle disease (Hanson, 1978; Lancaster, 1981). In addition different strains of Newcastle disease virus, age, level of immunity and species of bird may influence the pathological picture. Rapid and accurate diagnosis is essential for effective control of an outbreak.

The introduction of immuno-histochemical techniques has become a powerful tool in many areas of biological research, including pathology since it is now possible to detect a variety of specific cellular and tissue components such as antigens, antibodies, enzymes, lipid, glycoprotein, etc (Kabawat, Preffer and Bhan, 1985). The ability to use paraffin - embeded tissue for immunohistochemical studies is extremely valuable to the pathologist because it does not require any special processing of tissue eg. the availability of cryostats and freezers.

Recently, immuno histochemical techniques have been used successfully in the diagnosis of viral diseases such as Aujesky's disease, bovine viral diarrheoa, distemper and Newcastle disease (Ducatelle et al, 1982; Ward and Kaeberle, 1984; Palmer, 1986; and Chulan, 1986).

This present study was conducted in an attempt to localise Newcastle disease viral antigen in formalin fixed paraffin - embedded tissue from an experimental pathogenesis study of the lentogenic strain (V4) NDV.

Materials and Methods

The indirect immunoperoxidase method used in this study was based on the method of Bourne (1983). Localization of the NDV antigen was attempted in five organs brain, lung, spleen, caecal tonsil and bursa, which were selected to represent the repiratory system, lymphoid organs and nervous system. Samples were obtained from chickens which were inoculated with lentogenic (V4) strain of NDV provided by Arthur Webster Co., Sydney. Each birds was inoculated with 0.05 ml of allantoic fluid containing $10^{10.14}$ EID50 in 1.0 ml by the oro-nasal route. After the first 3 days two birds were killed each day except that four birds were killed on days9, 7, 14, 21 and 28. Tissues were collected at post mortem and fixed in 10% Normal Buffered Formalin (BNF) for 48 hours and processed for paraffin - embedding.

(a) Preparation of primary antibody.
Cell culture supernatant containing the V4 strain of NDV was collected from hybridoma cells grown in stationary flasks. The virus was purified from the allantoic fluid by ultra centrifugation and was inoculated intraperitonealy to balb c mice three times at weekly intervals. Four days after the third inoculation, splenocytes were fused with NSI myeloma cells using polyethylene glycol. The cell suspension was incubated in medium RPMI 1690 containing HAT and observed for the presence of clones of cells. The supernatant from wells containing clones of cells was screened in an indirect ELISA and by the indirect immunoperoxidase method using acetone fixed MDBK cells infected with NVD. Cells from wells which

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reacted in either test were subcloned and selected on their ability to produce specific reactions and cell culture supernatant was used as primary antibody in this study.

(b) Technique
Sections were cut at 3 mu and placed on slides coated with 0.5% polyvinyl acetate (Fulabond, H.B. Fuller Company) to improve adhesion of the sections. They were then treated with 2.5% H_2O_2 in methanol for 30 minutes to inhibit the activity of endogenous peroxidase then gently washed with PBS for 1 minute. The slides were pre-warmed in a water bath at exactly 37°C before being placed in 0,1% protease (Sigma Chemical Co. Missouri, USA Type VII) in PBS at 37° C for 10 seconds. They were transferred immediately to cold water and washed with PBS.

For blocking antigen the slides were treated with normal rabbit serum diluted to 5% in PBS for 20 minutes. Undiluted NDV V4 supernatant was added as a primary antibody for 60 minutes and then washed off three times with PBS. Sections were treated with secondary antisera (peroxidase - conjugated rabbit anti mouse immonoglobulin, DAKO, Denmark) diluted 1:100 in PBS for 30 minutes and washed twice in PBS.

The reaction was developed in 3.3-diamino-benzidine hydrochloride (DAB) (Fluka 32750), 0.05% in PBS with 5 drops of H_2O_2 for 7 minutes then counterstained with haematoxylin for 1 minute. The specimens were dehydrated, cleared and mounted in DPX.

Results

Localisation of antigen in five different tissues was obtained in this study of experimental infection with the lentogenic V4 strain. Positive reactions obtained by indirect immunoperoxidase staining in paraffin sections were seen as medium to dark brown, fine granular material clearly confined to the cytoplasm of cells. Non-specific background staining and endogenous peroxidase activity were very weak or absent and did not interfere with the interpretation of the specific staining, for example, in the lung. Virtually none was detected in the spleen, caecal tonsil, bursa or brain.

In the negative control sections, specific background staining and auto endogenous peroxidase were absent as were immunoperoxidase positive cells.

In the lung positive cells were found in small numbers in bronchiolar epithelial cells and in sub-epithelial mononuclear cells 7 days after birds were infected with the V4 strain of NDV. The reaction was variable in intensity. Antigen was also detected at the para-bronchial levels, and in the walls of air vesicles. It appeared that macrophages and reticular cells were the majority of the immunoperoxidase positive cells. Antigen was not found in the lung in earlier stages of infection.

Disseminated immunoperoxidase positive cells were detected initially on day 3 after infection in the caecal tonsil with a peak of antigen distribution on day 7. The small triangular and larger immunoperoxidase positive cells were probably reticular and macrophage type cells, respectively. Usually the positive cells were scattered in the lymphoid tissue and none could be detected in the epithelium. Antigen was still present on day 28 after infection, although there were a reduced number of positive cells. The caecal tonsil was the best organ for detecting the antigen with the indirect immunoperoxidase technique.

Similar types of immunoperoxidase positive cells were observed in the spleen. A few were detected on day 3 after infection. The amount of antigen was slightly increased on day 7 with the appearance of immunoperoxidase positive cells external to the ellipsoidal areas and splenic nodules but none was observed in the peri-ellipsoidal reticular cells. Only rarely were positive cells found in the germinal centres. There was little evidence of antigen after day 14 of infection.

No immunoperoxidase positive cells could be seen in the brain or bursa at different stages of infection in this study.

Discussion

The immunoperoxidase method used in this study allowed specific localization of

lentogenic NDV antigen in the caecal tonsil, spleen, and lung. The peak of antigen distribution was detected on day 7 post-infection. By day 28 the virus was still detectable in the caecal tonsil. There was a close correlation between microscopic findings and tissue culture isolation of virus.

A preferential tissue for this method appears to be the caecal tonsil, possibly because of the tropism of the NDV lentogenic (V4) strain (Beveridge, 1981), but spleen and lung at least might also be included. With velogenic strains a wider range of tissue may be necessary.

In comparison with the other immunoenzyme techniques such as direct immunoperoxidose staining, peroxidase anti-peroxidase (PAP) and immuno-flourescent antibody (IFA) techniques, the indirect immunoperoxidase method is more convenient and simple than other techniques in terms of the reagents, procedures and the equipment required (Chulan, 1986). The indirect immunoperoxidase method should be developed more widely as a means for the specific pathological diagnosis of NDV using formalin fixed-tissue.

In conclusion the indirect immuno-peroxidase method is a useful tool for diagnostic pathologists. This method has proved useful in infections with one lentogenic strain but there is an urgent need to develop this for velogenic strains so that it can be used as a rountine tool in diagnostic laboratories which receive significant numbers of avian specimens in endemic areas.

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