

RAPID CONFIRMATION OF RABIES ANTIGEN IN FORMALIN-FIXED PARAFFIN-EMBEDDED TISSUE BY IMMUNOPATHOLOGIC DIAGNOSIS

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ABSTRACT

Skunks experimentally infected with street virus rabies intramuscularly were used to demonstrate rabies viral antigen in paraffin-embedded tissue by immunopathology diagnosis approach, the avidin biotin complex (ABC) method. The use of ABC method provides a simple and sensitive method to localize rabies virus antigen in formalin-fixed tissues. The results of the present study also demonstrated that a procedures using formalin-fixed paraffin-embeded tissue and stained immunopathologically by the ABC method was an excellent method for both preservation of morfological details of cells/tissues and demonstration of rabies virus antigen.

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ABSTRAK

Skunk yang diinfeksi dengan *street rabies virus* intramuskuler digunakan untuk mengidentifikasi antigen virus rabies pada spesimen jaringan yang dicetak parafin melalui pendekatan diagnosis imunopatologi, metode *avidin biotin complex*. Aplikasi metode ABC yang digunakan untuk menentukan/melokasi adanya antigen virus rabies pada sediaan jaringan cetak parafin merupakan metode yang sederhana dan sensitif. Hasil penelitian ini juga menunjukkan bahwa prosedur fiksasi jaringan dengan formalin dan kemudian dicetak parafin, yang selanjutnya diwarnai secara imunopatologi dengan metode ABC merupakan metode yang baik dan tepat untuk preservasi rincian morfologik sel/jaringan dan identifikasi antigen virus rabies.

INTRODUCTION

Rabies virus causes lethal infection in humans and animals with the occurrence of acute encephalitis in most cases and paralysis in some cases. Until recently, the postmortem diagnosis of rabies has been based mainly on conventional histopathologic examination to demonstrate intracytoplasmic inclusions, Negri bodies (Negri, 1903). If fully developed Negri bodies were not observed, the diagnosis of rabies by this method was difficult or inconclusive. Negri bodies, however, were not detected in any tissues which were found to be positive by virus isolation in mice or in cell culture (Koprowski, 1973; Fekadu and Smith, 1986). The development of Negri bodies is related to the length of time a rabies victim lives after signs and symptoms of the diseases appear. It is also extremely difficult to detect rabies antigen in tissues that may no longer contain infectious viral particles because of postmortem autolysis. The fluorescent antibody (FA) technique is widely accepted as the best method to visualise fine particles of rabies viral antigen in rabies infected tissues. In FA technique, however, it is difficult to identify surrounding tissue structures and cellular morphological details. The FA technique of rabies antigen could be accomplished only by examining fresh-frozen or glycerin-preserved tissues.

Consequently, immunopathologic diagnosis has been developed that used monospecific antisera to rabies virus antigen in formalin-fixed embeded tissue sections with enzyme immunoassay approaches. In the

present study, avidin biotin complex (ABC) technique was developed to examine the distribution of street rabies virus antigen in the brain of skunk after inoculation by intramuscular route.

MATERIALS AND METHODS

Tissue preparation

Tissue from skunk experimentally infected with street virus rabies were used in trials of a formalin fixative and a processing method for the ABC technique. The glass slides of formalin-fixed paraffin-embedded brain tissues of the skunk and polyclonal antibody: rabbit hyperimmune serum to rabies virus was kindly provide by Prof. Dr. Roger K. Maes, Department of Microbiology and Public Health, and Animal Health Diagnostic Laboratory, Michigan State University, E. Lansing, MI, USA.

Skunk were inoculated intramuscularly with street rabies virus by methods previously described (Black and Lawson, 1970; Charlton and Casey, 1979). They were killed in terminal stages of the diseases, or when moribund. The brains were removed and pieces of brain were placed in 10% buffered formalin pH 7.0.

Brain tissues were dehydrated in ethanol, cleared in xylene and embedded in low-melting point paraffin (52-54°C). Section were cut at 6 μ m, floated on gelatin-coated slides, deparaffinized in 3 x 2 minutes changes of cold xylene, followed by 3 x 15 seconds changes of 95%, 70% and 50% ethanol, respectively, and rehydrated in distilled water.

Immunopathologic diagnosis assay procedure

The avidin biotin complex (ABC) technique was used. After deparaffinization and rehydration, brain tissue section were rinsed with 0,05 M phosphate buffered (PBS) saline pH 7.5, following which they were blocked for endogenous enzyme activity by incubation in 0,3% hydrogen peroxide in absolute methanol for 30 minutes. They were then treated with normal goat serum for 30 minutes to reduce non-specific binding. After the slides were washed with PBS, the primary antibody was applied and the slides were incubated in a moist chamber for 2 hours at 37°C. Control slides were treated with normal rabbit sera instead of the primary antibody. After incubation, the slides were washed and then treated with biotinylated goat anti-rabbit IgG diluted 1:200 for 30 minutes at room

temperature. After washed, they were incubated with horseradish peroxidase-labelled streptoavidin biotinylated complex form 15 minutes. The slides were again washed with PBS, after which they were treated with 0.5 mg/ml 3-3'diaminobenzidine tetrahydrochloride and 0.005% hydrogen peroxide in PBS, counterstained with hematoxylin, and examined under a light microscope.

RESULTS

Fresh-formalin-fixed tissues were very well stained by the avidin biotin complex (ABC). In skunks inoculated with street rabies virus, large Negri's bodies were mainly in the perikarya of neurons while neuronal processes contained viral antigen in the form of very fine particles of filaments. Avidin biotin complex staining was very intense and extensive with formalin-fixed tissues. Antigen occurred as large and small bodies, granules and filaments. Large clumps or aggregate viral antigen stained dark brown. Some bodies were brownish-yellow at the centre with a dark brown ring at the periphery. Small bodies or granules were present in most of neurons of the challenge virus standard infected brain (Fig. 1). Also morphological of the brain tissue was well preserved, being similar to that of routine histopathological sections. In the cerebellum, the dendrites of Purkinje cells were well outlined by their content of rabies antigen. The controls were negative and free of endogenous peroxidase (Fig. 2).

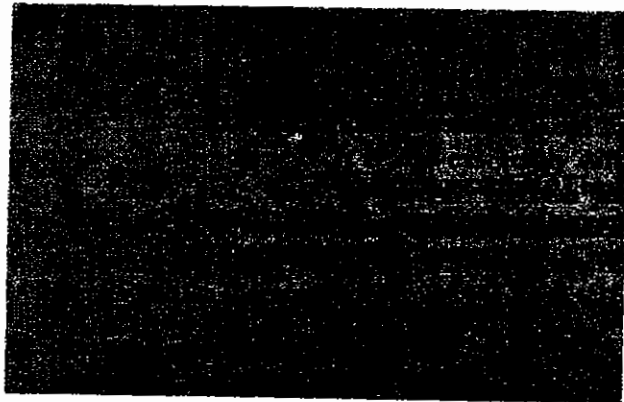


Fig. 1. Avidin biotin complex staining of rabies viral antigen in large and small inclusion bodies in the cytoplasm of a neuron (ABC, 500x).

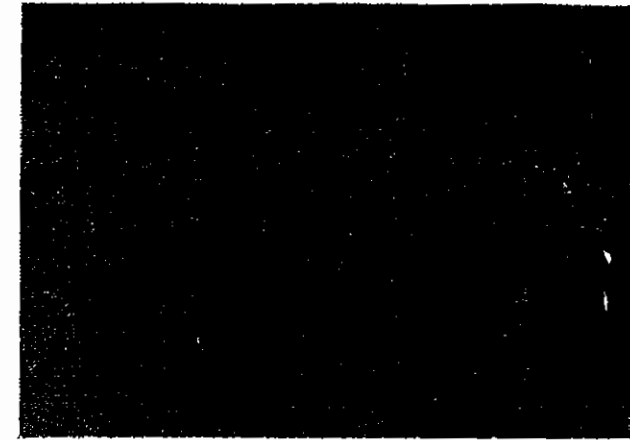


Fig. 2. Avidin biotin complex staining of non-challenge street rabies virus antigen standard. The control was negative and no rabies antigen in neuronal processes (ABC, 500x).

DISCUSSION

Immunocyto(histo)chemical methods are valuable tools for both routine histopathology and research. Permanence of the reaction product and usefulness in fixed tissue section together with the facility for simultaneous pathological diagnosis, make the immunoperoxidase methods the technique of choice in histopathology at the present time (Balachandran and Charlton, 1994).

In this trial, the avidin biotin complex (ABC) technique is a good method for rabies diagnosis in sections of 10% neutral buffered formalin. The both morphological features and details of the tissue and demonstration of fine as well as large particles of antigen were well preserved. Based on our observation, fine particles of rabies viral antigen in formalin-fixed, paraffin-embedded tissues, similar to immunofluorescence (Johnson *et al.*, 1980; Barnad and Voges, 1982). However, the sensitivity of viral antigen detection by immunofluorescence method tends to decrease after long preservation (Palmer *et al.*, 1985), so that its sensitivity in such samples is not as high as that in ABC method.

Besides the ABC method, peroxidase-antiperoxidase (PAP) immunohistochemical staining technique has also been developed that use specific antisera to rabies virus (Tsiang, 1982; Torres-Angel *et al.*, 1984; Palmer *et al.*, 1985; Bourgon and Charlton, 1987). Using these ABC and PAP methods, rabies viral distribution has been examined in natural cases of rabies in humans and animals after death (Feiden *et al.*, 1985; Feiden *et al.*, 1988).

Negri bodies which consist of viral nucleoproteins accumulated in the cytoplasm, were found in this study, especially in skunks at the moribund stage. The mechanism by which rabies virus disseminates within the CNS are poorly understood, even though rabies virus replication is known to be strictly neurotropic (Rupprecht and Dietzschold, 1987). In the experimental infection of rats, it has been demonstrated that rabies virus entered from the peripheral nerves and spread through axonal transport and the cerebrospinal fluid (CSF) pathway (Tsiang, 1982; Gillet *et al.*, 1986). In mice inoculated intracranially with a high dose of rabies virus, viral antigens were found in the ependymal cells and in neurons adjacent to the central canal of the spinal cord (Jackson and Reimer, 1989).

CONCLUSION

In conclusion, the ABC methods in formalin-fixed paraffin-embedded tissues was a good method for both demonstration of antigen and preservation of tissue structure. Our method is well suited for research on rabies. Since this method is simple (not time consuming) and sensitive, it is considered a desirable approach for routine rabies diagnosis on fixed tissue. It is therefore useful in cases where the only tissue available was fixed in formalin.

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