Journal of Virological Methods 237 (2016) 40–46

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Research article

Localization of the rabies virus antigen in Merkel cells in the follicle-sinus complexes of muzzle skins of rabid dogs

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Article history:
Received 19 March 2016
Received in revised form 10 June 2016
Accepted 29 August 2016
Available online 30 August 2016

Keywords:
Follicle-sinus complexes
Immunohistochemistry
Merkel cells
Postmortem diagnosis
Rabies

ABSTRACT

The direct fluorescent antibody test (dFAT) on fresh brain tissues is the gold standard for rabies virus antigen detection in dogs. However, this method is laborious and holds a high risk of virus exposure for the experimenter. Skin biopsies are useful for the diagnosis of humans and animals. In mammals, the tactile hair, known as the follicle-sinus complex (FSC), is a specialized touch organ that is abundant in the muzzle skin. Each tactile hair is equipped with more than 2,000 sensory nerve endings. Therefore, this organ is expected to serve as an alternative postmortem diagnostic material. However, the target cells and localization of rabies virus antigen in the FSCs remain to be defined. In the present study, muzzle skins were obtained from 60 rabid dogs diagnosed with rabies by dFAT at the Research Institute of Tropical Medicine in the Philippines. In all dogs, virus antigen was clearly detected in a part of the outer root sheath at the level of the ring sinus of the FSCs, and the majority of cells were positive for the Merkel cell (MC) markers cytokeratin 20 and CAM5.2. Our results suggest that MCs in the FSCs of the muzzle skin are a target for virus replication and could serve as a useful alternative specimen source for diagnosis of rabies.

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1. Introduction

Rabies is a zoonotic disease caused by the rabies virus. The virus is transmitted to humans via rabid animals and causes acute encephalomyelitis after a variable incubation period. More than 150 countries and territories currently report rabies infections in humans, with over 55,000 deaths annually, of which approximately 34,500 occur in Asian countries (WHO, 2013). In the Philippines, domestic dogs are the primary reservoir of rabies and more than 98% of human rabies deaths are due to dog bites (Dimaano et al., 2011).

The most widely used test for rabies diagnosis is the direct fluorescent antibody test (dFAT), which is recommended by both the OIE and WHO (OIE, 2009; WHO, 2013). The sensitivity of the dFAT is 100% when the brain samples are in a fresh state, and definitive diagnosis can be completed within 2 h. However, the sensitivity lowers and can be unsuitable when the brain samples have started to decompose under warm temperature (David, 2012; Kamolvarin et al., 1993; McElhinney et al., 2014). In addition, sampling of the dog brain is laborious, carries a high risk of virus exposure, and requires expensive equipment. Therefore, alternative specimens for postmortem diagnosis of rabid dogs by low-cost, simple tests with low virus-exposure risk are needed in rabies-endemic Asian countries.

Skin biopsies are useful for ante- and postmortem diagnosis of humans (Blenden et al., 1986) and animals (Blenden et al., 1983; Smith et al., 1972). The virus antigen can be detected in the peripheral nerves surrounding the hair follicles, and the positive rate

Abbreviations: CNS, central nervous system; dFAT, direct fluorescent antibody test; FSC, follicle-sinus complex; MC, Merkel cell.

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http://dx.doi.org/10.1016/j.jviromet.2016.08.021
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increases as infection progresses (Blenden et al., 1986). In animals, the tactile hair, known as the follicle-sinus complex (FSC), is a specialized touch organ that is abundant in the muzzle skin. The tactile hair is embedded in the blood sinus, which plays an important role in tactile sensitivity and assists the animal in exploring its environment (Esteves et al., 2009; Marshall et al., 2014). Each tactile hair is equipped with more than 2,000 sensory nerve endings (Halata, 1993). After infection of the central nervous system (CNS), the rabies virus centrifugally spreads to the peripheral tissues via the sensory nerve. Therefore, tactile hairs are expected to serve as an alternative postmortem diagnostic material. In addition, obtaining tactile hair follicles from the muzzle skin of dead dogs is easy and practical, and does not require any specialized and expensive equipment. However, the target cells and localization of rabies virus antigen in the FSCs remain to be defined. The present study were undertaken to provide information on the target cells and localization of rabies virus antigen in the FSCs in the muzzle skins of rabid dogs.

2. Materials and methods

2.1. Animals

Muzzle skin samples were obtained from 60 rabid dogs, which had been submitted to the Research Institute for Tropical Medicine of the Philippines for postmortem diagnosis of rabies. Forty dogs were found dead, whereas 20 dogs had been subjected to euthanasia. The 60 dogs (25 males, 17 females, and 18 of unknown sex) ranged in age from 1 month to more than 16 years, with 15 dogs of unknown age. Thirty-five of the 60 dogs had no history of rabies vaccination, and no information on rabies vaccination status was available for the other 25 dogs. Almost all of the dogs were free-roaming or strays. The primary clinical symptoms of canine rabies infection, such as unprovoked aggressiveness, mad biting of inanimate objects, aimless running, and excessive salivation, were observed in 25 of the 60 dogs, and there was no information available for the remaining dogs.

2.2. Direct fluorescent antibody test (dFAT)

Small transverse sections (2–3 mm in thickness) of Ammon's horn and the brain stem of the 60 dogs were cut, and a slide was touched against the cut surfaces after which the slides were placed on cold acetone overnight for fixation. After fixation, the slides were air-dried at room temperature. Then, 450 μl of fluorescence isothiocyanate-conjugated anti-rabies monoclonal antibody (Fujirebio, Malvern, PA, USA) was added. The slides were incubated for 30 min at 37 °C in a high-humidity chamber. The slides were dipped and rinsed 20–25 times in phosphate-buffered saline (PBS) twice followed by a wash in distilled water. Small amounts of mounting medium, 20% glycerol in Tris-buffered saline (pH 9.0), were placed on the slides before covering with coverslips for examination. The slides were examined under a fluorescence microscope (80i, Nikon, Tokyo, Japan).

2.3. Histopathological examination

Muzzle skin samples of the 60 rabid dogs and 3 rabies-vaccinated domestic mixed-breed dogs (8–10 years old) were fixed in 10% neutral buffered formalin at room temperature for more than 72 h. Muzzle skins including FSCs (Fig. 1A) were cut into transverse (Fig. 1B) and longitudinal (Fig. 1C, D) sections. The transverse sections were made at the level of the ring sinus of the FSCs identified via stereoscopic microscopy (MZ7.5; Leica). The samples were embedded in paraffin, sectioned at a thickness of 3 μm, and mounted. The sections were stained with hematoxylin and eosin and serial sections were subjected to immunohistochemistry as described below.

2.4. Immunohistochemistry

For the detection of the rabies virus antigen in tissues, sections were stained using the streptavidin-biotin-peroxidase complex method with rabbit anti-phosphoprotein (P) as described previously (Boonsriroj et al., 2016). For the identification of the cell type, the following primary antibodies were used: mouse anti-cytokeratin 20 (CK 20; Nichirei Biosciences, Tokyo, Japan) and cytokeratin CAM5.2 (CAM5.2; Becton-Dickinson, San Jose, CA, USA) as markers of Merkel cells (MCs) (Moll et al., 2005; Narisawa et al., 1992), and mouse anti-neurofilament protein (NF; Dako, Kyoto, Japan) as a nerve cell marker. Briefly, tissue sections were treated for antigen with 0.25% trypsin at room temperature for 30 min for anti-P antibodies, and microwaved at 170 W for 10 min for CK 20, 15 min for proteinase K, or 10 min for CAM 5.2 and NF. To remove endogenous peroxidase, sections were treated with 3% H_2O_2 in methanol. To block nonspecific reactions, sections were treated with 10% normal goat or rabbit serum. Primary antibodies were diluted 1:1000 (anti-P) in PBS and incubated at 4 °C overnight in a humidified chamber. As a secondary antibody, anti-rabbit IgG (Nichirei Biosciences) was used for anti-P. The EnVision+ System Labeled Polymer-HRP anti-mouse antibody (Dako) was used for CK 20 and CAM 5.2. Histofine Simple Stain MAX-PO anti-mouse (Nichirei Biosciences) was used for NF. Antibodies were visualized using 3-3′-diaminobenzidine (DAB; Dako). Finally, the slides were counterstained with hematoxylin.

2.5. Double immunofluorescence staining

To identify cell types that express virus antigen, double staining of a single tissue section was conducted with an immunofluorescent antibody. To evaluate the relationship between antigen-positive cells and MCs, a combination of anti-P antibody and CAM 5.2 staining was used as described in section 2.3. As a secondary antibody, FITC conjugate goat anti-rabbit IgG (H + L) (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) and Alexa Fluor 564 conjugate goat anti-mouse IgG (H + L) (Thermo Fisher, Waltham, MA, USA) were used at 1:200 dilutions. DAPI (Thermo Fisher) was used for counterstaining.

2.6. Colocalization of virus antigen and MCs

To analyze the colocalization of the virus antigen and MCs, 97 FSCs from 15 rabid dogs were double-stained with anti-P and anti-CAM 5.2 antibodies, and the numbers of each positive cell type and merged cells were counted. The correlation coefficient and curve approximation were calculated using SPSS statistical software (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. dFAT results of Ammon's horn and brain stem tissues

All Ammon's horn and brain stem specimens of the 60 dogs were positive for the rabies virus antigen in the dFAT.

3.2. Histopathological and immunohistochemical findings of muzzle skin biopsies

In 7 of the 60 rabid dogs, focal infiltration of lymphoplasmacytic cells and necrosis of the epithelial cells were observed in the outer root sheath located above the ring-wulst (Fig. 2A, B). In the remaining dogs, there were no marked histopathological findings

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