IMMUNOHISTOCHEMICAL RECOGNITION OF A WIDE SPECTRUM OF LYSSAVIRUSES IN FORMALIN-FIXED TISSUES BY ONE MONOCLONAL ANTIBODY

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Automated indirect immunoperoxidase (avidin-biotin complex) staining using monoclonal antibody #5DF12-3B6, directed against rabies N-protein, was used to detect rabies antigen in tissue samples from animals either naturally or experimentally infected with rabies. This monoclonal antibody recognized all 16 strains of rabies virus tested, as well as rabies-related lyssaviruses including Duvenhage, Lagos Bat, and Mokola. The sample infected with Mokola virus initially showed only weak staining, however, deletion of protease digestion resulted in stronger stain uptake. The test was sensitive and specific, correctly identifying rabies antigen in all but one of the samples tested (37/38), and no apparent staining in any of the negative samples tested (23/23). Tissues from 16 mammalian species were tested, including one rabies infected human tissue sample. The utility of the immunoperoxidase staining method described in this study lies in the ability of one monoclonal to recognize a broad spectrum of lyssaviruses in formalin-fixed tissues.

Rabies is a highly fatal, zoonotic viral disease of worldwide concern. Several regional rabies eradication programs have been proposed. A reliable diagnostic test for rabies antigen that recognizes a wide variety of rabies virus variant, as well as rabies-related viruses, that can be performed on formalin-fixed tissue, would be a useful screening tool for such a program. Formalin-fixation of tissues simplifies collection, storage and transport of samples, and eliminates hazards and need for biocontainment. It also facilitates retrospective studies specially if all tissues have already been fixed.

Rabies antigen has been detected in formalin-fixed tissue by both immunofluorescent

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antibody test (IFA) (*1,2,12-14*) and immunoperoxidase staining (2-4, 9-11). However, the advantage of immunoperoxidase staining over IFA is that it allows for concurrent histopathologic examination of the tissue sections(*2,13*).

Both monoclonal (MAb) and polyclonal antibodies have been used successfully in immunohistochemistry to detect rabies antigen in formalin-fixed tissue. Immunoperoxidase staining using MAb has yielded better results than staining with polyclonal antibodies(*8*). One study tested a series of MAbs against a variety of rabies epitopes, and defined a panel that could recognize a wide spectrum of lyssaviruses using immunofluorescence(*20*). MAbs have also been used extensively to differentiate between lyssavirus variants of different serotypes (19,20), and have been a valuable tool in the epidemiologic study of rabies (16).

The primary purpose of this study was to document the recognition of a wide spectrum of rabies and rabies-related viruses by a single MAb using an automated avidin-biotin complex (ABC) immunoperoxidase staining procedure. This MAb has already been used to identify rabies antigen in infected tissue samples(*4*). The MAb used in this study, #5DF12-3B6, recognizes rabies protein N, which is a component of rabies ribonucleoprotein (RNP). The RNP is the most highly conserved portion of the virion among lyssavirus serotypes.

MATERIALS AND METHODS

Monoclonal antibody

The hybridoma 5DF12-3B6 was originally developed in 1984 at the Swiss Rabies Center in Bern, Switzerland, by fusing P3U1 myeloma cells with spleen cells of a mouse immunized with nucleoprotein preparations of the SAD-Bern strain of rabies virus (H. Gerber, AI Wandeler, personal communication). Cloning and maintenance of hybridomas followed standard procedures(*7*). The MAb 5DF12-3B6 binds to N-protein of rabies viruses as documented by Western blotting and immunoprecipitation. In indirect IFA 5DF12-3B6 stains inclusion bodies in cells infected with lyssaviruses. The tissue culture supernatants used in the present investigations were produced on recloned 5DF12-3B6 hybridoma cultures grown in the Monoclonal Antibody Unit of Animal Disease Research Institute, Nepean, Canada.

Tissue samples

To analyze the ability of the MAb to detect the viruses, a total of 60 brain tissue samples were examined for rabies antigen using ABC immunoperoxidase staining. Of these, 44 samples were from a variety of mammalian species that were obtained in different degrees of preservation, fixed in 10% formalin and saved by the New York State Department of Health, Albany, NY. One half of these samples (22 samples) were street rabies positive (21 mid-atlantic raccoon variant (MAR), and 1 Ontario red fox variant) by in vitro viral isolation using mouse neuroblastoma cell culture(*15*) and IFA(*18*); the other half were rabies negative. The MAR positive samples included tissues from dog, cat, raccoon, rabbit, beaver, and white tail deer, and 1 dog was positive for the Ontario red fox variant. The negative samples included the species mentioned above plus muskrat, guinea pig, opossum, goat, skunk, horse and cattle.

Fifteen samples were from mice experimentally infected at the New York State

Department of Health, Albany, NY with different strains of rabies virus or rabies-related lyssavirus. Infection was verified by IFA and virus isolation. One murine tissue sample was included as a rabies negative control. All samples were numbered and stained blindly.

In addition, brain tissue from mice inoculated with Duvenhage, Lagos bat and Mokola were kindly supplied by Dr. C. Rupprecht (Centers for Disease Control and Prevention, Rabies Unit, Atlanta, GA). Furthermore, human brain tissue infected with silver-haired bat virus was also stained.

Immunohistochemistry

The ABC immunoperoxidase staining procedure was developed(*8*) and tested using known positive (4 horse and 2 bovine tissue samples) and negative formalin-fixed brain tissue samples obtained from the pathology files at Cornell University, Ithaca, NY. These samples were formalin-fixed and paraffin-embedded for up to 6 years. The rabies negative control tissues were from dog, horse and cattle infected with distemper, equine herpes virus 1 and bovine herpes virus 1 respectively.

ABC immunoperoxidase staining was performed with an automated slide stainer. Tissue sections of 4 um thickness were mounted on ProbeOn glass slides, and were deparaffinized using d-limonene and butylated hydroxanisole[a]. Endogenous peroxidases were inactivated with a solution of 0.5% H_2O_2 in methanol for 12 minutes at room temperature. The sections were then digested with a solution of proteases[b] for 20 minutes at 42°C. Nonspecific protein adherence was blocked using 4% horse serum in PBS. The primary murine MAb was incubated for 55 minutes at 42°C. The secondary biotinylated horse anti-mouse antibody[c] was incubated for 22 minutes at 42°C. The avidin biotin peroxidase solution[d] was incubated for 35 minutes at 42°C and the chromogen, 3,3-diaminobenzidine-4HCl, for 5 minutes at 42°C. The sections were counterstained using Gills # 2 hematoxylin, dehydrated and coverslipped.

The staining procedure described above was repeated without protease digestion on all of the samples.

The sections were observed by light microscopy and samples were identified as negative in the absence of specific staining.

RESULTS

ABC immunoperoxidase staining with MAb #5DF12-3B6 identified rabies antigen in neurologic tissue of 21 of the 22 naturally infected animals (Table 1). This staining procedure did not identify antigen in 1 sample taken from a raccoon that was infected with rabies MAR strain.

Brain tissue from 22 animals that were not infected with rabies and 1 rabies negative sample from an experimental mouse showed no apparent staining.

ABC immunoperoxidase staining was able to identify rabies antigen in a variety of different mammalian hosts. In all, tissue samples taken

Sources and manufacturers

[a]Hemo-de, Fisher Scientific, Pittsburgh, PA.
[b]Pronase E, Sigma Chemical Co., St Louis, MO.
[c]Biotinylated horse anti-mouse antibody, Vector Laboratories, Burlingham, CA.
[d]VECTASTAIN Elite ABC Kit, Vector Laboratories, Burlingham, CA.

from 15 different animal species and 1 human sample were examined for rabies antigen. The only misdiagnosed sample was one taken from a raccoon, which was the most frequently examined animal (n=18). Misdiagnosis was unlikely due to species considerations.

The MAb was able to identify all the rabies strains that were tested (Table 2). All strains stained strongly with minimal nonspecific staining. Tissues infected with Lagos Bat and Duvenhage, 2 of the 3 rabies related viruses tested, showed strong, specific stain uptake as well. Tissue infected with Mokola rabies-related virus showed a weak staining reaction, however, deletion of protease treatment of the Mokola infected tissue sample resulted in much stronger stain uptake.

No cross reactivity was observed in tissues infected with distemper, equine herpes virus 1 and bovine herpes 1.

DISCUSSION

This study has successfully demonstrated that automated ABC immunoperoxidase staining with MAb #5DF12-3B6 can recognize a wide spectrum of lyssavirus serotypes in formalin fixed paraffin embedded tissues. It is this wide spectrum recognition of lyssavirus serotypes, without cross reaction with other antigens, that makes this study noteworthy.

This staining procedure did not identify antigen in one sample taken from a raccoon that was infected with MAR strain. There was

TABLE 1. ABC Immunoperoxidase staining of brain tissue from naturally infected animals

Species	No. animals tested	Virus strain	IFA^ positive	IHC| positive
Dog	4	MAR*	2	2
Cat	6	MAR	3	3
Raccoon	18	MAR	12	11
Rabbit	3	MAR	1	1
Beaver	2	MAR	1	1
Whitetail deer	2	MAR	2	2
Dog	1	ORF#	1	1
Muskrat	1	-	0	0
Cattle	2	-	0	0
Goat	1	-	0	0
Horse	1	-	0	0
Guinea pig	1	-	0	0
Opossum	1	-	0	0
Skunk	1	-	0	0
Total	44		22	21

* MAR - Mid-Atlantic Raccoon.
# ORF - Ontario Red Fox.
^ IFA - Immunofluorescent antibody test.
| IHC - ABC Immunoperoxidase staining

TABLE 2. ABC Immunoperoxidase staining of brain tissue from mice experimentally infected with different strains of rabies virus

Virus strain	Immunofluorescent antibody test	Immunoperoxidase
Ontario red fox	+	+
Mexican dog	+	+
Oklahoma skunk	+	+
ERA	+	+
Silver-haired bat	+	+
Denmark bat	+	+
Hoary bat	+	+
Eptesicus bat I	+	+
Eptesicus bat II	+	+
Vampire bat	+	+
Myotis bat	+	+
Holland bat	+	+
Red bat	+	+
Poland raccoon dog	+	+
Mexican freetail bat	+	+

nothing unususal about the host or the strain of rabies virus. This failure of recognition is most likely due to the minimal antigen content of the sample, as it was only weakly IFA positive, and the tissue sample itself was exceptionally small.

There are 6 lyssavirus serotypes: (1) Rabies, (2) Duvenhage, (3) Mokola, (4) Lagos Bat, (5) kotonkan, and (6) Obodhiang. The first three produce clinical disease in man, and the last two are arthropod-borne. Only rabies has been recognized as having worldwide distribution. It was previously believed that the rabies-related viruses were restricted to shrews and mice of sub-Saharan Africa. However, atypical rabies virus similar to Mokola virus were isolated from cats and a dog in Zimbabwe(*5*), and the recent realization that a "Duvenhage-like" virus is present in bats in Europe, may pose a question regarding past rabies diagnostic screening procedures(*17*). This illustrates the importance for stringent screening for rabies-related viruses in all rabies diagnostics, control programs, or epidemiology studies, regardless of their location.

The weak stain uptake of tissue heavily infected with Mokola virus in this study is of concern, although deletion of protease digestion did improve stain uptake. Most rabies strains are so antigenically similar that it is difficult to distinguish one from another. However, there is significant antigenic variation between rabies and rabies-related viruses. The arbo lyssaviruses show only a distant antigenic relationship to rabies, whereas, the other three rabies-related viruses are antigenically similar to rabies virus(*17*). Of these three, only Mokola virus shares common determinants with the arbo lyssaviruses(*6*) making it a genetic-link between rabies virus and the arbo lyssaviruses. However, this also suggests that of the similar lyssaviruses, rabies, Mokola, Duvenhage, and Lagos Bat, Mokola is the most antigenically

distinct. This might account for the weak reaction of the Mab tested in tissue containing abundant Mokola virus antigen.

In addition to the wide spectrum of lyssavirus recognition, the MAb ABC immunoperoxidase test described in this study has several other advantages over previously described rabies diagnostic tests. Previously immunoperoxidase staining has been time consuming, and the number of samples processed per day was limited to approximately 50 slides per day(*8*). Using an automated slide stainer has greatly alleviated this restraint. The machine used in this study can stain 60 slides using a 3 1/2 hour protocol. In addition, automation decreases the amount of variability between slides by keeping constant all variables, including temperature, reagent amounts and incubation times.

Immunoperoxidase staining was performed on formalin-fixed tissue. In many cases it is not practical to transport fresh or frozen tissue to the appropriate laboratory facility for IFA staining or viral isolation. Fixing tissues in formalin is easily done, as it requires no special preparations or technical skills. In addition, fixing tissue in formalin reduces the risk of human exposure. Furthermore, the ability to detect rabies antigen in formalin-fixed tissues allows for retrospective studies, as well as diagnostic use.

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RESUMEN

El reconocimiento inmunohistoquímico de un amplio espectro de lyssaviruses en tejidos fijados en formalina por un anticuerpo monoclonal.

Una tinción de inmunoperoxidasa indirecta (complejo avidina-biotina) usando el anticuerpo monoclonal 5DF12-3B6, que reconoce la proteína N del virus de la rabia, se usó para detectar antígeno del virus de la rabia en muestras de tejido de 15 especies animales y una muestra humana infectadas con rabia naturalmente o experimentalmente. Este anticuerpo monoclonal reconoció todas las 16 cepas de virus de la rabia que se usaron en este estudio, como también lyssavirus relacionados a rabia como Duvenhage, Lagos Bat y Mokola. La muestra infectada con Mokola inicialmente sólo demostró una tinción débil, la que se hizo más fuerte cuando se eliminó el tratamiento con Pronase E. La tinción es sensible y específica, identificando correctamente el antígeno de rabia en todas las muestras usadas con excepción de una muestra (37/38) que era débilmente positiva por IFA y muy pequeña. Además no se detectó tinción específica en las muestras negativas (23/23). La utilidad del método de tinción de inmunohistoquímica descrito se basa en la habilidad de un anticuerpo monoclonal para reconocer un amplio espectro de lyssavirus en tejidos fijados en formalina.

RESUMO

O reconhecimento imunohistoquímico de um amplo espectro de lyssaviruses em tecidos fixados em formalina por um anticorpo monoclonal.

Uma tinção de imunoperoxidasa indireta (complexo avidina-biotina) usando o anticorpo monoclonal 5DF12-3B6, que reconhece a proteína N do vírus da raiva, se usou para detectar antígeno do vírus da raiva em mostras de tecido de 15 espécies animais e uma mostra humana infectadas com raiva naturalmente ou experimentalmente. Este anticorpo monoclonal reconheceu todas as 16 cepas do vírus da raiva que foram usadas em este estudo, como também lyssavirus relacionados à raiva como Duvenhage, Lagos Bat e Mokola. A amostra infetada com Mokola demonstrou inicialmente uma tinção que ficou mais forte quando se eliminou o tratamento com Pronase E. A tinção é sensível e específica, identificando corretamente o antígeno de raiva em todas as amostras usadas, com excessão de uma amostra (37/38) que era debilmente positiva por IFA e muito pequena. Contudo não se detectou tinção específica nas mostras negativas (23/23). A utilidade do método de tinção de imunohistoquímica descrito se baseia na habilidade de um anticorpo monoclonal para reconhecer um amplo espectro de lyssavirus em tecidos fixados em formalina.