JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1986, p. 573–580
0095-1137/86/100573-08$02.00/0

Vol. 24, No. 4

Demonstration of Antigenic Variation among Rabies Virus Isolates by Using Monoclonal Antibodies to Nucleocapsid Proteins

JEAN S. SMITH,[1]* FRANCES L. REID-SANDEN,[1] L. F. ROUMILLAT,[2] CHARLES TRIMARCHI,[3] KEITH CLARK,[4] GEORGE M. BAER,[1] AND WILLIAM G. WINKLER[5]

Rabies Laboratory, Viral and Rickettsial Zoonoses Branch, Division of Viral Diseases, Center for Infectious Diseases, Centers for Disease Control, Lawrenceville, Georgia 30246[1]; Viral Exanthems and Herpesvirus Branch[2] and Global Epidemiology,[5] Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia 30331; Rabies Laboratory, Center for Laboratories and Research, State of New York Department of Health, Albany, New York 12201[3]; and Division of Zoonoses Control, Texas State Health Department, Austin, Texas 78756[4]

Received 31 March 1986/Accepted 23 June 1986

Rabies virus isolates from terrestrial animals in six areas of the United States were examined with a panel of monoclonal antibodies to nucleocapsid proteins. Characteristic differences in immunofluorescence reactions permitted the formation of four antigenically distinct reaction groups from the 231 isolates tested. The geographic distribution of these groups corresponded well with separate rabies enzootic areas recognized by surveillance of sylvatic rabies in the United States. Distinctive reaction patterns were also identified for viral proteins from four infected bat species, and identical patterns were found in eight isolated cases of rabies in terrestrial animals. These findings suggest that monoclonal antibodies can be used to study the prevalence, distribution, and transmission of rabies among wildlife species.

Rabies in the United States is primarily a disease of wildlife species. Of the 5,000 to 7,000 cases of rabies reported annually, 80 to 90% occur in wildlife hosts (7).

Although the disease in each of the affected species has been well studied, little is known of the epidemiology of rabies virus transmission between wildlife species, particularly the failure of sylvatic hosts to transmit rabies to other susceptible species in the same area (18). The compartmentalization of the disease in this manner has resulted in the predominance of rabies in a single host species in several areas of the United States: a large region of skunk rabies that extends from Minnesota to Texas across the central United States and a separate skunk rabies area in northern California, two separate raccoon rabies areas in the southeastern and mid-Atlantic states, and a region of red fox rabies in Maine and New York (7). Within these areas, single-species involvement is almost universal and the cases that occur in other animals are generally regarded as spillover or accidental infection from the major reservoir animal. For example, 964 of the 1,035 cases of rabies in terrestrial wildlife reported from Maryland in 1984 occurred in raccoons. The remaining cases were isolated, distributed among eight species (7). The compartmentalization of the disease within one animal species observed in this and other areas of the United States does not seem to be caused by a lack of sufficient numbers of other species in any area but may reflect the ecologic isolation of species, unrecognized virus strain differences, or a combination of these and other unidentified factors (18).

Also a factor in the study of disease transmission between wildlife species is recognition of the role played by rabid insectivorous bats. Since the first report of rabies in bats in Florida in 1953 (16), rabies has been found in bats throughout the continental United States and in 30 of the 39 indigenous bat species (7, 8). Although bats are suspected as a source of rabies infection in terrestrial mammals, particularly as the source of rabies that has suddenly appeared in areas or species that previously were unaffected (12), the role of bats as reservoirs for rabies maintenance in nature has not been proven.

We recently developed a panel of monoclonal antibodies to the nucleocapsid proteins of rabies virus that can identify antigenic differences among some strains of virus (15). In the present study we have used this panel to examine isolates from each of the terrestrial rabies enzootic areas of the United States and adjacent areas of Canada and Mexico and isolates from the four bat species which are most often found rabid in these areas. The identification of distinctive antigenic differences in the nucleocapsid proteins of these viruses was then used as an epidemiological marker system for the study of strain prevalence and distribution. The long-term use of such a system could lead to new perspectives of the epidemiology of sylvatic rabies.

MATERIALS AND METHODS

Hybridoma monoclonal antibodies. Monoclonal antibodies induced by the immunization of BALB/c mice with ERA rabies virus were produced and characterized as described previously (14). All antibodies used in this study stained intracytoplasmic inclusions of ERA-rabies-virus-infected, acetone-fixed brain impression slides or cell cultures and were considered to be specific for the nucleocapsid antigenic complex (13). Antibodies were used in the form of mouse ascites.

Immunofluorescent-antibody staining for nucleocapsid antigen. The indirect immunofluorescent-antibody staining procedure was used to detect nucleocapsid antigen with acetone-fixed touch impressions of brain material or cell monolayers treated with monoclonal antibodies for 30 min at 37°C, washed to remove unbound antibody, and restained with fluorescein-conjugated goat antibody to mouse immunoglobulin G (Tago Inc., Burlingame, Calif.). Slides were examined at a magnification of ×200 to ×400 with a standard universal microscope with vertical illumination (Carl Zeiss Inc., New York, N.Y.). The light source was an XBO-150-W xenon bulb with KP490 and LP510 filters.

* Corresponding author.

TABLE 1. Virus isolates from major terrestrial rabies enzootic areas

Area (major host [species])	Specific location	No. tested	Yr(s)	Source of virus isolate (species)
1. Northeastern United States and Canada (fox [*Vulpes fulva*])	New York	6	1983–1985	Fox (*Vulpes fulva*)
	Maine	3	1983	Fox (*Vulpes fulva*)
	Ontario	2	1978, 1983	Fox (*Vulpes fulva*)
	New York	2	1982, 1985	Fox (*Urocyon cinereoargenteus*)
	New York	1	1985	Raccoon
	Ontario	3	1983	Raccoon
	Ontario	10	1983	Skunk
	New York	1	1985	Coyote
	New York	2	1985	Cat
2. Southern United States (raccoon [*Procyon lotor*])	Georgia	6	1983	Raccoon (*Procyon lotor*)
	Florida	2	1983	Raccoon (*Procyon lotor*)
	Alabama	2	1978	Raccoon (*Procyon lotor*)
	South Carolina	2	1984	Raccoon (*Procyon lotor*)
	South Carolina	3	1984	Skunk
	South Carolina	1	1983	Chipmunk
3. Mid-Atlantic United States (raccoon [*Procyon lotor*])	Maryland	3	1982	Raccoon (*Procyon lotor*)
	Virginia	4	1982	Raccoon (*Procyon lotor*)
	Pennsylvania	1	1985	Raccoon (*Procyon lotor*)
	Maryland	3	1982	Skunk
	Maryland	1	1982	Fox
	West Virginia	3	1984	Fox
	Maryland	2	1983	Cat
	Virginia	2	1983, 1985	Cat
	Washington, D.C.	2	1983, 1985	Cat
	Pennsylvania	1	1984	Dog
	Virginia	2	1983	Cow
	Maryland	1	1982	Cow
	Maryland	1	1985	Horse
	Washington, D.C.	1	1983	Panda[a]
	Maryland	2	1981	Groundhog
	Maryland	1	1984	Squirrel
	Pennsylvania	1	1984	Squirrel
4. South central United States (skunk [*Mephitis mephitis*])	Texas	33	1983–1984	Skunk (*Mephitis mephitis*)
	Arkansas	16	1983–1984	Skunk (*Mephitis mephitis*)
	Kansas	4	1975–1977	Skunk (*Mephitis mephitis*)
	Missouri	3	1983	Skunk (*Mephitis mephitis*)
	Texas	2	1984	Skunk (*Spilogale* sp.)
	Texas	1	1984	Skunk (*Conepatus* sp.)
	New Mexico	1	1984	Horse
	Texas	3	1984	Horse
	Texas	1	1983	Raccoon
	Texas	1	1984	Cow
	Texas	2	1984	Goat
	Texas	8	1984	Cat
	Texas	5	1984–1985	Dog
	Texas	1	1983	Rat
5. North central United States and Canada (skunk [*Mephitis mephitis*])	Montana	6	1983–1984	Skunk (*Mephitis mephitis*)
	Minnesota	6	1983	Skunk (*Mephitis mephitis*)
	Iowa	5	1981	Skunk (*Mephitis mephitis*)
	Wisconsin	8	1983–1984	Skunk (*Mephitis mephitis*)
	Ohio	5	1982–1984	Skunk (*Mephitis mephitis*)
	Kentucky	3	1984	Skunk (*Mephitis mephitis*)
	Tennessee	5	1984	Skunk (*Mephitis mephitis*)
	Virginia	1	1983	Skunk (*Mephitis mephitis*)
	Alabama	1	1978	Skunk (*Mephitis mephitis*)
	Arkansas	4	1983–1984	Skunk (*Mephitis mephitis*)
	Missouri	5	1983	Skunk (*Mephitis mephitis*)
	Manitoba, Canada	2	1983–1984	Skunk (*Mephitis mephitis*)
	Minnesota	1	1983	Groundhog
	Illinois	1	1983	Dog
	Tennessee	1	1983	Dog
6. Northern California (skunk [*Mephitis mephitis*])	California	3	1974–1983	Skunk (*Mephitis mephitis*)
	California	2	1982–1983	Raccoon

Continued on following page

TABLE 1—*Continued*

Area (major host [species])	Specific location	No. tested	Yr(s)	Source of virus isolate (species)
7. Central Texas (fox [*Urocyon cinereoargenteus*])	Texas	8	1984–1985	Fox (*Urocyon cinereoargenteus*)
	Texas	1	1983	Raccoon
	Texas	1	1984	Cow
8. Mexico and Mexican border counties in Texas and California (dog)	Mexico	5	1978	Dog
	Texas	6	1985	Dog
	California	1	1977	Cat

[a] Lesser red panda (part of exhibit at National Zoological Park in which rabid raccoons had been reported).

Virus strains. (i) **Laboratory strain.** The ERA vaccine strain was obtained from the American Type Culture Collection, Rockville, Md.

(ii) Animal field strains. Virus isolates from diagnostic specimens submitted to the Centers for Disease Control or to individual state public health laboratories were selected to represent a range of species, geographic areas, and collection times. Isolates were collected from the predominant host species and from other species only sporadically infected in each of the currently recognized species-defined enzootic areas of the United States: the red fox (*Vulpes fulva*) rabies area of the northeastern United States, the raccoon (*Procyon lotor*) rabies area of the southeastern and mid-Atlantic states, and the striped skunk (*Mephitis mephitis*) rabies area of the central United States from Minnesota to Texas, as well as the separate outbreak area in northern California (Table 1). Virus isolates from red fox rabies areas of Ontario and Quebec were also included because of surveillance data indicating that fox rabies in the northeastern United States is the result of the southeastward movement of fox rabies from these provinces in the early 1960s (3).

In addition to the acquisition of isolates from these large, easily recognized rabies enzootic areas, virus isolates were collected from rabid dogs in the Mexican border counties of Texas, where an increased number of cases was observed in 1985 (Centers for Disease Control, Rabies surveillance annual summary: 1985, in press). For comparison, virus isolates from dog rabies areas of Mexico (the states of Chihuahua, Coahuila, and Mexico) were also included. Additionally, isolates were collected from gray foxes (*Urocyon cinereoargenteus*) in an area of central Texas which for 20 years has consistently reported more rabid foxes than any other area of Texas (2, 7).

TABLE 2. Terrestrial rabies isolates not associated with established enzootic area

Source of virus isolate (species)	Location	Yr(s)
Fox (*Vulpes fulva*)	Rhode Island	1986
Fox (*Urocyon cinereoargenteus*)	New York	1983–1984[a]
Fox	Oregon	1982
Rat	Texas	1984
Horse	New Hampshire	1983
Cow	Pennsylvania	1983
Cow	Virginia	1983
Cat	Oregon	1984
Cat	California	1983

[a] Four isolates.

Isolates were collected from a variety of terrestrial animals found rabid in areas without established enzootics in terrestrial species (Table 2). Also, rabies samples were obtained from four bat species which are frequently reported rabid in the United States: *Eptesicus fuscus*, *Lasiurus cinereus*, *Lasiurus borealis*, and *Tadarida brasiliensis mexicana* (Table 3).

Experimental approach. Each antibody was initially diluted and tested for reaction with the ERA strain of rabies virus. The highest dilution giving 4+ staining intensity against ERA was then tested for reactivity to rabies virus isolates from each of the represented bat species and from the predominant host species in each of the major terrestrial-species-defined rabies enzootic areas. A negative or diminished reaction was confirmed by further tests of the virus sample with 10-fold-less-dilute antibody, and common patterns of reactivity were used as a basis for grouping isolates. To determine the homogeneity of virus antigenic types within each of the terrestrial-species-defined rabies enzootic areas, we tested isolates from a variety of species only occasionally found rabid in each outbreak area. All isolates from a specific outbreak area, regardless of species of origin, were then grouped by a common reaction pattern.

TABLE 3. Virus isolates from bat species

Source of virus isolate (region)	State	No. tested	Yr(s)
E. fuscus (eastern United States)	Maryland	7	1982–1983
	New York	15	1981–1985
	Virginia	1	1984
	Pennsylvania	8	1984
E. fuscus (western United States)	Colorado	6	1984
	Nevada	1	1983
T. brasiliensis mexicana	Texas	13	1980–1983
	Nevada	3	1983
	Colorado	1	1984
	New Mexico	1	1983
L. cinereus	New York	2	1982–1983
	Maryland	2	1982, 1984
	Colorado	9	1984
L. borealis	Florida	9	1984
	New York	1	1981
	Kentucky	1	1983
	Tennessee	1	1983
	Virginia	1	1984
	Georgia	1	1981
	Texas	2	1982

TABLE 4. Reaction patterns of virus isolates from major terrestrial-rabies enzootic areas

Area (major host)	Reaction[a] to hybridoma no.:																No. of isolates
	143	146	97-3	3	97-1	62	52-1	71	22	52-2	141	24-10	61	8	24-1	41	
1. Northeastern United States and Canada (fox)	−	−	−	+	−	+	+	+	+	+	+	+	+	+	+	−	30
2. Mid-Atlantic and southeastern United States (raccoon)	+	+	+	−	+	+	+	+	+	+	+	+	+	+	+	+	31 16
3. South central United States (skunk)	+	+	+	0	+	−	+	+	+	+	+	+	−	−	−	+	81
4. North central United States (skunk)[b]	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	54

[a] Positive (+), negative (−), or diminished (0) immunoreactivity of hybridoma antibodies to the nucleocapsid antigens of street and laboratory rabies viruses.
[b] It was not possible to distinguish this reaction pattern from those of skunk, fox, or dog isolates from northern California, central Texas, or Mexico (and border areas in the United States), respectively.

Virus strains were first tested as touch impressions of infected brain sections. If insufficient antigen for reactivity with the antibodies was present, samples of a 20% brain suspension were allowed to infect mouse neuroblastoma cell cultures (obtained from T. J. Wiktor, Wistar Institute, Philadelphia, Pa.), which were then grown in eight-well cell culture chamber slides (Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.). In most instances, sufficient antigen for testing was obtained after 2 days in culture.

RESULTS

Characteristics of virus isolates from terrestrial rabies enzootic areas. Rabies isolates from the major terrestrial-species-defined rabies enzootic areas of the United States (Table 1) could be identified as belonging to one of four groups on the basis of isolate reaction to the panel of antibodies (Table 4). A single reaction pattern was found in 11 of 11 virus isolates collected over a period of 8 years from red foxes in areas of the northeastern United States and Ontario and Quebec, Canada, where this species is the predominant rabies host. The reaction patterns of 19 isolates from less commonly infected species in this area were identical to those of isolates from red foxes. Line 1 of Table 4 reflects the reaction pattern of all 30 isolates (group 1) collected from this enzootic area. This reaction pattern was not found in isolates from any other area of the United States.

Another distinctive reaction pattern characterized isolates (group 2) collected over a period of 8 years from two geographically separate areas of the United States in which the predominant rabid wildlife host is the raccoon. Viruses from 12 raccoons in the southeastern states and 8 raccoons in the mid-Atlantic states reacted identically to the panel, and the same reaction pattern was found for 27 isolates from less frequently infected species in these two areas. Line 2 of Table 4 reflects the pattern of reactivity of all 47 isolates collected from this enzootic area. No other outbreak area produced isolates reacting to the antibody panel in this manner.

Virus isolates of reaction groups 3 and 4 were collected in enzootic areas defined by three different animal hosts and in several distinct geographic areas. The geographic distribution of these isolates (Fig. 1) can be summarized as follows.

(i) Skunk rabies virus isolates from the large skunk rabies band extending through the central United States could be divided into two reaction groups. One (reaction pattern 3 of Table 4) comprised 40 of 40 skunk virus isolates collected over a period of 10 years from the south central states of Texas and Kansas, 16 of 20 isolates from Arkansas, and 3 of 8 isolates from Missouri. This same reaction pattern was found in the isolates from four horses, one raccoon, one cow, two goats, eight cats, five dogs, and one rat collected in this skunk rabies enzootic area. A separate reaction group (reaction pattern 4 of Table 4) could be formed from 25 of 25 skunk rabies isolates from the north central states (Montana, Minnesota, Wisconsin, and Iowa), 15 of 15 isolates from the skunk rabies band stretching from Ohio to northern Alabama, 5 of 8 isolates from Missouri, and 4 of 20 isolates from Arkansas. This same reaction pattern was also found for virus isolates collected from one groundhog and two dogs in this skunk rabies enzootic area.

(ii) The reaction pattern of skunk isolates from the south central states was not found in isolates collected from any other enzootic area of the United States.

(iii) The reaction pattern of isolates from the skunk rabies area of the north central states was found for five isolates (from three skunks and two raccoons) from the separate skunk rabies area of northern California and for a single cat isolate from a Mexican border area of California. Additionally, this same reaction pattern was observed for isolates from eight gray foxes, six dogs, one cow, and one raccoon collected in central and Mexican border areas of Texas and was also found in dog rabies isolates from Mexico.

Characteristics of virus isolates from bats and associated terresterial species. Although the widespread occurrence of bat rabies in the United States did not permit the identification of discrete geographic outbreak areas, it was possible to form reaction groups for isolates collected throughout the country from three migratory and one nonmigratory bat species (Tables 3 and 5). A common reaction pattern was observed for 18 of 18 virus isolates from *T. brasiliensis mexicana* bats from four states. Reaction group 2 comprised 15 of 16 *L. borealis* bat isolates collected in seven states. Of the 13 *L. cinereus* bat isolates collected in three states, 11 had a common reaction pattern and formed reaction group 3. Although reaction group 4 could be formed from 26 of 31 isolates from *E. fuscus fuscus* bats in the eastern United States, two different reaction patterns were found in the remaining 5 isolates, and 6 of 7 isolates from *E. fuscus pallidus* bats from Colorado and Nevada displayed different reaction patterns. The observed diversity of reaction patterns of bat isolates is listed in Table 6.

Although none of the observed reaction patterns of the bat

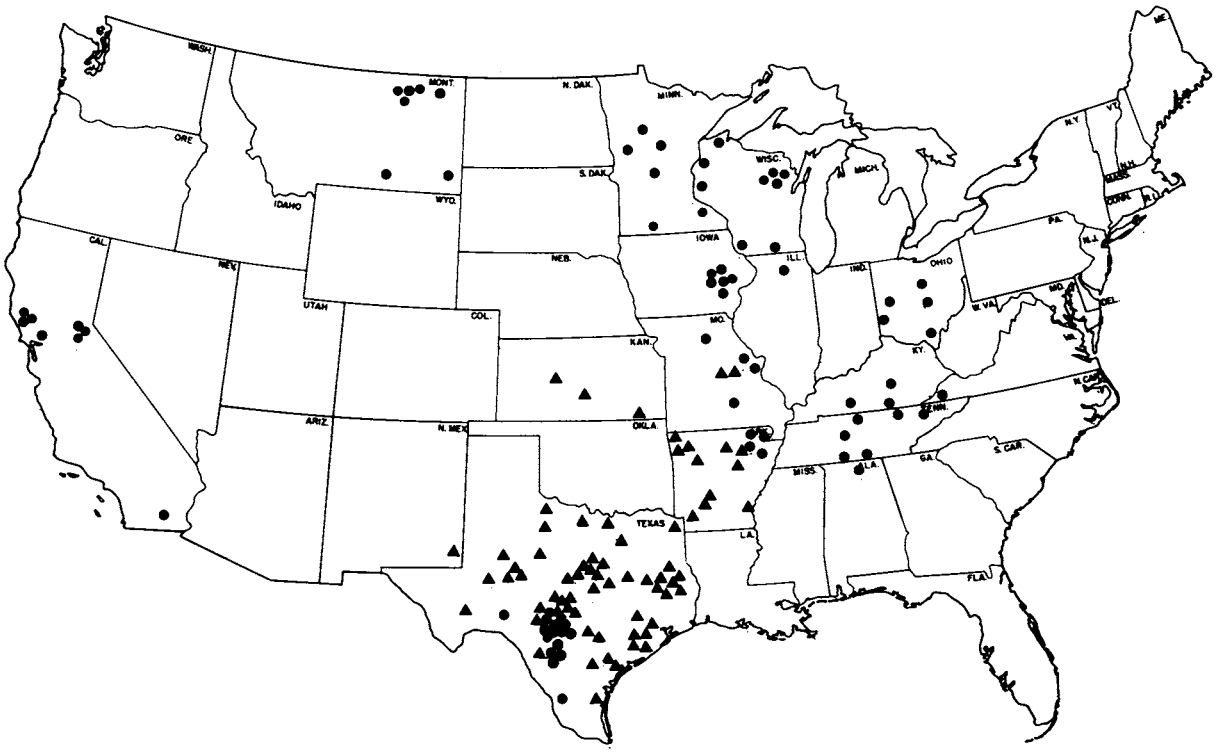

FIG. 1. Counties of collection of rabies virus isolates identified by a distinct reactivity pattern with a panel of monoclonal antibodies. ▲, Isolates which were reactive with all antibodies except 3, 62, 61, 8, and 24-1 (reaction pattern 3 of Table 4); ●, isolates which were reactive with all antibodies except 143 (reaction pattern 4 of Table 4).

isolates was represented in the patterns for the major terrestrial rabies enzootics, there was evidence that sporadic infection of terrestrial animals may occur after contact with infected bats (Table 5). An isolate collected from a rat in Fort Worth, Tex., displayed a reaction pattern different from that of virus isolates collected from 73 other terrestrial animals in Texas but shared a common reaction pattern with 13 virus isolates collected from *T. brasiliensis mexicana* bats in Texas. Additionally, viruses indistinguishable from those found in 26 eastern *Eptesicus* bats were also isolated from six rabid terrestrial animals in areas of the eastern United States which were free of significant terrestrial rabies: a cow

TABLE 5. Reaction patterns of virus isolates from bat species and associated terrestrial animals

Source of isolate	Reaction[a] to hybridoma no.:																No. of isolates
	143	146	97-3	3	97-1	62	52-1	71	22	52-2	141	24-10	61	8	24-1	41	
1. *T. brasiliensis* bat	−	−	−	+	−	+	+	+	+	+	+	+	+	+	+	+	18
Associated animal (rat; Texas)	−	−	−	+	−	+	+	+	+	+	+	+	+	+	+	+	1
2. *L. borealis* bat	−	−	−	−	0	−	+	+	+	+	+	+	+	+	+	+	15
Associated animal (fox [*Urocyon* sp]; New York)	−	−	−	−	0	−	+	+	+	+	+	+	+	+	+	+	1
3. *L. cinereus* bat	−	−	−	0	0	+	+	+	+	+	+	+	+	+	+	+	11
4. *E. fuscus fuscus* (eastern United States)	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	26
Associated animals																	
Fox (*Urocyon* sp.; New York)	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	3
Fox (*Vulpes* sp.; Rhode Island)	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	1
Cow (Pennsylvania)	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	1
Horse (New Hampshire)	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	1

[a] Positive (+), negative (−), or diminished (0) immunoreactivity of hybridoma antibodies to the nucleocapsid antigens of street and laboratory rabies viruses.

TABLE 6. Reaction patterns of virus isolates from bat species for which no grouping could be made

Bat species (state, yr)	Reaction[a] to hybridoma no.:															
	143	146	97-3	3	97-1	62	52-1	71	22	52-2	141	24-10	61	8	24-1	41
L. cinereus (Maryland, 1982)	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+
L. cinereus (New York, 1983)	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+
L. borealis (Kentucky, 1983)	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	+
E. fuscus (Maryland, 1983)	−	−	−	−	+	+	+	+	0	−	−	−	+	+	+	+
E. fuscus (Pennsylvania, 1984)	−	−	−	−	+	+	+	+	0	−	−	−	+	+	+	+
E. fuscus (Pennsylvania, 1984)	−	−	−	−	+	+	+	+	0	−	−	−	+	+	+	+
E. fuscus (New York, 1981)	−	−	−	−	+	+	+	−	0	−	−	−	+	+	+	+
E. fuscus (New York, 1985)	−	−	−	−	+	+	+	−	0	−	−	−	+	+	+	+
E. fuscus (Colorado, 1984)	−	−	−	+	+	+	+	+	−	−	−	−	+	+	+	+
E. fuscus (Colorado, 1984)	+	+	−	−	−	+	+	+	+	−	−	−	+	+	+	+
E. fuscus (Colorado, 1984)	+	+	−	−	−	+	+	+	+	−	−	−	+	+	+	+
E. fuscus (Colorado, 1984)	+	+	−	−	−	+	+	−	+	−	−	−	+	+	+	+
E. fuscus (Colorado, 1984)	+	+	−	−	+	+	+	−	0	−	−	−	+	+	+	+
E. fuscus (Colorado, 1984)	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	+
E. fuscus (Nevada, 1983)	+	+	−	−	+	+	+	−	−	−	−	−	+	+	+	+

[a] Positive (+), negative (−), or diminished (0) immunoreactivity of hybridoma antibodies to the nucleocapsid antigens of street and laboratory rabies viruses.

in Pennsylvania (6), a horse in New Hampshire (9), a red fox in Rhode Island, and three gray foxes in southern New York. A fourth rabid fox from New York contained a virus indistinguishable from that found in *L. borealis* bats.

The reaction patterns of additional isolates submitted for testing because they occurred unexpectedly in areas previously free of terrestrial rabies are listed in Table 7. No grouping of these isolates was possible.

DISCUSSION

In the present study we present a panel of monoclonal antibodies which can identify antigenic differences on the nucleocapsid proteins of rabies virus isolates from the major terrestrial-species-defined enzootic areas of the United States. These isolates can then be distinguished from rabies virus found in four of the most commonly affected bat species in the United States. We also present data to show that this panel can be used to study the distribution, prevalence, and transmission of rabies variants between wildlife species.

The geographic distribution of the antigenic groups identified for isolates from the red fox, raccoon, and striped skunk rabies enzootic areas of the United States corresponded well with the geographically separate enzootic areas defined by rabies surveillance and with what is known about the movement of rabies into these areas. For example, the antigenic pattern associated with rabies viruses in red foxes in New York and Maine was not found in any other area of the United States. The observation of this same rabies variant in virus isolates from Ontario and Quebec agrees with surveillance data from the early 1960s which reported the movement of rabid foxes from Ontario across the Saint Lawrence River into the northeastern United States (3) and supports the suggestion that enzootic rabies in the northeastern United States is the continuation of an outbreak which began in red foxes in Canada in the 1950s (18). Enzootic rabies in raccoons and the antigenic pattern associated with this variant were found in both the southeastern and mid-Atlantic states. This finding is also compatible with surveillance and epidemiologic data from this area (5). Rabid raccoons have been identified in shipments of raccoons from the Southeast to private hunting clubs in the mid-Atlantic states (10). This has led to the suggestion that the interstate transport of infected animals from rabies-endemic areas of the Southeast was responsible for the introduction of disease into the previously unaffected mid-Atlantic area (15). Although two antigenically different rabies variants were found in what now appears as a single large skunk rabies enzootic area in the central United States, surveillance reports of the early 1960s had recognized increased activity in two well-separated skunk rabies areas: an older well-established focus in Minnesota and Iowa and a newly emerging epizootic in Texas (2). The expansion of the two areas during the next 10 years resulted in their merger in parts of Missouri and Arkansas (4), an observation supported by the finding of two different rabies variants in skunks in these states.

As a characteristic reaction pattern for the major host species within each of these enzootic areas was identified, those cases in less commonly affected species, which were or were not the result of spillover infection from the major host species, could be predicted by a comparison test of isolates. In most of the rabies enzootic areas examined, the reaction pattern of all isolates, regardless of the host of origin, was identical to that of the major host species, with one interesting exception. A clustering of rabies cases in gray foxes and an increase in dog rabies in central and Mexican border areas of Texas (enzootic areas 7 and 8 of Table 1) had suggested disease transmission separate from the active skunk rabies enzootic in Texas (enzootic area 4 of Table 1) (7). Monoclonal antibody analysis of the isolates from these areas supported this surveillance. The reaction

TABLE 7. Reaction patterns of virus isolates from terrestrial animals for which no grouping could be determined

Animal (state, yr)	Reaction[a] to hybridoma no.:															
	143	146	97-3	3	97-1	62	52-1	71	22	52-2	141	24-10	61	8	24-1	41
Cow (Virginia, 1983)	−	−	−	−	−	0	+	−	+	+	+	+	+	+	+	+
Cat (California, 1983)	+	+	−	−	−	+	+	−	0	−	−	−	+	+	+	+
Cat (Oregon, 1984)	−	−	−	−	−	+	+	+	+	−	−	−	+	+	+	+
Fox (Oregon, 1982)	−	−	−	0	0	+	+	0	+	+	+	+	+	+	0	+

[a] Positive (+), negative (−), or diminished (0) immunoreactivity of hybridoma antibodies to the nucleocapsid antigens of street and laboratory rabies viruses.

patterns of the nucleocapsid proteins of eight of eight isolates from gray foxes in central Texas (Gillespie, Kimble, and Uvalde counties) were identical (pattern 4 of Table 4). This pattern easily distinguished these isolates from isolates from 36 striped skunks collected throughout the state (the collection sites illustrated in Fig. 1 encompassed 26 Texas counties) which displayed reaction pattern 3 of Table 4. These two separate groups persisted even when isolates from the two species were obtained within the same county. For example, the reaction pattern of virus from skunks in Uvalde and Kimble counties was identical to all other skunk isolates collected in Texas but could easily be distinguished from the patterns of viruses collected from foxes in these same counties. Virus isolates from a variety of species collected in Texas counties where the skunk is the predominant rabies host reflected the virus variant found in rabid skunks in these areas. The reaction pattern found in virus isolates from the eight gray foxes was also found for a cow isolate and a raccoon isolate in this area and also in six dog rabies isolates from Mexican border areas of Texas. Five dog rabies isolates from Mexico also contained virus with this same reaction pattern. It was not possible to distinguish isolates from dogs and foxes in this area, even when they were reacted with an expanded panel of 76 nucleocapsid and glycoprotein monoclonal antibodies (17; the generous gift of T. J. Wiktor, Wistar Institute, Philadelphia, Pa.).

An examination of rabies cases which occurred unexpectedly in terrestrial animals in areas without an ongoing enzootic in terrestrial species revealed a variety of reaction patterns, some of which were also found in rabid bats in the same area. An analysis of rabies isolates from New York provided evidence for four instances in which transmission from a bat to a terrestrial animal may have occurred. They occurred among gray foxes, a species only rarely found rabid in the northeastern states, and in an area removed from the established enzootic in red foxes. These cases were of particular interest because of concern that an expansion of red fox rabies into a new area and species had occurred. The first rabid gray fox was reported in December 1983 in a county (Dutchess) which had been free of significant terrestrial rabies for 25 years. In October 1984 three other rabid gray foxes were diagnosed from this same area (one from Rensselaer county and two from Ulster county). Monoclonal antibody analysis of the nucleoproteins of these viruses could easily distinguish them from the virus proteins associated with the disease in red foxes in the Canadian border counties of New York. Three of the isolates exhibited a reaction pattern identical to that found for rabid *Eptesicus* bat isolates collected in 1984 from Ulster, Albany, and Rensselaer counties, whereas that of another fox was indistinguishable from the pattern of *L. borealis* bat isolates (Tables 4 and 5). Additionally, the reaction pattern of isolates from two gray foxes found in the red fox rabies enzootic area (collected in Saint Lawrence County in 1982 and 1985) was identical to that of red fox virus isolates collected in this area. These findings indicate that the observed differences in the nucleocapsid proteins of isolates from these two areas of New York were most likely not the result of passage of red fox rabies virus to gray foxes but could indicate the transmission of rabies from bats to terrestrial animals.

The identification among terrestrial animals in New York of a cluster of rabies cases apparently separate from those of the major terrestrial host species is illustrative of the potential usefulness of monoclonal antibodies in the prediction of the role played by rabid bats in the emergence of new epizootics in terrestrial species (12). Although the absence of additional rabies cases in terrestrial species in areas where these isolated cases have occurred would indicate that enzootic conditions are not established, the possibility that such conditions may arise makes this an important area for study.

The finding of distinctive reaction patterns for viruses from four bat species suggests that it may also be possible to use the antibody panel to study virus transmission within and between bat species. For example, individuals of the solitary, migratory bat species may share a common pool of variants during their long and stressful annual migration. This may have been the reason for a common reaction pattern among isolates of rabies from *L. borealis* bats collected in six states or similar data for isolates from *L. cinereus* bats in three states. The single virus strain found in isolates from *T. brasiliensis mexicana* bats could reflect their migration patterns or their extremely colonial habits (2×10^7 or more bats may occupy a single nursery cave [1]). It is also possible that the greater availability of shelter in the eastern United States allows more segregation of sedentary, colonial bat species such as *E. fuscus fuscus* and thus provides a means for efficient intraspecific virus transfer. Conversely, the heterogeneity of the virus strains found in six rabies isolates collected in a few months from colonial, resident *E. fuscus* bats in Colorado may reflect the interspecies roosting observed among some western bat species. *Eptesicus*, *Tadarida*, *Antrozous*, and *Myotis* bats frequently share living quarters in the western states (1), and one rabid silver-haired bat (*Lasionycteris noctivagans*, a solitary, migratory bat) was found roosting in an *E. fuscus* maternity colony in Alberta, Canada (D. B. Schowalter and J. R. Gunson, Rabies surveillance in Alberta, Rabies Inf. Exchange **1**:17, 1979). Study of additional isolates from these and other bat species should be helpful in the identification of the relative importance of migration patterns, activity during the formation of colonies, and roosting behavior in the inter- and intraspecific transfer of rabies virus among the bat species.

In the present study we present a typing scheme based on the ability of the rabies nucleocapsid protein-directed monoclonal antibodies to detect characteristic differences among isolates collected in separate species-defined rabies enzootics. The accumulation of the nucleocapsid proteins in brain tissue allows their antigenic analysis on impression slides prepared directly from most field samples, thus avoiding the laboratory culture of virus. Although the activity of antibodies to viral glycoproteins may also be useful in detecting differences in rabies variants, these antigens are not easily tested directly in virus samples and require the adaptation of isolates to cell cultures (a process which may in itself select for antigenic variation [11]). Because any study of the epidemiology of rabies in wildlife species requires the testing of large numbers of isolates, it may be possible to use an initial grouping of isolates by the nucleocapsid reactivity presented here as a basis for the selection of a smaller number of isolates from each of these enzootic areas to be evaluated with antibodies to glycoprotein antigens. This approach should expand the usefulness of monoclonal antibodies in any effort to learn more about rabies virus transmission and maintenance in nature.

Although antigenic differences not detected by this panel of antibodies may be observed as new antibodies are produced and characterized, the present results define reaction groups which are consistent with the terrestrial rabies enzootic areas currently recognized by disease surveillance

in these areas and suggest that separate cycles of rabies transmission may occur in bat species. It is also clear from these studies that much work is needed to characterize isolates of rabies virus from bat species and to determine the mechanism by which a diversity of variants have evolved within wildlife populations in the United States.

ACKNOWLEDGMENTS

We thank the following for providing rabies virus isolates for testing: Steve Waldron, Arkansas Bureau of Laboratories; Richard W. Emmons and Mary J. Martins, California Department of Health Services; John Emerson, Colorado Department of Health; Faye Sorhage, Florida Department of Health; Keith Sikes, Georgia Department of Human Resources; Leonard G. Wood, Illinois Department of Public Health; Nancy Hall, Iowa University Hygenic Laboratory; Dennis Howard, Kansas State University; Robert S. Clark, Kentucky Department for Health Services; Howard E. Lind, Maine Public Health Laboratories; William Dixon, Maryland Department of Health and Mental Hygiene; Lloyd E. Boyd, Minnesota Department of Health; Loretu Kuraru and Don Blenden, College of Veterinary Medicine, University of Missouri; R. P. Myers and Jen'e M. Daniel, Diagnostic Laboratory, Montana Department of Livestock; Agnes Lapham, Nevada Department of Agriculture; Joyce Cournoyer, New Hampshire Public Health Laboratory; Richard Steece, New Mexico Department of Health; George Bear and J. Ronald Genevie, Ohio Department of Health Laboratories; W. A. Webster, Animal Diseases Research Institute, Nepean, Ontario; Bob Sokolow, Oregon Department of Human Resources; John N. Ersoz, Pennsylvania State Veterinary Diagnostic Laboratory; Harold Dowda, South Carolina Bureau of Laboratories; Gary L. Swinger, Tennessee Department of Public Health; Suzanne Jenkins and Sandy Currin, Virginia Department of Microbiological Science; James B. Thomas, Washington, D.C., Department of Human Resources; Keith Martin, West Virginia State Hygienic Laboratory; and Donald B. Nelson, Wisconsin State Laboratory of Hygiene. We also thank Joe McDade for many thoughtful discussions in the preparation of this manuscript.

LITERATURE CITED

1. **Barbour, R. W., and W. H. Davis.** 1969. Bats of America, p. 120–130. University Press of Kentucky, Lexington.
2. **Center for Disease Control.** 1962. Rabies surveillance annual summary: 1961. Center for Disease Control, Atlanta.
3. **Center for Disease Control.** 1964. Rabies surveillance annual summary: 1963. Center for Disease Control, Atlanta.
4. **Center for Disease Control.** 1965. Rabies surveillance annual summary: 1964. Center for Disease Control, Atlanta.
5. **Centers for Disease Control.** 1982. Rabies: mid-Atlantic states. Morbid. Mortal. Weekly Rep. **31**:592–593.
6. **Centers for Disease Control.** 1983. Exposure to a rabid cow: Pennsylvania. Morbid. Mortal. Weekly Rep. **32**:128–129.
7. **Centers for Disease Control.** 1985. Rabies surveillance annual summary: 1984. Centers for Disease Control, Atlanta.
8. **Constantine, D. G.** 1979. An updated list of rabies-infected bats in North America. J. Wildl. Dis. **15**:347–349.
9. **Fuller, J. E.** 1984. A case of equine rabies. N. Engl. J. Med. **310**:525–526.
10. **Nettles, V. F., J. H. Shaddock, R. K. Sikes, and C. R. Reyes.** 1979. Rabies in translocated raccoons. Am. J. Public Health **69**:601–602.
11. **Prabhakar, B. S., N. F. Parks, and H. F. Clark.** 1983. Altered expression of rabies virus antigenic determinants associated with chronic infection and virulence. J. Gen. Virol. **64**:251–254.
12. **Scatterday, J. E., N. J. Schneider, W. J. Jennings, and A. L. Lewis.** 1960. Sporadic animal rabies in Florida. Public Health Rep. **75**:945–953.
13. **Schneider, L. G., B. Dietzschold, R. E. Dierks, W. Matthaeus, P.-J. Enzmann, and K. Strohmaier.** 1973. Rabies group-specific ribonucleoprotein antigen and a test system for grouping and typing of rhabdoviruses. J. Virol. **11**:748–755.
14. **Smith, J. S., J. W. Sumner, and L. F. Roumillat.** 1984. Enzyme immunoassay for rabies antibody in hybridoma culture fluids and its applicaion to differentiation of street and laboratory strains of rabies virus. J. Clin. Microbiol. **19**:267–272.
15. **Smith, J. S., J. W. Sumner, L. F. Roumillat, G. M. Baer, and W. G. Winkler.** 1984. Antigenic characteristics of isolates associated with a new epizootic of raccoon rabies in the United States. J. Infect. Dis. **149**:769–774.
16. **Venters, H. D., W. R. Hoffert, J. E. Scatterday, and A. V. Hardy.** 1954. Rabies in bats in Florida. Am. J. Public Health **44**:182–185.
17. **Wiktor, T. J., A. Flamand, and H. Koprowski.** 1980. Use of monoclonal antibodies in diagnosis of rabies virus infection and differentiation of rabies and rabies related viruses. J. Virol. Methods **1**:33–46.
18. **Winkler, W. G.** 1975. Fox rabies, p. 3–22. *In* G. M. Baer (ed.), The natural history of rabies, vol. 2. Academic Press, Inc., New York.