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Antigenic and Molecular Characterization of Bat Rabies Virus in Europe

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The predominant role of *Eptesicus serotinus* in the epizootic of bat rabies in Europe was further outlined by the first isolation of the rabies virus from this species in France. The distribution of the virus was studied in naturally infected *E. serotinus* bats at the time of death and suggested that the papillae of the tongue and the respiratory mucosa may play a role in virus production and excretion. The analysis of 501 French rabies virus isolates from various animal species by antinucleocapsid monoclonal antibodies indicated that transmission of the disease from bats to terrestrial animals is unlikely. The antigenic profile of two isolates from French bats corresponded to that of European bat lyssavirus type 1 (EBL1). Comparisons of 12 different isolates from bats with antinucleocapsid and antiglycoprotein monoclonal antibodies and by direct sequencing of the polymerase chain reaction amplification product of the N gene indicated that EBL1, EBL2, Duvenhage virus (serotype 4 of lyssavirus), and the European fox rabies virus (serotype 1) are phylogenetically distant. They formed four tight genetic clusters named genotypes. EBL1 was shown to be antigenically and genetically more closely related to Duvenhage virus than to EBL2. We propose that EBL1 and EBL2 constitute two distinct genotypes which further serologic characterization will probably classify as new serotypes. We also report a simple method for the rapid characterization of EBL based on the digestion of the polymerase chain reaction product of the N gene by three restriction endonucleases.

Over the years, investigators have found rabies virus in nonhematophagous bats in America, Africa, and Europe (2, 13, 16, 21, 33). Between 1954 and 1984, bat rabies appeared to be rare in Europe, with only 14 recorded cases (15). The isolation in 1985 of two rabies-related viruses from an *Eptesicus serotinus* bat in Denmark (26) and from a Finnish zoologist who was bitten by a bat (24) led to a large epidemiological investigation. As expected, the result was an increase in the number of cases, from 15 in 1985 to 122 in 1986 and 142 in 1987, indicating a possible spread of the virus among European bats. Since 1988, the number of recorded cases has stabilized at lower levels of 53, 42, and 40 cases in 1988, 1989, and 1990, respectively. The temporal evolution of the cases infers a rabies outbreak among the insectivorous bat population during the years 1985 to 1987. The declining number of cases in the past few years suggests that the conditions required for the epizootic are no longer maintained, and the disease now appears to be enzootic among bats in Europe, probably as it was before 1985. Considering the lack of precise epidemiological data (in particular, the total number of bats analyzed), however, this may also correspond to a decrease in the number of bats that were investigated after 1987.

Although rabies virus isolates from hematophagous, insectivorous, and frugivorous bats from America all belong to the serotype 1 of the *Lyssavirus* genus, the rabies virus isolates from European and African insectivorous bats are classified as rabies-related viruses because of the antigenic differences found by serological studies (33, 35). The African isolates are classified in the following two distinct serotypes: serotype 2, of which the prototype strain is the Lagos bat virus isolated from *Eidolon helvum*, and serotype 4, of which the prototype strain is the Duvenhage virus isolated from a man and from *Miniopterus* sp. (25). The European isolates were initially thought to belong to serotype 4 on the basis of their antigenic relationships (33, 34), but they were subsequently recognized as independent isolates (11). These viruses, called the European bat lyssaviruses (EBL), are now subdivided into 2 biotypes, EBL type 1 (EBL1) and EBL2, which were proposed to constitute serotype 5 (17, 27).

In the present study, we analyzed the first recorded case of a rabid bat in France. The virus distribution at the time of death in this naturally infected *E. serotinus* bat was determined in an attempt to analyze the pathogenicity for the host and to identify the peripheral tissues involved in producing infectious secretions. In order to discuss the possible natural transmission of rabies virus from bats to terrestrial mammals, a large epidemiological survey of 501 French rabies virus isolates was performed with a reduced panel of antinucleocapsid monoclonal antibodies (NC-MAbs). The reactivities of selected isolates of EBL, European fox rabies virus, and the African bat Duvenhage virus to a large panel of MAbs directed against epitopes on the rabies virus nucleocapsid and glycoprotein were also compared (10, 22, 23, 43). The relationships deduced from this MAb study were further assessed at the nucleotide level by transcribing cDNA, amplifying the transcribed DNA by the polymerase chain reaction (PCR), and sequencing and comparing the nucleoprotein genes. This approach was shown to be particularly suitable for a precise epidemiological investigation (31, 32) and was not subjected to any bias because of virus culture in vivo or in vitro. It allowed an investigation of the evolutionary relationships within or between serotypes and biotypes, leading to new classification proposals at the taxonomic level. Finally, a very rapid and easy method of typing European rabies virus isolates from bats and foxes as

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TABLE 1. Origin of the viruses

Type	Virus	Country of isolation[a]	Species isolated from:	Year	Reference or source	Strain number
Serotype 1	Rabies virus	France	*Vulpes vulpes*	1989	Institut Pasteur	8910
				1986	Institut Pasteur	9004
	ERA vaccinal strain	United States	Dog	1935	1	
Serotype 4	Duvenhage virus 1	Republic of South Africa	Human	1971	25	86132
	Duvenhage virus 2	Republic of South Africa	*Miniopterus* sp.	1981	41	9020
EBL	EBL1	Hamburg, F.R.G.	Bat	1968	33	
		Stade, F.R.G.	Bat	1970	33	
		Bremerhaven, F.R.G.	Bat	1982	33	
		Poland	*E. serotinus*	1985	21	8615
		Denmark	*E. serotinus*	1985	26	
		G.D.R.	*E. serotinus*	1985	H. Sinnecker	
		Russia	Human	1986	36	
		Briey, France	*E. serotinus*	1989	Institut Pasteur	8918
		Bainville, France	*E. serotinus*	1989	Barrat et al., 1989	8919
	EBL2	Finland	Human	1986	24	9007
		The Netherlands	*Myotis* sp.	1986	Haagsma, 17	9018

[a] F.R.G., former Federal Republic of Germany; G.D.R., former German Democratic Republic.

well as Duvenhage virus based on restriction polymorphism analysis is proposed.

MATERIALS AND METHODS

Rabies virus detection in and isolation from bats. Tissue specimens and organs were divided for direct detection of the rabies virus nucleocapsid by the fluorescent-antibody test and for homogenization. For the fluorescent-antibody test (FAT), each tissue specimen was sliced at multiple levels in a cryostat. Sections (4 μm) were dried, fixed in acetone for 30 min, and examined after staining with fluorescein isothiocyanate-labeled rabbit anti-rabies nucleocapsid immunoglobulins G (Diagnostics Pasteur, Marnes la Coquette, France) as described elsewhere (4). Sections were viewed with an epifluorescence Zeiss microscope equipped with an ultra-high-pressure mercury UV lamp. After homogenization, the clarified supernatants were tested for rabies virus nucleocapsid detection (RREID) by enzyme-linked immunosorbent assay and for rabies virus isolation by the rapid tissue culture infection test (RTCIT) (4) and the suckling mouse inoculation test (SMIT) (18).

Virus. Representative isolates of rabies and rabies-related viruses were used in this study. The original hosts and geographic sources are summarized in Table 1. Isolation of rabies virus from brain tissue and propagation of the viruses in cell cultures have been described previously (4, 42).

Antigenic analysis. Forty-four NC-MAbs (5, 23, 43) and 40 antiglycoprotein MAbs (G-MAbs) (20) were used. They describe five antigenic sites on the ERA virus nucleoprotein (N) and phosphoprotein (M1) (10, 22) and six antigenic sites on the ERA virus glycoprotein. Most of the MAbs were kindly provided by the late T. J. Wiktor; MAb 187.5 was kindly provided by L. G. Schneider (34), and MAbs 59-1, PVA-3, 26-9, 183-14, M-30, PVB-1, 69-9, and 11-1 were produced at the Pasteur Institute (Paris, France). The reactivity patterns with NC-MAbs were determined by an indirect fluorescent-antibody staining technique performed on brain smears of infected suckling mice by using a fluorescein isothiocyanate-labeled anti-mouse immunoglobulin G (Biosys, Compiègne, France) (43). Suspect reactions were confirmed on infected BSR cells. The reactivities toward G-MAbs were measured by the virus neutralization index with a constant amount of each MAb and 10- or 5-fold serial dilutions of virus. A minimum reduction of 100-fold in virus titers was considered a positive result.

Genetic analysis. Viral RNA was purified after a single passage on suckling mouse brain (32). cDNA synthesis was performed with 15 pmol of primer PVN7 and H^- super reverse transcriptase (Bethesda Research Laboratories), and the DNA was amplified by PCR by using 30 pmol of primers PVN7 and PVN8, as described by Sacramento et al. (32). The program was one cycle (denaturation at 94°C for 60 s, annealing at 45°C for 90 s; ramping 6, and elongation at 72°C for 150 s) followed by 36 cycles (denaturation at 94°C for 50 s, annealing at 45°C for 90 s, ramping 6, and elongation at 72°C for 150 s), the ultimate elongation being completed at 72°C for 5 min. PVN7 (5′-ATGTAACACCTGTACAATG-3′; plus sense) and PVN8 (5′-AGTTTCTTCAGCCATCTC-3′; minus sense) primers mapped at positions 55 to 73 and 1585 to 1565 of the PV rabies genome, respectively (40). For restriction analysis, 20 μl of the amplified DNA sample was digested in 40 μl of the appropriate buffer for 2 h at 37°C by using 10 U of *Pst*I, *Pvu*II, or *Dde*I. The products obtained by restriction enzyme digestion were fractionated by horizontal agarose (0.8% LSM and 2% NuSieve GTG) gel electrophoresis in TEA buffer. Gels were stained with ethidium bromide (1 μg/ml) and photographed under UV light. The nucleotide sequencing was performed directly on the amplified fragment that was purified on NuSieve GTG agarose (32). Convenient primers, which allowed the sequence to be obtained on both strands of the amplified DNA, were chosen (data not shown). Sequences were analyzed by using the University of Wisconsin GCG package (9).

RESULTS

Necropsy findings and virus isolation. A total of 22 dead bat specimens were submitted to the French National Reference Centre for Rabies for rabies diagnosis between 1988 and 1991. They consisted of seven *Eptesicus serotinus*, one *Eptesicus nilssoni*, six *Pipistrellus pipistrellus*, one *Pipistrellus nathusii*, one *Pipistrellus khuli*, three *Plecotus austriacus*, one *Plecotus auritus*, one *Nyctalus noctula*, and one of

TABLE 2. Virus antigen detection by the FAT or by enzyme-linked immunosorbent assay (RREID) and virus isolation attempts[a]

Site of isolation	Antigen detection by:				Virus detection by:				
	FAT		RREID[b]		RTCIT		SMIT		
	On smears	On tissue section	g/ml[c]	OD	g/ml	Result	g/ml	Day of death of FAT-diagnosed rabid mice	No. of mice alive 28 days after inoculation/no. inoculated
Heart and blood	0[d]	0	0.06	0.012	0.12	0	0.12	12	9/11
Spleen	0	0	0.004	0.039	0.015	0	0.015		10/11
Liver	0	NT	0.24	0.077	0.478	0	0.478		0/12[e]
Lungs	0	+	0.06	0.087	0.114	0	0.114	7, 14, 19, 21, 25, 27	2/10
Brown fat	0	NT	0.037	0.078	0.073	0	0.073		8/10
Medulla	+	NT	0.015	0.331	0.029	0	0.029	8, 13, 13, 16, 16, 16, 19	0/10
Salivary glands	0	0	0.047	0.008	0.047	0	0.047		7/9
Pancreas	0	0	0.04	0.027	0.040	0	NT	NT	NT
Kidneys	0	0	0.026	0.005	0.105	0	0.105		9/10
Brain	+	NT	0.05	0.474	0.05	+	0.05	9, 10, 16, 22	2/10
Tongue	NT	+	NT	NT	NT	NT	NT	NT	NT

[a] On cell culture (RTCIT) or from suckling mice (SMIT). NT, not tested.
[b] The optical density (OD) cutoff value for RREID was 0.100.
[c] Grams of tissue specimens homogenized per milliliter.
[d] No virus isolated or antigen detected.
[e] All the suckling mice were eaten by their mother.

undetermined genus and species. The only bat found to be positive for rabies virus (isolate 8918) was an *E. serotinus* bat caught on 11 September 1989 in Briey (Meurthe et Moselle) in the northeast of France. Virus was isolated from the brain by RTCIT and by SMIT from the brain, the medulla, the lungs, and the heart but not from the spleen, the liver, the brown fat, the salivary glands, the pancreas, or the kidneys (Table 2). Rabies virus antigen was also detected by FAT or RREID in the brain, the medulla, the lungs, and the tongue (Table 2). Direct immunofluorescence on cryosections showed few foci of infection in the lung alveoli (Fig. 1A). The infection was probably localized in the nerves and not in

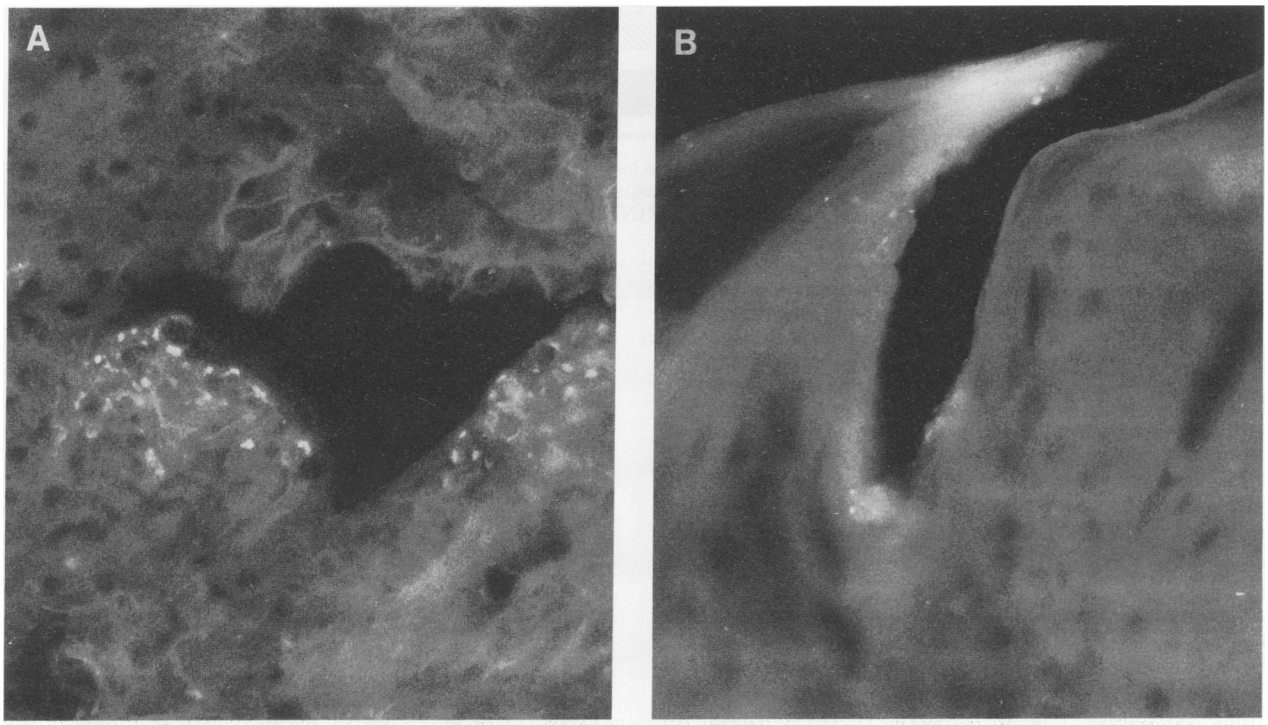

FIG. 1. (A) Section of lung from *E. serotinus* with antinucleocapsid immunofluorescence staining. Few foci of infection are seen in the lung alveoli. (B) Section of filiform papillae of the tongue from *E. serotinus* with antinucleocapsid immunofluorescence staining. Virus antigen is revealed in the filiform papillae of the lingual epithelium. Magnifications, ×1,180.

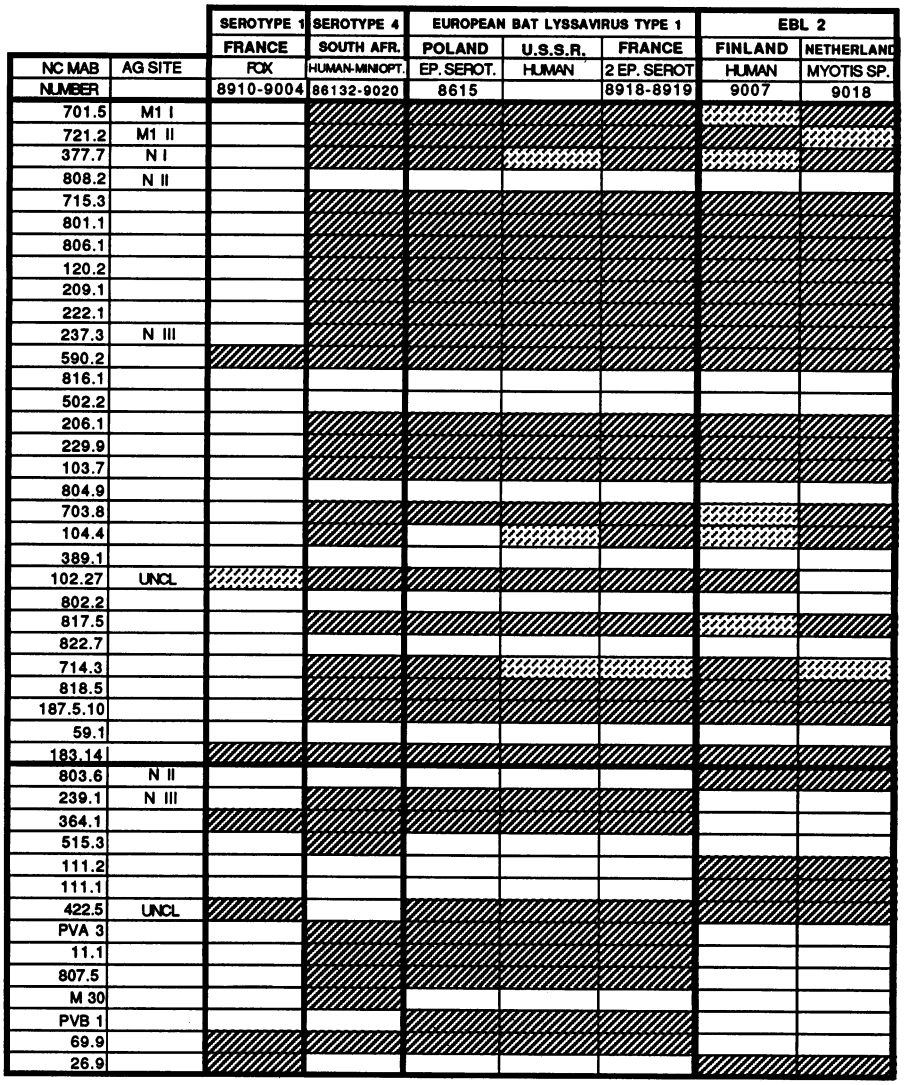

FIG. 2. Reaction pattern of NC-MAbs of serotype 1 and 4 viruses as well as EBL1 and EBL2. The 14 discriminant antibodies discussed in the text are grouped in the lower part of the figure. Abbreviations: AG, antigenic site; M1, phosphoprotein; N, nucleoprotein; UNCL, antigenic site unclear or not determined; EP SEROT, *E. serotinus*; MINIOPT., *Miniopterus* sp. open boxes, positive staining; lightly shaded boxes, variable staining; heavily shaded boxes, negative staining.

the lung tissue as such. Immunofluorescence also revealed detectable amounts of virus antigen in the filiform papillae of the lingual epithelium (Fig. 1B).

MAb study. A large number (501) of French virus isolates submitted to the routine diagnosis procedure were checked for EBL identification by using two reduced panels of NC-MAbs, A (MAbs 187.5 and 502.2) and B (MAbs 59-1, 26-9, 69-9) (35). MAb 187.5 was negative for virus isolates from European bats, MAbs 502.2 and 59.1 served as positive controls (43), and MAbs 26.9 and 69.9 were specific for EBL1 and EBL2, respectively. Of the 501 rabies virus isolates obtained from terrestrial mammals, 349 were from cats or wild carnivores (fox, stone marten, and marten) whose habits may bring them into contact with bats. None of the virus isolates corresponded to EBL, but they all showed a profile typical of that of serotype 1 lyssaviruses. In contrast, the rabies virus isolate 8918 from a bat was characterized as EBL1 with a reduced panel of eight NC-MAbs (26). A more complete profile of this isolate was determined with a panel of 44 NC-MAbs (Fig. 2). An identical antigenic profile was obtained with three other lyssaviruses that were directly isolated from *E. serotinus* in Gdansk, Poland, in 1985 (21) and in Bainville-sur-Madon, France, in 1989 or from a child who died in Belgorod, Russia, after being bitten by a bat (36). To complete this analysis, the nucleocapsid antigenic profiles of two isolates from foxes (serotype 1), two Duvenhage viruses (25), and two EBL2 virus isolates were determined with the panel of 44 NC-MAbs. The numerous discrepancies found between the serotype 1 virus and the viruses from bats affected all the antigenic sites described on the M1 and N proteins without any preferential localization (Fig. 2). This study allowed selection of a large panel of 14 NC-MAbs (422.5, 239.1, 364.1, PVA3, 11.1, 807.5, 69.9, PVB1, 513.3, M30, 803.6, 111.1, 111.2, 26.9), all of them clearly discriminating between rabies virus isolates from bats belonging to serotype 4, EBL1, or EBL2. The results suggested that EBL1 is more related to serotype 4 than to EBL2, according to their

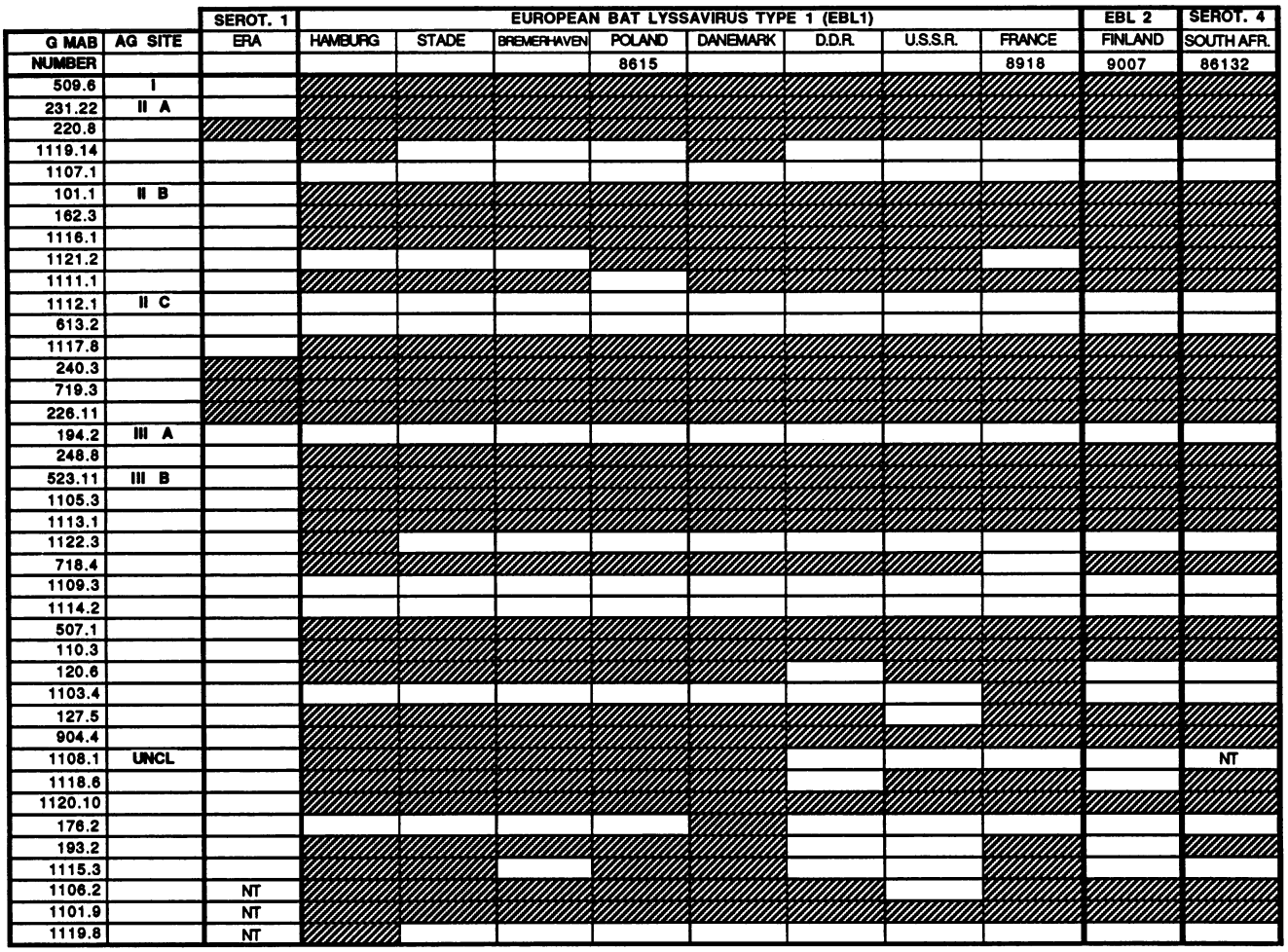

FIG. 3. Comparison of the neutralization pattern of the ERA vaccinal strain (serotype 1), the Duvenhage virus (serotype 4), and EBL1 and EBL2. Abbreviations: G MAb, antiglycoprotein MAbs; AG, antigenic site; UNCL, antigenic site unclear or not determined; NT, not tested; open boxes, virus neutralization; shaded boxes, no virus neutralization.

nucleocapsid antigenic profiles. Ten isolates of EBL1, EBL2, and serotype 4 were further studied with 40 G-MAbs (Fig. 3). Unlike the NC-MAbs, G-MAbs did not clearly discriminate between isolates from bats belonging to serotype 4, EBL1, or EBL2. Most of the rabies virus isolates gave a negative reaction whatever the bat virus. According to these results, EBL1 cannot be subdivided into three minor groups (the former Federal Republic of Germany, Poland, and Denmark) as proposed previously (30), but each isolate can be distinguished on the basis of specific reactions with at least one G-MAb. Nevertheless, a striking identity was noticed between the reactivity profiles obtained with G-MAbs of EBL1-former German Democratic Republic and EBL2-Finland (isolate 9007).

Genetic analysis. Primers PVN7 and PVN8 were chosen because they fulfilled the following criteria. (i) They are located within conserved regions between the Mokola virus genome (serotype 3) and the PV genome (serotype 1) (32), which are presumably among the two most distant serotypes within the *Lyssavirus* genus. These primers are capable of detecting most of the lyssavirus isolates. (ii) The amplified sequence encompasses the totality of the N gene. (iii) The expected PCR product is 1,531 bp long, a convenient size for restriction polymorphism. The PVN7-PVN8 primer set succeeded in the amplification of the N gene of the European fox rabies virus (serotype 1 of lyssavirus), as expected, and, in addition, all of the EBL1, EBL2, and Duvenhage virus (serotype 4) isolates studied (Fig. 4). We selected one Duvenhage virus (isolate 86132), two EBL2 viruses (isolates 9018 and 9007), two EBL1 viruses (isolates 8918 and 8615), and one isolate from a French fox (isolate 9004) for further analysis. From the complete sequence of the amplified products (data not shown), the restriction maps of the six N genes were deduced and three restriction endonucleases (*Dde*I, *Pst*I, and *Pvu*II) were selected for their ability to easily characterize each type of virus (Fig. 4). The absence of *Pvu*II digestion characterized the isolate of serotype 4 (isolate 86132). The *Pst*I cleavages differentiated between the EBL2 isolates (isolates 9007 and 9018) that gave products of 1,305 and 240 bp and the other isolates that lacked this restriction site. The *Dde*I digestion of the amplified products allowed the distinction between EBL1 and the isolate from a French fox (isolate 9004). It must be noted that viruses belonging to the same serotype or biotype show identical restriction patterns. In contrast, the patterns were clearly different, both by the number and the size of the fragments, from one serotype (biotype) to another. Several differences were observed, however, between *Dde*I and *Pvu*II diges-

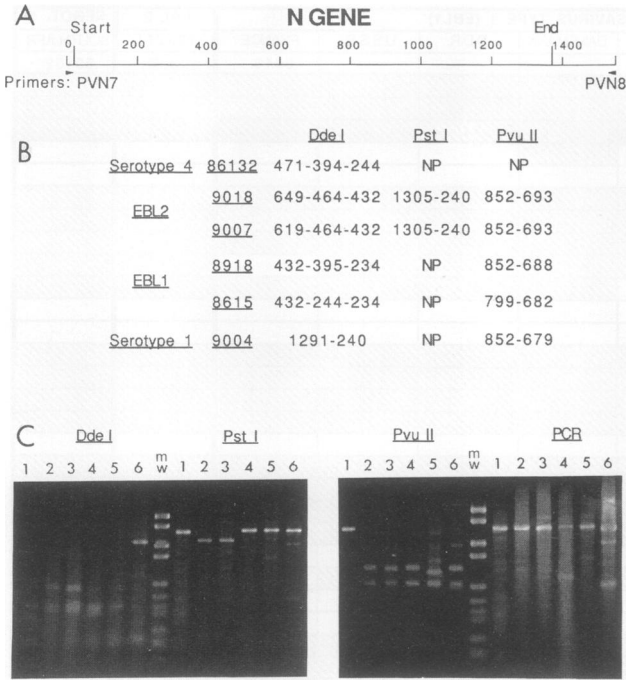

FIG. 4. PCR analysis of the N gene of Duvenhage virus isolate 86132 (serotype 4), EBL2 isolates 9018 and 9007, EBL1 isolates 8918 and 8615, and rabies virus isolate 9004 from a European fox (serotype 1) and endonuclease digestion patterns in 0.8% LSM and 2% NuSieve GTG agarose after digestion with *Dde*I, *Pst*I, and *Pvu*II. (A) Genomic region amplified by PVN7-PVN8 primer set amplification. (B) Sizes of the restriction fragments visible on the gel after digestion with *Dde*I, *Pst*I, and *Pvu*II. NP, the endonuclease site is not present. (C) Amplification products (PCR) and restriction fragment length polymorphism after digestion of isolates 86132 (lanes 1), 9018 (lanes 2), 9007 (lanes 3), 8918 (lanes 4), 8615 (lanes 5), and 9004 (lanes 6) with *Dde*I, *Pst*I, and *Pvu*II. The molecular weight markers (mw) were pBR328 digested with *Bgl*I and pBR328 digested with *Hin*fI (2176, 1766, 1230, 1033, 653, 517, 435, 394, 298 × 2, 234 × 2 bp).

tions of the EBL2 and EBL1 viruses. Isolate 9018 lacked a *Dde*I cleavage site and gave a larger fragment of 649 bp, which was in contrast to a fragment of 619 bp for isolate 9007; isolate 8918 lacked *Dde*I and *Pvu*II cleavage sites and gave larger fragments of 395 and 852 bp instead of 244 and 799 for isolate 8615, respectively (Fig. 4).

For a better comprehension of the phylogeny of rabies virus in bats in Europe, pairwise comparisons between the nucleotides and the deduced amino acid sequences of the N-coding regions of the selected isolates and the other previously published strains of serotype 1, PV (40), SAD B19 (8), and AVO1 (29), were performed. The resulting similarity matrix (Table 3) indicates that the grouping of isolates into genotypes 1, 4, EBL1, and EBL2 is mostly concordant with the serologic analysis. Sequences derived from the same serotype or biotype are more closely related, with average nucleotide and amino acid similarities of 94.2 and 98%, respectively. This resulted in four distinct phylogenic clusters named genotype 1, genotype 4, genotype EBL1, and genotype EBL2. Intergenotypic relationships showed that genotypes 1, 4, and EBL2 are equidistant from one another, with average nucleotide and amino acid similarities of 75.7 and 87.8%, respectively. However, if genotype EBL1 shows an equal divergence with genotypes 1 and EBL2, it is, surprisingly, more related to genotype 4 (79.3 to 79.8% and 92.7 to 93.3% nucleotide and amino acid similarities, respectively. This definitively excludes its previous grouping with EBL2.

DISCUSSION

Until 1985, bat rabies was described only in the northeastern part of Europe: northern Germany, Poland, the former Soviet Union as well as Turkey and the former Yugoslavia (15). Since 1985, it has progressively been reported in many other parts of Europe: Denmark in 1985, The Netherlands in 1987, the southwest of Germany in 1987, the south of Spain in 1987, and Czechoslovakia and the east of France in 1989. All these reports concern six bat species: *E. serotinus*, *Myotis myotis*, *Myotis daubentoni*, *Myotis dasycneme*, *Pipistrellus pipistrellus*, and *Pipistrellus nathusii*. These bats are nocturnal animals that feed almost exclusively on insects and frequently share the same daytime roosts. *E. serotinus* is the species that is most commonly found to be infected; this is followed by species belonging to the genus *Myotis*, with nine cases of rabies virus infection found in this genus between 1985 and 1989 (15). The prevalence of rabies disease in the serotine bat, which is also supported by the two French cases, should, however, be moderated by the fact that the habits and the roosts of these bats more often bring

TABLE 3. Similarity matrix[a]

Virus	Strain	Amino acids and nucleotide similarity[b]								
		EBL1		EBL2		Serotype 4	Serotype 1			
		8615	8918	9007	9018	86132	9004	AVO1	PV	SAD
EBL1	8615		98.7	87.3	86.4	**92.7**	87.6	88.0	88.0	89.4
	8918	95.7		88.0	87.6	**93.3**	87.8	88.2	88.2	88.7
EBL2	9007	77.2	78.1		97.8	86.2	86.9	87.6	86.9	87.3
	9018	76.1	76.9	96.0		85.8	86.4	86.7	86.4	86.9
Serotype 4	86132	**79.3**	**79.8**	76.0	75.8		87.1	88.0	87.6	88.0
Serotype 1	9004	75.6	75.5	74.4	74.5	74.9		97.8	97.1	97.6
	AVO1	75.2	75.4	74.9	75.1	74.9	91.8		97.6	98.0
	PV	75.4	75.3	74.4	74.6	74.5	92.8	92.8		99.1
	SAD	75.8	75.7	74.6	74.9	74.7	93.0	99.0	93.0	

[a] Pairwise comparison of the N coding region of serotype 1, serotype 4, and EBL1 and 2 viruses.
[b] Percentage of amino acid (upper part) and nucleotide (lower part) similarity are given. Numbers squared by dotted lines correspond to intraserotype pairwise comparisons. Boldface numbers correspond to serotype 4-EBL1 comparisons.

them into contact with humans and then to a diagnosis of rabies.

The pathogenicity of EBL in the natural host has not yet been studied because of the protection of bats, which are endangered species in Europe. Therefore, the necropsy data presented here, although statistically limited, are useful. In order to maintain the disease, a latent infection of bats during hibernation is discussed. Although it has been proposed that the brown fat of bats acts as reservoir tissue (3, 39), no infection was detected in the brown interscapular adipose tissue of the *E. serotinus* bats that were necropsied. This finding corroborates the proposal that the presence of the virus in the brown fat may occasionally proceed from the centrifugal virus spread but does not correspond to any special tissue tropism (12). Centrifugal spread of virus to sites that are capable of yielding infectious secretions is also necessary for the maintenance of the enzootic. The salivary gland epithelium is a prime site of viral proliferation in the North American insectivorous bat *Tadarida brasiliensis mexicana* (7). In contrast, we failed to isolate the virus from the salivary glands of the *E. serotinus* bats that we necropsied. The only sites of virus replication were the medulla and the brain, as expected, but the lungs and the taste buds of the tongue were also sites of virus replication. The lungs of experimentally infected mice and hamsters (12, 14) and the lungs and tongues of naturally infected *T. brasiliensis mexicana* (7) were also shown to harbor rabies virus antigen. This suggests that the lungs and the taste buds of the tongue could also participate in the production of infectious oral secretions (6, 28). Insectivorous bats may occasionally transmit rabies to carnivorous animals in North America (37). To investigate whether such transmission is possible in Europe, 501 rabies virus isolates from terrestrial animals in France were analyzed for the presence of bat rabies virus by using specific NC-MAbs. This epidemiological survey gave negative results, indicating that although two cases of infection were reported in humans (24, 36), transmission of the disease from bats to terrestrial mammals is exceptional.

It was first proposed that viral nucleocapsids could be used to determine the genus either by the complement fixation method or by direct immunofluorescence with hyperimmune antisera (35). The genus members were then further divided into serotypes by seroneutralization, whose specificity is supported by the glycoprotein. However, by using MAb studies, it rapidly appeared that considerable antigenic variations exist between lyssavirus nucleocapsids, making the concept of a genus-specific antigen highly unlikely. In fact, rabies virus-related strains were later directly identified on the basis of their reactivities with NC-MAbs (mostly anti-N but several anti-M1) (43) before being classified into the previously determined serotypes 2, 3, and 4. The results presented here show that EBL1 shares more epitopes with Duvenhage virus than with EBL2. The antigenic differences in the nucleocapsids of EBL1 and EBL2 confirm that at least two different biotypes of lyssavirus infect European bats (17, 27). EBL1 is predominant in *E. serotinus*, whereas EBL2 is exclusively found in *Myotis* spp. The finding of two biotypes in European bats was not unexpected, since in North America five major antigenic patterns were observed, depending on the insectivorous bat species, *Eptesicus* spp., *Lasiurus* spp., and *Tadarida* spp. (37). Nevertheless, in contrast to the European situation, these North American isolates all belong to serotype 1.

The usefulness of the N antigen in the distinction between serotypes prompted us to undertake a comparative analysis of its nucleotide and deduced amino acid sequences. This technique was found to be suitable for precise epidemiological investigations (31). The aim was both to determine the evolutionary relationship of the lyssaviruses at the molecular level and to evaluate the consistency and precision of the resulting genotypic classification in comparison with the previous serologic one. One of the most difficult tasks of this experiment was the selection of appropriate primers for amplification. The PVN7-PVN8 primer set succeeded in amplifying the N gene of isolates belonging to serotypes 1 and 4 and biotypes EBL1 and EBL2. This was not the case with primer sets described previously (32, 38). The specificities of the previously described primer sets was more limited to serotypes 1 and 3 (data not shown). The PVN7-PVN8 set of primers has a large spectrum for the amplification of lyssaviruses, whatever their serotype, although its range of activity is still under investigation. The sequence analysis provided evidence of four main phylogenetic clusters named genotype EBL1, genotype EBL2, genotype 1, and genotype 4, which corresponded to the previous serotypes and biotypes. An equidistant phylogenetic relationship was observed among genotypes 1, 4, and EBL2, while EBL1 appeared to be more closely related to genotype 4 than to genotype 1 and EBL2. Therefore, the two biotypes of EBL should be considered totally distinct genotypes. As shown previously (31, 38), the restriction polymorphism was also found to be useful for identifying and distinguishing rabies virus isolates from bats. Nevertheless, more extensive studies are required to establish definitively its clinical application by taking into account the eventual mutations in the restriction endonuclease sites. Our results indicated that such mutations may occur and demonstrated the necessity of performing several different restriction endonuclease digestions to confirm the typing.

In summary, the classification criteria of the genus *Lyssavirus* has evolved over the years. Today, sequencing of defined genomic areas may identify lyssavirus variants more precisely than does the use of MAbs and appears to be a useful tool for precise epidemiological investigations. Consequently, the procedure for grouping isolates should be reinvestigated, especially when new isolates such as EBL still remain to be classified. These results indicate that the conservation between members of the same genotype is never lower than 91.8 and 97.1% at the nucleotide and amino acid levels, respectively. It establishes that the homology at the nucleotide level in the N gene is higher than that in the Ψ gene, in which the conservation between isolates from foxes in France and strains used in vaccines (serotype 1) is only 83.5 to 86.7% (31). Conversely, the conservation at the nucleotide and amino acid levels between genotypes is never higher than 79.8 and 93.3%, respectively. The exact N gene similarity limits, below which a new genotype should be defined, remain to be further evaluated and discussed. More comparative sequence analyses of other wild-type isolates and prototype strains from other serotypes will help to adjust this value. A subsequent analysis of the protective activities of these isolates may classify them as true variants or as newly recognized serotypes against which conventional vaccines may not fully protect an animal, as demonstrated for EBL (19).

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