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Rabies: Epidemiological Tendencies and Control Tools

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Abstract: Rabies encephalitis still generates 50,000 human deaths/year. It is due to neuron infection by lyssaviruses. Seven genotypes (GT) are currently distinguished within the *Lyssavirus* genus which segregate in two phylogroups (PG). This classification is constantly evolving due to isolation of new lyssaviruses within bat populations. Functional differences exist between GTs in term of neurotropism, pathogenesis, induction of apoptosis, immunogenicity, and their molecular basis are starting to be elucidated. Lyssavirus vectors are mammals, preferentially from the *Carnivora* and *Chiroptera* orders. Phylogenetic reconstruction strongly supports that lyssaviruses evolved in chiropters long before the emergence of carnivoran rabies which very likely occurred through host-switchings from bats to carnivores. If dog rabies control is possible by vaccination and population control, if oral vaccination demonstrated its potential to eliminate rabies from a terrestrial wildlife reservoir (fox in Western Europe), it is unrealistic today to clear lyssaviruses from bats, while bat rabies is a growing concern for both public and animal health. As bat transmit divergent lyssavirus GTs which are not well prevented by available vaccine strains, there is a need to increase the protection spectrum of vaccines. DNA-based immunization with plasmids expressing chimeric G proteins (fusion of two halves from different GTs) was shown to be effective in inducing a complete immune response and to broaden the spectrum of rabies vaccines toward lyssavirus vaccines. Further, the lyssavirus G protein can carry foreign epitopes/antigens in the perspective of multivalent vaccines against various zoonoses of carnivores.

INTRODUCTION

According to a recent re-evaluation of both public health and economic burden, rabies encephalitis remains responsible annually for 55,000 human deaths corresponding to 1.74 million disability-adjusted life years (DALYs) [1]. If Asia still

pays the heaviest tribute (31,000 deaths), the death toll in Africa is also alarming (24,000 deaths). On the other hand, under the impulsion of PAHO over the last 15 years, Latin American governments have implemented strict control measures (mass vaccination of dogs and treatment of exposed people) resulting in a stringent reduction of canine rabies, followed by an immediate benefit for human health (< 50 cases/year) [2]. This success clearly demonstrates that, with political will and financial support, efficient tools are available today to reduce human and animal rabies incidence. In humans, safe and potent vaccines produced in cell culture, recommended by WHO, are progressively replacing old vaccines prepared from animal nervous tissue that are less immunogenic and occasionally suspected of adverse effects; in animals, classical vaccines in use under domestic conditions are complemented by a panel of recombinant and attenuated vaccines administrable by the oral route to vaccinate non-accessible dogs or wildlife.

RABIES AND THE LYSSAVIRUS INFECTIOUS CYCLE

The etiologic agents of rabies encephalitis belong to the order *Mononegavirales*, family *Rhabdoviridae*, genus *Lyssavirus* [3]. Lyssaviruses are most generally transmitted by the bite of an infected animal although transmission through aerosols could play a role in bat colonies. Even if inoculation generally occurs in non-nervous tissues, such as muscles, lyssaviruses are naturally neurotropic and propagate through the neuronal network up to the central nervous system (CNS). The incubation period, which corresponds to transfer up to the CNS, varies from 2 weeks to several years (average: 2 months) depending on the place of inoculation and the virulence of the variant. The symptomatic period, which results from neuronal dysfunctions still poorly understood, is shorter (1 week on average). It leads invariably to death although there are growing efforts to develop efficient therapies [4]. In the late stages, non-neuronal tissues such as the salivary glands are infected, allowing for virus transmission through biting.

The molecular bases for lyssavirus neurotropism are not fully understood but certainly implicate neuro-specific receptor recognition, first at the neuromuscular junction, then at inter-neuronal connexions. Three cellular proteins have been proposed to play some role in lyssavirus attachment [5]: the nicotinic acetylcholine receptor (nAChR), the neuronal cell adhesion molecule (NCAM) and the low affinity neurotrophin receptor (p75NTR). These receptors are bound by the viral transmembrane glycoprotein (G) which forms spike-like projections out of the lipid envelope of the bullet-shaped virion [6]. Then, the particule is internalized in an endosome, acidification provokes G mediated fusion of viral and endosomal envelopes and the internal helical ribonucleocapsid (or RNP) is delivered into the cytoplasm. The RNP is composed of the genomic RNA (12 kilobases, non-segmented, negative polarity) intimately associated with the nucleoprotein (N) and with a polymerase complex comprising the RNA-dependent RNA polymerase (L) and its cofactor the phosphoprotein (P, formally named M1). This RNP complex mediates viral transcription and replication in the cytoplasm [7]. The fifth viral structural protein, the matrix protein (M, formally named M2) is notably responsible for virus budding and bullet-shaped morphology. It occupies an intermediate position between the RNP and the envelope and interferes in both structures. In the neuron, between entry at the synapse and transcription/replication in the perinu-

clear space, the virus is actively transported along the axon and dendrites via retrograde or anterograde transport. It is unclear whether the RNP (upon release at the synapse) or the endosome vesicule is transported. A strong interaction between the P protein and the dynein light chain 8 (DLC8, a key element of the retrograde axonal motors) could play for the first hypothesis [8,9].

Natural History and Evolution of Lyssaviruses

Because the majority of human rabies infections worldwide are consecutive to dog bites in developing countries, rabies transmission has long been attributed to dogs only. Remnants of this profound dog/rabies association have integrated into common language, such as with the French word "canicule" designating the hot summer periods when dogs were presumed more effective at transmitting rabies due to the bad influence of Sirius, the dog constellation. However, with the progressive control of rabies in dogs and increased activity in field isolation, it has becoming clear that lyssaviruses are preferentially transmitted by wildlife vectors from two mammalian orders, *Carnivora* and *Chiroptera* (Fig. 1). Carnivoran vectors are members of the families *Canidae* (dog, fox, and raccoon dog), *Procyonidae* (raccoon), *Mustelidae* (skunk), and *Herpestidae* (mongoose). Chiropteran vectors have been found in more than 150 species of 50 genera including both megachiropters and microchiropters, frugivorous, insectivorous and hematophageous. The development of both antigenic and molecular methods for virus identification have progressively allowed for lyssavirus classification. Cross-reactivity between antisera against internal antigens (RNP in which N is highly conserved) first served to
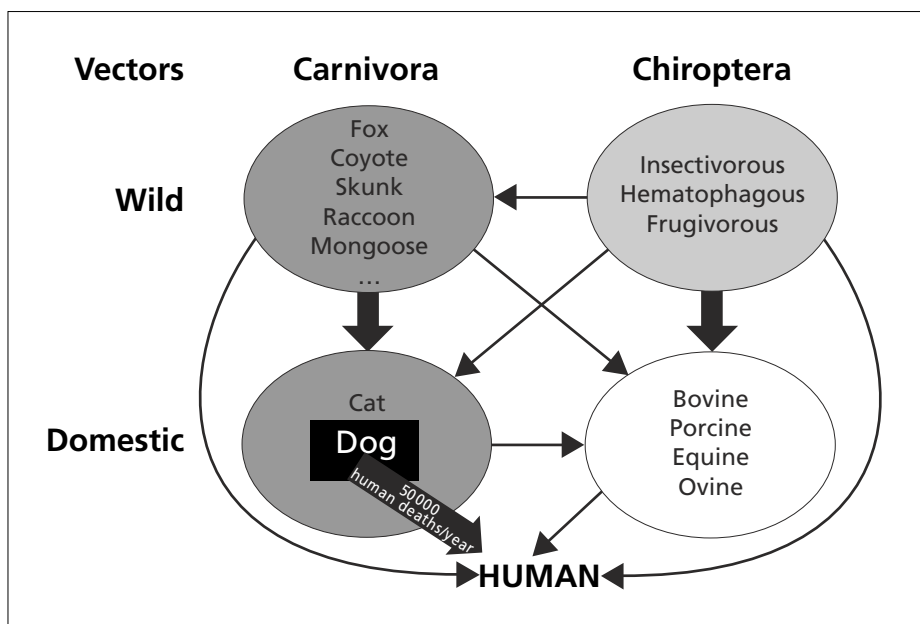

Fig. 1 : Dynamics of transmission of lyssaviruses from *Carnivora* and *Chiroptera* vectors to humans.

distinguish the *Lyssavirus* genus from the *Vesiculovirus* genus (VSV) within the *Rhabdoviridae* family. Virus-neutralising antibodies (VNAbs), which mainly recognized the G protein (less conserved), subdivided the *Lyssavirus* genus into three serotypes, and MAb studies further refined the classification into four serotypes: 1 (Rabies virus, RABV), 2 (Lagos bat virus, LBV), 3 (Mokola virus, MOKV) and 4 (Duvenhage virus, DUVV). Finally phylogenetic comparison of the N, G and P genes have delineated seven genotypes [10-12] (Table 1): the first four matched the four serotypes, and three additional genotypes were created for European bat lyssavirus type 1 (EBLV-1), type 2 (EBLV-2) and Australian bat lyssavirus (ABLV). The seven genotypes are distributed into two phylogroups [11]: phylogroup I includes genotypes 1 and 4 to 7; phylogroup II comprises genotypes 2 and 3 (Fig. 2). This current classification is bound to constantly evolve, particularly as surveillance of bat lyssaviruses is reinforced. Already, four additional divergent lyssaviruses have been isolated in bats of Central Asia, East Siberia and the Black Sea region and are proposed as new genotypes [13,14]: Aravan virus (ARAV), Khujand virus (KHUV), Irkut virus (IRKV) and West-Caucasian bat virus (WCBV). ARAV, KHUV and IRKV are more closely related to viruses of genotypes 4 (DUVV), 5 (EBLV-1) and 6 (EBLV-2) which altogether could form a distinct lineage of Old World (Africa-Europe-Asia) bat lyssaviruses. Although WCBV would appear phylogenetically closer to phylogroup II (LBV and MOKV) it is one of the most divergent lyssavirus isolated so far. It is predictable that increased surveillance of bat lyssaviruses in Asia will soon suggest a more complex classification [15-17].

Each genotype encompasses variants with distinct genetic patterns typical of the geographical area and/or the species of isolation. Each variant is primarily maintained in a single species under specific ecological conditions, but is also able to infect a variety of other species. These regular transmissions across the species barrier are more frequently dead-end infections, but may exceptionally result in variant adaptation and emergence of a new vector. In genotype 1 (classical rabies), a cosmopolitan lineage illustrates human influence in rabies epidemiology through the worldwide spread of the ancient European dog variant during colonization periods [11,18]. This variant has undergone successful adaptation to wildlife species (skunk, coyote, fox in North-America, etc.). With the increasing amount of genetic data on viral field isolates, the evolution mechanisms of the lyssavirus genome and their epidemiological consequences in nature have been analyzed [19-21]. Evolution primarily proceeds by point mutations (limited insertion/deletion, no visible role for recombination) at a low rate for an RNA virus estimated at about 4.10^{-4} synonymous mutations/site/year. There is a high prevalence of synonymous versus non-synonymous mutations suggesting strong selective constraints and purifying selection. It is, however, possible to identify limited regions or residues that seem subjected to some positive selection, particularly in G but also in N or P proteins. Although this local positive selection could result from virus/vector adaptation, no precise residue has been unambiguously pointed out as a signature of the variant adaptation to a specific vector. At the evolutionary level, phylogenetic reconstruction strongly supports an ancient evolution of lyssaviruses in chiropteran vectors with regular spillover and host-switching to carnivoran vectors [19]. Such bat/carnivore spillover is clearly predicted to have originated the raccoon lineage in the Eastern USA as well as the skunk lineages in South-Central USA and North-Central Mexico [22]. As a confirmation of these phylogenetic predictions, natural bat/carnivore host-switchings are regularly observed in nature. In most cases, they

Table 1 : Toxonomy classification of the *Lyssavirus* genus using phylogenetic and serologic characteristics.

| Phylogroup	Geno-sero types	Species Tentative species	Abrev. (ICTV)	Geographical origin	Potential vector(s)
I	1-1	Rabies virus	RABV	World (except several islands)	Carnivores (world) Bats (Americas)
I	4-4	Duvenhage virus	DUVV	Southern-Africa	Insectivorous bats
I	5-?	European bat lyssavirus type 1	EBLV-1	Europe	Insectivorous bats *Eptesicus serotinus*
I	6-?	European bat lyssavirus type 2	EBLV-2	Europe	Insectivorous bats *Myotis sp*
I	7-?	Australian bat lyssavirus	ABLV	Australia (Philippines ?)	Frugivorous/insectivorous bats *Pteropus sp./Microchiroptera*
II	2-2	Lagos bat virus	LBV	Sub-Saharian Africa	Frugivorous bats *Megachiroptera*
II	3-3	Mokola virus	MOKV	Sub-Saharian Africa	**Unknown** (isolated from shrews)
?	?-?	*Aravan virus*	ARAV	Central Asia	Insectivorous bats (isolated from *Myotis blythi*)
?	?-?	*Khujand virus*	KHUV	Central Asia	Insectivorous bats (isolated from *Myotis mystacinus*)
?	?-?	*Irkut virus*	IRKV	East Siberia	Insectivorous bats (isolated from *Murina leucogaster*)
?	?-?	*West Caucasian bat virus*	WCBV	Caucasus region	Insectivorous bats (isolated from *Miniopterus schreibersi*)
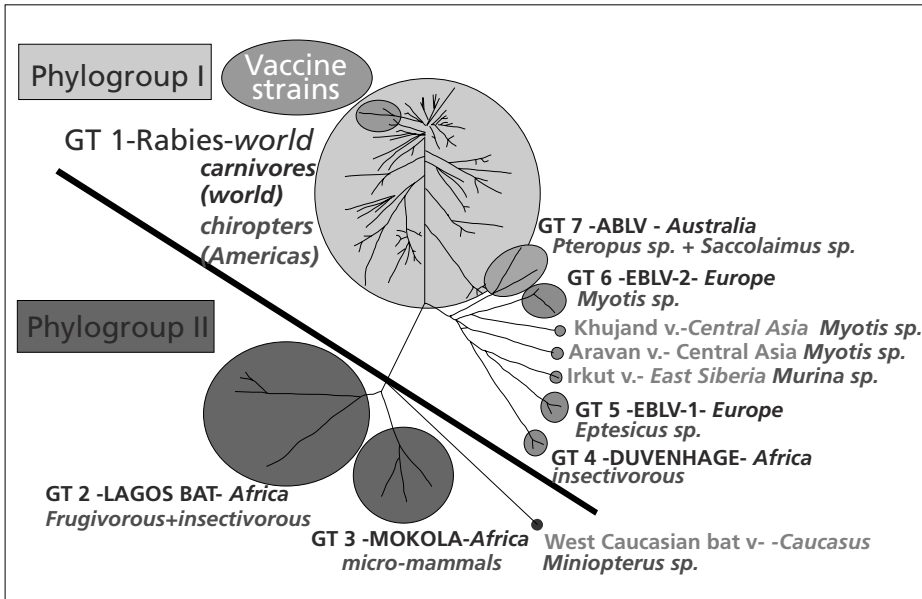
Fig. 2: **Diversity of the *Lyssavirus* genus:** A radial phylogenetic tree has been obtained by comparing partial nucleotide sequences of the G protein ectodomain using the Neighbour Joining Method (CLUSTAL-W; PHYLIP). The transversal bold line distinguishes phylogroups I and II. Circles illustrate the current genetic dispersion for each genotype (GT) which geographical location and principal vector species are indicated (bats in light, carnivores in dark). The vaccine strain position is indicated at the top of GT1.

only lead to dead-end infections like for EBLV1 transmission to sheep in Denmark [23] or to a stone-marten in Germany (24). More rarely, their adaptation is successful like for a RABV *Myotis* variant which circulated for a while among foxes in the Prince Edward Island, Canada [25] or more recently for a RABV *Eptesicus* variant which has been circulating among skunks since 2001 in Arizona [26].

PATHOGENESIS AND PERSPECTIVES OF CONTROL

Efforts are currently underway worldwide to experimentally evaluate the pathogenicity of lyssaviruses, and particularly the transmissibility of bat lyssaviruses to terrestrial mammals including potential carnivore vectors. Lyssaviruses from different genotypes have been inoculated to various animal models such as the bat, dog, cat, red fox, sheep, ferret, hamster, and mouse [11,27-29]. Although the relevance of the rodent model for rabies infection can be discussed, it is the only one in which infection by a limited number of isolates from all genotypes of lyssaviruses has been tested. Globally, lyssaviruses of phylogroup I are pathogenic for mice when injected by the intracerebral or intramuscular routes, while lyssaviruses of phylogroup II are less pathogenic by the intramuscular route [11,29]. In non-rodent mammals, the pathogenicity is more variable depending on the species and the lyssavirus genotype. The molecular bases for these differences have been studied in

vitro and in mice. Pathogenicity by the peripheral route results in the loss of responsiveness of T-cells specific for lyssavirus antigens [30]. Apoptosis is also thought to play a key role in pathogenicity [31,32]. In vitro, it is more intensively induced in mouse neuroblastoma cells infected with lyssaviruses of phylogroup II [33]. By provoking more cell death, viral spread and pathogenesis in vivo is reduced. Coherently, a strong induction of apoptosis is also observed during human neuroblastoma cell infection with non-pathogenic mutants of phylogroup I [34,35]. Lyssaviral induction of apoptosis is probably multigenic and involves at least M and G proteins [33,35]. Concerning viral markers of pathogenicity, all lyssaviruses of phylogroup II sequenced so far, including the recently isolated WCBV, possess an acid residue (D or E) in position 333 of the G protein [11,14], while the maintenance of a basic residue (mostly R, sometimes K) at this position is an absolute prerequisite to maintain pathogenicity of phylogroup I laboratory strains [36,37]. R333D mutation could impair receptor recognition and penetration into neurons [38]. In this context, it is of note that G proteins from phylogroup II lyssaviruses (as well as from genotypes 4 (DUVV) and 5 (EBLV-1) in phylogroup I) are unable to specifically bind p75NTR, one potential receptor for rabies virus [39]. Other differences between G proteins from rabies virus (phylogroup I) and Mokola virus (phylogroup II) may also contribute to their different pathogenicity : RABV G protein trimers show greater stability and induce fusion at a lower pH than MOKV G protein trimers [40].

The constant potential of bat lyssavirus variants to spill-over and invade new carnivore vectors is currently impairing any perspective of global rabies eradication. It is clear that canine rabies can be controlled by systematic vaccination and population control, as has been demonstrated over the last century in many European countries, and as it is today successfully performed in Latin America [2]. In addition, it has also been beautifully demonstrated in Western Europe that the rational use of oral vaccination is a powerful tool to eliminate rabies from a terrestrial wildlife reservoir like the red fox [41]. Once adapted to each specific ecological context, a similar approach could be reasonably applied to many regions in the world still faced with terrestrial rabies : in Central, Eastern, and Northern Europe for fox rabies as well as for the emergent raccoon dog rabies ; in the Americas, for the multiple existing terrestrial vectors : skunk, raccoon, fox, coyote, and mongoose [42]. However, it still appears unrealistic, today, to clear lyssaviruses from bats. This will remain a constant concern for both public and animal health, with the added potential of the virus to acquire new carnivore vectors. In the USA, over the last decades, RABV bat variants have progressively become the principal cause of indigenous human rabies cases [43]. Even in countries fulfilling the OIE rabies-free status (no terrestrial case for two consecutive years with effective surveillance) bat rabies remains present. In France, 26 EBLV-1 positive bats have been diagnosed so far with an increasing number since vulpine rabies elimination in 1998. In UK and Australia, which have been considered as a rabies-free countries for one century, EBLV2 and ABLV have been responsible for one (Scottland, 2002) and two human cases (since 1995), respectively [44]. Facing this situation, it is of note that all the current rabies vaccine strains belong to genotype 1 and were isolated a long time ago [45]. They are clearly protecting against RABV and ABLV, and they are more or less efficient against other genotypes in phylogroup I (EBLVs, DUVV), but they are unable to protect against lyssaviruses from phylogroup II (MOKV, LBV, WCBL) [11,29,46-48]. This absence of cross-neutralisation is globally inversely related to the genetic distance between each genotype and the vaccinal strain used

[11,29]. Assuming continued emergence in rabies epidemiology, especially in bats hosting divergent lyssaviruses, there is a need for a broader spectrum of protection. Perspectives in this direction have been opened using novel strategies such as DNA-based immunization, which has been shown to induce a complete immune response and protection in the mouse as well as in other target species like the dog [46,47,49,50]. Plasmids expressing chimeric G proteins were constructed by fusing the COOH-half of one genotype with the NH2-half of another genotype (Fig. 3). Upon intramuscular injection in mice, protection was obtained against the two parental genotypes but also against other genotypes, demonstrating the possibility of increasing the spectrum from anti-rabies to anti-lyssavirus vaccines. In addition, the capability of the lyssavirus G protein to carry foreign epitopes/antigens was also demonstrated in the perspective of a future DNA multivalent vaccine against various zoonoses for carnivores [51].
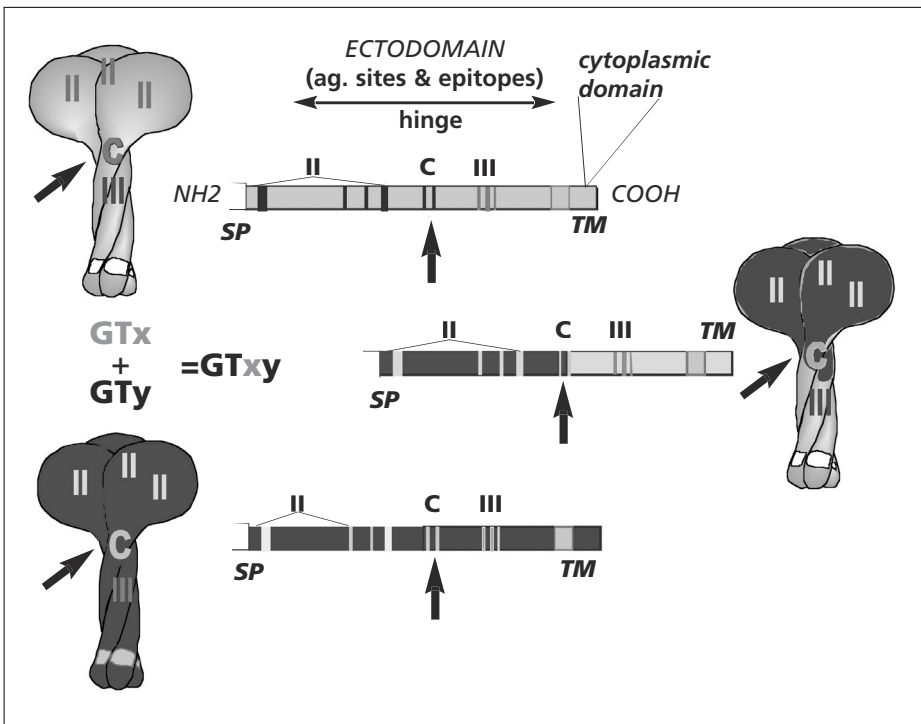

Fig. 3: **Chimerical Lyssavirus G proteins to increase the vaccine spectrum:** The transmembrane G protein comprises from the NH2 to the COOH extremity: a signal peptide (SP) removed from the mature protein; a glycosylated ectodomain carrying the antigenic sites and epitopes; a transmembrane peptide (TM); a cytoplasmic domain. There are 2 majors antigenic sites: site II is composed of epitopes dispersed along the NH2-half of the ectodomain that are put into contact by the protein folding (disulfide bridges); site III is more compact and limited in size (about 10 amino acids). Most G epitopes are conformational except one located between the 2 main antigenic sites and which constitutes a hinge region (region C). This region has been used to generate chimerical G proteins composed by the fusion of NH2- and COOH halves from divergent genotypes. The G protein structure proposed is purely hypothetical since the exact structure has not been solved so far.

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