Cross-neutralization of antibodies induced by vaccination with Purified Chick Embryo Cell Vaccine (PCECV) against different Lyssavirus species

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Keywords: cross-neutralization, lyssavirus, PCECV, rabies, vaccine

Background: Rabies is a neglected zoonotic disease caused by viruses belonging to the genus lyssavirus. In endemic countries of Asia and Africa, where the majority of the estimated 60,000 human rabies deaths occur, it is mainly caused by the classical rabies virus (RABV) transmitted by dogs. Over the last decade new species within the genus lyssavirus have been identified. Meanwhile 15 (proposed or classified) species exist, including Australian bat lyssavirus (ABLV), European bat lyssavirus (EBLV-1 and -2), Duvenhage virus (DUVV), as well as Lagos bat virus (LBV) and Mokola virus (MOKV) and recently identified novel species like Bokeloh bat lyssavirus (BBLV), Ikoma bat lyssavirus (IKOV) or Lleida bat lyssavirus (LLBV). The majority of these lyssavirus species are found in bat reservoirs and some have caused human infection and deaths. Previous work has demonstrated that Purified Chick Embryo Cell Rabies Vaccine (PCECV) not only induces immune responses against classical RABV, but also elicits cross-neutralizing antibodies against ABLV, EBLV-1 and -2.

Material & Methods: Using the same serum samples as in our previous study, this study extension investigated cross-neutralizing activities of serum antibodies measured by rapid fluorescent focus inhibition test (RFFIT) against selected other non-classical lyssavirus species of interest, namely DUVV and BBLV, as well as MOKV and LBV.

Results: Antibodies developed after vaccination with PCECV have neutralizing capability against BBLV and DUVV in the same range as against ABLV and EBLV-1 and -2. As expected, for the phylogenetically more distant species LBV no cross-neutralizing activity was found. Interestingly, 15 of 94 serum samples (16%) with a positive neutralizing antibody titer against RABV displayed specific cross-neutralizing activity (65-fold lower than against RABV) against one specific MOKV strain (Ethiopia isolate), which was not seen against a different strain (Nigeria isolate).

Conclusion: Cross-neutralizing activities partly correlate with the phylogenetic distance of the virus species. Cross-neutralizing activities against the species BBLV and DUVV of phylogroup 1 were demonstrated, in line with previous results of cross-neutralizing activities against ABLV and EBLV-1 and -2. Potential partial cross-neutralizing activities against more distant lyssavirus species like selected MOKV strains need further research.

Introduction

According to latest estimates by the World Health Organization, every year worldwide about 60,000 people die of rabies.1 The majority of these human deaths occur in developing countries in Asia and Africa, where the majority of the estimated 60,000 human rabies deaths occur, it is mainly caused by the classical rabies virus (RABV) transmitted by dogs.1,2 In Europe, rabies is mainly transmitted by foxes. In Central and Western Europe over the past decade, rabies has successfully been eliminated in dogs and foxes in a variety of countries and consequently many European countries are free of terrestrial rabies.3,4 Bat rabies, however, remains endemic and it is commonly accepted that bats are the primary evolutionary hosts of lyssaviruses.5,6 Together with other (classified or proposed) lyssavirus species, the genus consists of 15 different species.2 In Europe lyssavirus species responsible for bat rabies are European bat lyssavirus 1 and 2 (EBLV-1 and -2).7 In 2010, a novel lyssavirus species has been identified, which after the place of its first
detection in Germany was designated *Bokeloh bat lyssavirus* (BBLV). Recently, BBLV was again isolated from a bat in Bavaria, Germany and in Northeastern France. Other novel species are *Ikoma bat lyssavirus* (IKOV) identified 2009 in an African civet and *Lleida bat lyssavirus* (LLEBV, not yet classified) identified 2013 in a bent-winged bat in Spain. In the Americas bat rabies is mainly transmitted by classical rabies virus (RABV) and in Australia, which is considered free of terrestrial rabies, bats are a reservoir of *Australian bat lyssavirus* (ABLV). In Africa *Duvenhage virus* (DUVV), *Shimoni bat virus* (SHIBV), and *Lagos bat virus* (LBV) are found in bats. *Mokola virus* (MOKV) – the only *lyssavirus* species not found in bats – has been identified in Africa in mainly shrews, and cats. Based on their genetic and antigenic differences, *lyssavirus* species have been proposed to form up to 4 distinct phylogroups (for phylogenetic relationship among *lyssavirus* species and phylogroup classification see Fig. 1). Of the 15 *lyssavirus* species, RABV, EBLV-1 and -2, ABLV, DUVV, IRKV (all phylogroup 1) and MOKV, (phylogroup 2) have caused human deaths. Studies have been conducted investigating cross-neutralization between sera against divergent members of the *lyssavirus* genus. Human rabies vaccines, which contain inactivated RABV strains, have been shown to elicit adequate rabies virus neutralizing antibody (VNA) concentrations, not only against the classical RABV but also against other species of phylogroup 1, i.e., EBLV-1 and -2 and ABLV. Cross-protection by vaccines is considered to exist within all species of phylogroup 1, but has not been shown for all species. In this study, we used the identical serum samples as in our previous study, in which we had demonstrated cross-neutralization against ABLV and EBLV-1 and -2, with the objective to investigate, whether the same cross-neutralizing activity is seen against the phylogroup 1 *lyssavirus* species BBLV and DUVV. As a proof of concept, LBV and MOKV as members of phylogroup 2, which is phylogenetically more distant to RABV, were also tested. While cross-neutralization for these virus species is not assumed, this to our knowledge has never been investigated and we included these 2 species in our current study.

**Results**

**Cross-neutralization against DUVV and BBLV**

As demonstrated in Figure 2, PCECV elicited cross-neutralizing activities against BBLV and DUVV. All serum samples with rabies virus (RABV) neutralizing activity ≥0.5 IU/mL (the concentration regarded as adequate immune response after vaccination) also neutralized DUVV (Fig. 2A, virus neutralizing activity ≥0.5 IU/mL; n = 94/94). Against BBLV 88 of the 94 sera (94%) had cross-neutralizing concentrations ≥0.5 IU/mL (Fig. 2B), while 6 serum samples (6%) did not reach the VNA concentration considered adequate (0.5 IU/mL). However, VNA concentrations were clearly elevated compared to non-vaccinated controls (data not shown). Comparison with RABV challenge virus strain (CVS) neutralization was graphically displayed and the

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**Figure 1.** Phylogenetic reconstruction by Bayesian inference of all representative lyssaviruses, modified after, based on the first 405 nt of the nucleoprotein gene. Node numbers indicate posterior probabilities. Dotted lines and numbers 1–4 represent phylogroups (*, proposed). ARAV, Aravan virus; ABLV, Australian bat lyssavirus; BBLV, Bokeloh bat lyssavirus; DUVV, Duvenhage virus; EBLV-1 and EBLV-2, European bat lyssavirus types 1 and 2; IRKV, Irkut virus; KHUV, Khujand virus; LBV, Lagos bat virus (lineages A, B, C, and D); MOKV, Mokola virus; RABV, rabies virus; SHIBV, Shimoni bat virus; WCBV, West Caucasian bat virus; IKOV, Ikoma lyssavirus; LLEBV, Lleida bat lyssavirus (proposed). Scale bar indicates expected number of substitutions per site.
coefficient of determination $R^2$ was calculated. For DUVV vs. CVS the comparison between VNA concentrations showed a coefficient of determination $R^2 = 0.54$, while for BBLV vs. CVS the coefficient of determination $R^2$ was lower, $R^2 = 0.32$ (Fig. 2A and B), which means that 54% and 32% of the total variation in DUVV and BBLV results can be explained by a linear relationship between DUVV or BBLV and RABV neutralization, respectively. The slope of the regression line was 0.71 (CI 95% 0.57–0.85) for DUVV vs. CVS and 0.52 (CI 95% 0.36–0.67) for BBLV vs. CVS.

Cross-neutralization against LBV and MOKV

All sera were negative when LBV was used as challenge strain in the RFFIT and no cross-neutralization was detected. When using MOKV, Lab-No. 12850 as challenge strain the majority of sera were negative as well. However, 15 serum samples (16%) tested positive against this isolate (titers ranging between 17.9 and 1078, GMT 169; see Fig. 3). This finding was confirmed by retesting with the same virus isolate. Neutralizing titers were about 60- to 70-fold lower than against RABV and MOKV positive samples were only seen in samples with a rabies virus VNA titer >1:1000. At an arbitrary cutoff at a titer of 1:5000, 13 of the 26 samples with high titers (50%) displayed positive titers against MOKV. This finding was confirmed by repeating the testing of the positive samples on a different day, leading to the same results. When using a different isolate of MOKV, 12868, no cross-neutralizing activity was seen.

Discussion

The vast majority of human deaths from lyssavirus infection is caused by classical RABV, mainly after exposure to dogs. Other lyssavirus species however can lead to human infection as well and tragically human deaths have occurred after exposure to ABV, EBLV-1 and -2, IRKV, DUVV and MOKV. Previously we demonstrated cross-neutralizing capabilities of antibodies induced by vaccination with PCECV against ABLV and EBLV-1 and -2. Recently a novel lyssavirus, designated Bokeloh bat lyssavirus (BBLV), was found in a Natterer’s bat (Myotis nattereri) in Germany and in the meantime further cases have been identified in Germany and in France.
While already demonstrated against EBLV-1 and -2 and ABLV,\(^{25}\) in this study for the first time cross-neutralizing activities of antibodies induced in humans by classical (RABV) vaccine have been tested against DUVV and BBLV. We used the same sera as in our previous study, which originated from a PEP study.\(^{31}\) For the purpose of this study, it is not of importance, whether the subjects received pre- or post-exposure prophylaxis regimen and at what time point serum samples were taken. Direct comparison with ABLV and EBLV-1 and -2 results obtained with identical testing of the same serum samples\(^{25}\) indicated that DUVV and BBLV results are of the same magnitude. All positive sera against RABV were shown to neutralize DUVV. The comparison between VNA showed a coefficient of determination \(R^2 = 0.54\), which is in the range of what has previously been seen between RABV and EBLV-1,\(^{25}\) while an inter-lab comparison of classical RFFIT using the same challenge virus CVS-11 resulted in a coefficient of determination \(R^2 = 0.65\).\(^{25}\) Lower antibody levels and less good correlation was seen for BBLV (6 sera had VNA concentrations below 0.5 IU/mL against BBLV, overall results were more scattering, \(R^2 = 0.32\)). However, VNA concentrations were specific and in a similar range, with the majority of samples reaching concentrations considered adequate for protection. In consequence, this supports the notion that vaccine made of inactivated RABV induces antibodies that have the capabilities to cross-neutralize also BBLV.

We also compared the respective slopes of the regression line for the lyssaviruses of phylogroup 1 (including EBLV-1 and -2 and ABLV from our previous study\(^ {25}\)). All slopes were below 1, ranging from 0.52 (BBLV) to 0.91 (ABLV). For all viruses except for ABLV the 95% CI did not include 1, suggesting a “true” slope of less than 1. In consequence, even if cross-neutralization is seen throughout the range of VNA concentrations, the neutralization against RABV seems to be more species-specific at higher VNA concentrations than in the lower range. Here antibody specificity, affinity and avidity may play a role. At the highest neutralizing serum concentrations, antibodies may be more specific to identical epitopes of the same virus compared to somewhat further related viruses. Antigenic comparison of the coding region for the ectodomain of the g-protein only partly correlates with cross-neutralizing activity. In fact, pairwise identities of nucleotides (Kimura-2-parameter, MEGA 5) revealed values of between 67% (BBLV, ABLV) and 60% (DUVV) compared to CVS. While the ectodomain of the g-protein of BBLV is closer to RABV than EBLV-1 or DUVV, the cross-neutralization is lower and more scattering. Against DUVV however, good correlation was found, despite the fact that within phylogroup 1 this virus is the most distant to RABV of the species tested. Avidity and affinity of the antibodies induced by the vaccine may play a role, as well as individual factors of the vaccinated individual.

In general, a cross-neutralizing activity of antibodies induced by vaccination with rabies vaccine against all species of phylogroup 1 has been shown or can be assumed.\(^ {24,26}\) However, for IRKV infection it was shown that only routine PrEP (3 vaccine doses, on days 0, 7, and 28) induced strong protection against IRKV infection, while only very high doses of RABV immunoglobulins conferred partial protection of animals.\(^ {27}\) Similar experimental studies are needed to confirm cross-protection for BBLV. In this context, it is worth highlighting that in the absence of reference sera for other lyssaviruses than RABV, for conversion into international units, a heterologous WHO international standard immunoglobulin (2nd human rabies immunoglobulin preparation, National Institute for Standards and Control, Potters Bar, UK) has been used. This reference serum was adjusted to 0.5 IU/mL for CVS-11 and 2.5 IU/mL for BBLV and 20 IU/mL for DUVV to achieve the minimal VNA concentration. This, however, should not be misinterpreted as a cutoff for VNA concentration. While sera may only neutralize other viruses at a lower dilution (titer), neutralization against DUVV or BBLV was seen at low serum concentrations of as low as 0.5 IU/mL. In consequence, it would not be appropriate to draw the conclusion that for an adequate RVNA concentration against DUVV a serum concentration of 20 IU/mL (measured by regular RFFIT) would be needed. Least of all should this be mistaken as a cutoff for ‘protection’. While in the classical RFFIT, using CVS-11 as challenge strain, a VNA concentration of 0.5 IU/mL is considered ‘adequate’, this does not necessarily mean that above this cutoff protection can be guaranteed or below this cutoff protection is not given. This also applies to the modified RFFIT using different lyssaviruses: there is no defined ‘level of protection’.

Against virus species of phylogroup 2, or even the newly introduced or proposed phylogroups 3 or 4, no cross-neutralization of antibodies has so far been demonstrated and was not assumed, as these viruses are antigenically distinct from RABV. For example, it was suggested that 72–74% amino acid sequence identity within glycoprotein ectodomains provides sufficient cross-neutralization between lyssaviruses.\(^ {28}\) Our study confirmed a lack of cross-neutralization against LBV, i.e., none of the sera tested was able to cross-neutralize LBV. In this context it is a highly interesting that we found partly cross-neutralizing activities against MOKV (12850, isolated from Ethiopia;\(^ {29,30}\) ). However, when using a different strain of MOKV (12858, isolated from Nigeria), the cross-neutralizing activity was not seen.

As the initial study from which sera were obtained was performed in Europe in Lithuania, a contact of the trial participant with MOKV (exposure to MOKV / MOKV infection) can be ruled out. A possible explanation of cross-neutralizing activity may lie in the fact that the glycoprotein in this MOKV isolate has some conserved areas, against which some subjects by chance developed antibodies with some affinity and in consequence neutralizing capacity. In fact, a comparison of the deduced amino acid sequence revealed that both in antigenic sites II (AA 34–42) and III (330–338) 2 amino acid substitutions each were found (data not shown). Affinity may be lower due to non-perfect match, explaining that (1) only a subset of sera displays cross-neutralizing activity against MOKV and (2) cross-neutralization is only seen in sera with almost 2 log-scales (60- to 70-fold higher titers necessary for neutralization). To our knowledge this partial
cross-neutralization between RABV and MOKV has never been demonstrated before and these findings need further research. It is reassuring that for at least some strains some level of cross-protection may exist, which in our opinion justifies the administration of rabies vaccine to subjects with suspected exposure to MOKV, i.e., contact with shrews in Africa, or researchers working with MOKV.

Conclusion

In summary, this study confirms that PCECV is capable of eliciting cross-neutralizing activities against a variety of lyssavirus species belonging to phylogroup 1. Interestingly, of the more distant lyssaviruses some cross-neutralizing activity against one specific MOKV isolate was found.

Material and Methods

In this follow-up serology study we used the same sera that had already been tested at the Friedrich-Loeffler-Institute for cross-neutralizing activity against EBLV-1 and -2 and ABLV. Originally 100 human serum samples were provided by Novartis, taken from a clinical trial conducted in healthy subjects in Lithuania, simulating post-exposure prophylaxis (PEP). All subjects had received a full course of PEP with PCECV (Rabipur®). Samples had been deliberately chosen to uniformly and continuously cover the range between 0.5 and 500 IU/mL. Of these, 94 serum samples had sufficient serum left to be included in this follow-up. Individual serum samples were tested for the presence of VNA using a modified rapid fluorescent focus inhibition test (RFFIT), similar to previous modification of RFFIT to test against EBLV-1, with BBLV (Lab-Nr: 21961; GenBank: JF311903), DUVV (12863; EU293119), LBV (12859; EU293110) and 2 MOKV isolates (12850; KF155005 and 12868; AY333111) as test virus. Other than using different challenge virus, the modified RFFIT was done as described by Cox et al.33 Lyssavirus species-specific test viruses used in the assay were isolated from the virus archive of the FLI. BBLV has been first isolated by our group. All viruses were cell culture supernatants obtained from original brain material stored at -80°C. For serological testing the viruses were passaged not more than 3 times on mouse neuroblastoma cells (NA 42/13) to yield sufficient titers. Specificity was determined using human serum samples from unvaccinated individuals, resulting in negative results for all challenge viruses (data not shown). In general, sera were tested in duplicate in 2-fold serial dilutions on mouse neuroblastoma cells (NA 42/13) with a starting dilution of 1:10. The VNA titer was expressed as the reciprocal of the serum dilution showing a 50% reduction in fluorescent foci of the test virus in vitro and the exact titer was calculated using inverse interpolation. For conversion into international units, a heterologous rabies immunoglobulin preparation, National Institute for Standards and Control, Potters Bar, UK) adjusted to 0.5 IU/mL for CVS-11 and 2.5 IU/mL for BBLV and 20 IU/mL for DUVV. Individual VNA concentrations were plotted in double-logarithmic graphs, with a coefficient of determination R2 as a measure of scattering. The 95% confidence intervals (Bonferroni corrected) of the regression line were calculated using R software.

For the comparison of the ectodomain of the glycoprotein sequences were obtained from GenBank, aligned and presented in topology tree prepared in MEGA 5.36 Comparison of the deduced amino acid sequence of different lyssavirus species is presented as Supplemental Figure 1.

Disclosure of Potential Conflicts of Interest

C.M., and A.G. are employees and D.G. is a former employee of Novartis Vaccines, manufacturer of PCEC rabies vaccine.

Acknowledgments

We thank Jeannette Kliemt (FLI) for excellent technical support. Additional thanks go to Noël Tordo (Institut Pasteur, Paris, France) and Tony Fooks (AHVLA, Weybridge, UK) for lively discussions on the results.

Funding

This study was supported, in part, by Novartis Vaccines.

Supplemental Material

Supplemental data for this article can be accessed on the publisher’s website.

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