EMBO Molecular Medicine

Development of broad-spectrum human monoclonal antibodies for rabies post-exposure prophylaxis

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Review timeline:

Submission date:	02 November 2015
Editorial Decision:	18 December 2015
Revision received:	28 February 2016
Accepted:	15 February 2016

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Céline Carret

1st Editorial Decision 18 December 2015

Thank you for the submission of your manuscript to EMBO Molecular Medicine. We have now heard back from two of the three referees whom we asked to evaluate your manuscript. As these two referees have very similar recommendations, we decided to go ahead and make a decision now.

Although referees do find the study suitable for publication in principle, referee 2 suggested expanding the discussion and providing additional explanations here and there.

We would welcome the submission of a revised version for further consideration and depending on the nature of the revisions, this may be sent back or not to the referees for another round of review.

In order to gain time, shall the manuscript be accepted I would also like you to address several editorial issues listed below.

I look forward to seeing a revised form of your manuscript as soon as possible and within 3 months.

***** Reviewer's comments *****

Referee #1 (Comments on Novelty/Model System):

The work is outstanding from a technical perspective. It is not particularly novel as several monoclonal antibodies have been tested previously for RIG replacement. The authors' correctly state

that the problem with MABs is lack of complete coverage against the multitude of lyssavirus. The described MABs have very broad neutralizing activity and may therefore allow for their clinical development. The animal model is appropriate.

Referee #1 (Remarks):

This manuscript, which in great detail describes the specificity of several MABs that may be suited to replace RIG, is timely and will be an important addition to our published arsenal of rabies biologics. The authors may wish to check the manuscript carefully for language - some of the sentences don't quite adhere to English rules of grammar (e.g., last two sentences of the introduction).

Referee #3 (Remarks):

The manuscript by De Benedictis describes the isolation and characterization of several monoclonal antibodies to lyssaviruses, with potential utility for rabies post-exposure prophylaxis. A total of 500 mAbs were isolated from memory B cells from vaccinees prescreened for anti-RABV activity. From these, two mAbs (RVC20 and RVC58) were shown to neutralize with greater breadth and potency than those mAbs currently under clinical development. This data is supplemented by partial epitope mapping and virological analysis. Finally, the authors show that relatively low doses of a combination of these 2 mAbs protects hamsters from a lethal RABV challenge, and assessed the effect of these mAbs on vaccine responses in hamsters. The potential superiority of these new mAbs over CR57, CR4098 and RAB1 is clearly shown here, providing the basis for future clinical studies.

Figure 2 and related text. There are some intriguing findings here. Although the reciprocal competition is very clean for mAbs to antigenic site I, it is more confusing for antigenic sites III and III.2, with mAbs RVB181, RVC56 etc. blocked by antigenic site III antibodies, but unable to block in the opposite assay. The authors speculate that these antibodies form a third cluster that recognizes a distinct site, but I am not sure this accounts for the timing. The opposite is true for RBV686. Are there potential other explanations e.g. conformational changes that could account for this?

Figure 3. the extraordinary breadth of RCV68 (despite low potency) suggests this target should be further investigated as a new conserved target. It would be worth adding this to the discussion.

Page 7. Can the authors comment on the discrepancy between neutralization of pseudotyped viruses compared to live viruses by RVC68? Is this simply a general reflection of the reduced potency of the mAb compared to others i.e. is the pseudovirus assay intrinsically more sensitive or does this reflect the use of IC50 versus IC90?

Figure 6. Why is the more detailed analysis (the pie charts) only performed for selected sites? For Ag site 1, only the 2 most polymorphic sites are described, whereas for Ag site II only one site is omitted.

Figure 7. In panel A (the challenge study) the high dose is 0.045, whereas for the vaccine responsiveness experiments in panels B-D, HD is 40mg/kg. I found this confusing and would more clearly label the axes in B-D.

Page 12. Can the authors comment on the observation that in the presence of the HD of mAbs, the binding responses are significantly lower, but neutralizing responses unaffected.

The discussion is weak. While it is important to place these data in the context of PEP, there are many other aspects (more scientific) that are not discussed at all e.g. potential new epitopes. A more thoughtful discussion would substantially strengthen this paper.

Minor:
Page 4, last line of intro - incomplete sentence

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Figure 5B is extremely hard to follow - the addition of a schematic showing how the chimeras are constructed might be useful to clarify this.

1st Revision - authors' response 28 February 2016

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We thank the reviewer for the positive comments on our manuscript. As suggested we have checked the manuscript for language. Several sentences have been now rephrased accordingly by the English-native scientists co-authoring the study.

Referee #3 (Remarks):

The manuscript by De Benedictis describes the isolation and characterization of several monoclonal antibodies to lyssaviruses, with potential utility for rabies post-exposure prophylaxis. A total of 500 mAbs were isolated from memory B cells from vaccinees prescreened for anti-RABV activity. From these, two mAbs (RVC20 and RVC58) were shown to neutralize with greater breadth and potency than those mAbs currently under clinical development. This data is supplemented by partial epitope mapping and virological analysis. Finally, the authors show that relatively low doses of a combination of these 2 mAbs protects hamsters from a lethal RABV challenge, and assessed the effect of these mAbs on vaccine responses in hamsters. The potential superiority of these new mAbs over CR57, CR4098 and RAB1 is clearly shown here, providing the basis for future clinical studies.

We thank the reviewer for the positive comments on our study.

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competition is very clean for mAbs to antigenic site I, it is more confusing for antigenic sites III and III.2, with mAbs RVB181, RVC56 etc. blocked by antigenic site III antibodies, but unable to block in the opposite assay. The authors speculate that these antibodies form a third cluster that recognizes a distinct site, but I am not sure this accounts for the timing. The opposite is true for RBV686. Are there potential other explanations e.g. conformational changes that could account for this?

We agree with the Referee's comments about the difficulty to define precisely antigenic sites solely on the basis of cross-competition binding studies. In interpreting competition results, it should be taken into account that when two epitopes overlap, or even when the areas covered by the arms of the two antibodies overlap, competition should be almost complete and mutually cross-competitive. Thus, only marked mutual cross-competition should be taken as unequivocal evidence of overlapping epitopes, since weak or one-way inhibition may simply reflect a decreased in affinity owing to steric or allosteric effects (see Epitope Mapping Protocols, chapter 6, Glen E. Morris, Humana Press). A more detailed definition of the epitopes of this antibody panel would require further investigation and this work could be part of a follow-up study.
Authors acknowledge that this point was not sufficiently explained in the previous version of the manuscript and have therefore addressed it accordingly (see Results page 6, lines 13-19).

Figure 3. the extraordinary breadth of RCV68 (despite low potency) suggests this target should be further investigated as a new conserved target. It would be worth adding this to the discussion.

We appreciate the Referee's point. The method used to isolate the monoclonal antibodies investigated in this study had already proven effective in identifying broadly neutralizing antiviral antibodies, which made it possible to discover conserved epitopes that may ultimately lead to design new vaccines capable of conferring broader protection (Corti and Lanzavecchia, Annual Review in Immunology 2013). In relation to rabies, the broadly neutralizing activity of the RVC68 antibody, in spite of its limited potency if compared to other antibodies isolated in the study, worth further investigation as it presumably recognizes a conserved and probably yet undetermined epitope. Possible applications of our findings may range from vaccine development, immune therapy or to the development of new diagnostic tools accounting for the wide lyssavirus diversity. Authors acknowledge that this finding was not sufficiently discussed in the previous version of the manuscript and have addressed the Referee's remark accordingly in the Discussion section (page 12, lines 15-17).

Page 7. Can the authors comment on the discrepancy between neutralization of pseudotyped viruses compared to live viruses by RVC68? Is this simply a general reflection of the reduced potency of the mAb compared to others i.e. is the pseudovirus assay intrinsically more sensitive or does this reflect the use of IC50 versus IC90?

The discrepancy in neutralisation observed between live virus and pseudotyped virus assays is likely due to more than one reason. Firstly, the density of the G protein on the surface of these viruses may differ. Data from previous studies that isolated potent and broadly neutralising influenza mAbs (Corti et al. JCI 2010) or assessed the neutralising potency of bat sera against lyssaviruses (Wright et al. Virology 2010) suggests that pseudotyped viruses have a lower density of viral envelope protein on their surface. This characteristic of PV allowed for the isolation of mAbs that bound to the HA2 stem region of influenza A viruses, which is not readily exposed on the live virus (Corti et al. JCI 2010), but also for a more accurate reflection of lyssavirus epidemiology in bats, thanks to the greater sensitivity of the PV assay (Wright et al. Virology 2010). Secondly, PV-based assays allow the study of entry inhibition however; in the case of live virus assays we also have the potential for viral replication and spread that could affect neutralisation titres. Finally, as the reviewer infers, due to the fact that the approved protocol for running each assay (PV, RFFIT and FAVN) is different this could also lead to variation in the final readout between the assays.

Figure 6. Why is the more detailed analysis (the pie charts) only performed for selected sites? For Ag site 1, only the 2 most polymorphic sites are described, whereas for Ag site II only one site is omitted.

We thank the Referee for the careful review and we agree that it would be more appropriate to show the complete analysis on all positions where the degree of conservation is not equivalent to 100%. We have therefore changed **Figure 6** accordingly by adding pie charts for residues at position 230 (panel a), 330 and 335 (panel b).

Figure 7. In panel A (the challenge study) the high dose is 0.045, whereas for the vaccine responsiveness experiments in panels B-D, HD is 40 mg/kg. I found this confusing and would more clearly label the axes in B-D.

We thank the Referee for the careful review. As suggested we have labeled the axes of panels B, C and D of **Figure 7** accordingly. The amount of monoclonals (in mg/kg) used in each experimental group has been therefore indicated in parentheses.

Page 12. Can the authors comment on the observation that in the presence of the HD of mAbs, the binding responses are significantly lower, but neutralizing responses unaffected.

Similarly to the work by Goudsmith et at (2006) for the CR4098+CR57 cocktail, we also assessed the neutralizing titres detectable more than 40 days following PEP, including the administration of vaccine and the RVC20+RVC58 antibody mixture. Of note, we have further assessed whether the peripheral neutralizing titers conferring protection to hamsters may be due to either hamster endogenous post-vaccination immune response, to exogenous human antibodies due to passive immunization or by a mixture of them. We found that viral neutralization was mainly due to hamster

endogenous response when HRIG or RVC20+RVC58 (0.045mg/kg) was administered, and that our cocktail had almost been fully cleared by the organism about 40 days after administration. As for the RVC20+RVC58 (40 mg/kg) antibody mixture (dubbed HD in the previous version), although the endogenous response elicited (as detected by ELISA by measuring the levels of hamster antibodies directed against the RABV G protein) indicated that an interference between the passively administered monoclonal antibodies and vaccine had somehow occurred, hamsters still had a high neutralizing titre in peripheral blood over 40 days post administration and were therefore still potentially protected against a lethal RABV challenge. These findings merit further investigations, as it has the potential to break the paradigm on which post-exposure prophylaxis approaches are based. Authors acknowledge that this important finding was not discussed in the previous version of the manuscript and for this reason a specific comment has been now added in the discussion section (page 13, lines 10-27).

The discussion is weak. While it is important to place these data in the context of PEP, there are many other aspects (more scientific) that are not discussed at all e.g. potential new epitopes. A more thoughtful discussion would substantially strengthen this paper.

Authors acknowledge that other aspects of the study were not discussed in the previous version of the manuscript; this is why the discussion section has been strengthened with a paragraph underlining some of the most important aspects:
(i) the potential for discovering new epitopes (i.e. that recognized by RVC68), and the relevance of identifying antigenic sites that are conserved among different lyssaviruses (pages 12, lines 6-17);
(ii) a possible explanation of unexpected pattern of previously characterized ASIII antibodies and, more generally, of ASIII antibodies as characterized in the present study (page 6, lines 13-19).

Minor:
Page 4, last line of intro - incomplete sentence

Thanks for the careful review. We have completed the sentence “the combination of two antibodies specific for distinct antigenic sites on the G protein and able to broadly neutralize both RABV and non-RABV
lyssaviruses…” that is now replaced with “the combination of two antibodies that bind to different antigenic sites on the RABV G protein and are able to broadly neutralize both RABV and non-RABV lyssavirus isolates, will significantly reduce the risk of PEP failure.” (page 4, lines 27-29).

Page 7 - last paragraph. It does not appear to me that CR57 has a greater range of IC50/90 values than RVC20, though undoubtedly it is less potent.

We agree with the Referee's comment and have changed the text accordingly by restricting the comment about the broader range of IC50s to the comparison between RVC58 vs RAB1 and CR4098. This sentence has been rewritten (page 8, lines 7-8) as follows: "CR4098 and RAB1 showed a broader range of IC50/IC90 values (0.7-23600 ng/ml, 1-4153 ng/ml, respectively), neutralizing six and three RABV isolates, respectively, with IC50 >1000 ng/ml, a concentration which is likely not to be effective in PEP."

Figure 5B is extremely hard to follow - the addition of a schematic showing how the chimeras are constructed might be useful to clarify this.

We agree with Referee's comment and acknowledge him for the suggestion to include a diagrammatic sketch showing how the chimeras were constructed. We have therefore included a schematic showing generation of epitope swapped G protein in the new **Figure EV1.**

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

Corresponding Author Name: Davide Corti
Journal Submitted to: Embo Molecular Medicine
Manuscript Number:

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The number of individuals in each experimental group (n=12 per group) was calculated using the Fisher's exact conditional test for two proportions (as implemented by Proc Power twosamplefreq, SAS software) and power 1-β=0.80 (α=0.05).
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Not applicable.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples nor animals were excluded from the analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Animals were randomly assigned to tretement or control groups.
For animal studies, include a statement about randomization even if no randomization was used.	Not applicable.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	In order to minimise the effect of subjective bias during allocation, animals were randomly assigned to tretement or control groups. No blinding of investigator was implemented.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done.
5. For every figure, are statistical tests justified as appropriate?	Statistics have been included for any figures regarding animal experiments.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	
Is there an estimate of variation within each group of data?	Yes, there is. The variation within each group has been shown in the figures.
Is the variance similar between the groups that are being statistically compared?	Yes, it is.

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tum

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Not applicable
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Cell lines were provided by the American Type Culture Collection (ATCC) and tested for mycoplasma contamination on a routine basis.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	All experiments were performed on female SPF Syrian hamsters (Mesocricetus auratus) of 6-7 weeks of age (average weight 105 grams) (Charles River Laboratories). Animals were housed in individually HEPA-filtered ventilated cages, three individuals per cage, at a temperature of 22±1°C, on a 12L:12D light cycle, with free access to water and food. Pressed cotton pads, mouse houses and litter bags were used as environmental enrichment, and the standard rodent feed was weekly integrated with autoclaved sunflower seeds.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	The entire study was performed in strict accordance with the relevant national and local animal welfare bodies [Convention of the European Council no. 123 and National guidelines (Legislative Decrees 116/92 and 26/2014)]. The protocol was authorized by the Italian Ministry of Health (Decrees 128/2011-B and 115/2014-PR) before experiments were initiated and approved by the Committee on the Ethics of Animal Experiments of the IZSVe.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	The information on animal experiments has been reported in compliance with the ARRIVE guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Cantonal Ethical Committee of Cantone Ticino, Switzerland.
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Blood samples were collected from participants vaccinated against rabies. All donors gave written informed consent for research use of blood samples, following approval by the Cantonal Ethical Committee of Cantone Ticino, Switzerland.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not applicable.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not applicable.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	not applicable.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	not applicable.
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not applicable.

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Not applicable.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	not applicable.
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	Not applicable.
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	Not applicable.