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Immunologic work up of subjects who didn't respond appropriately to a rabies post exposure prophylaxis

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With my heartfelt tanks to all the research collaborators and advisors who contributed toward the smooth running of this study

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1. Abstract

Background

New recommendations for rabies postexposure prophylaxis (rPEP) were published by the Centers for Disease Control and Prevention and the World Health Organization in 2010. In view of these new recommendations, the adequacy of rPEP among patients consulting the travel clinic of the University Hospital of Lausanne has been investigated and 6,8% of patients have been identified as non-responders with the new rPEP regimen. In this study we have selected the non-responders for a complete immunologic work up.

Method

Clinical and paraclinical immunologic investigations have been done to the non-responders patients. Those investigations have been conducted to look for an increased susceptibility to infections and an immunodeficiency. The investigations included a clinical evaluation, a full blood count, measurement of the immunoglobulin levels, a numeration of the subpopulations of the lymphocytes, a HIV test and an evaluation of the humoral response to tetanus, pneumococcal, and hepatitis B vaccinations. A lymphocyte proliferation assay with rabies antigen was performed to assess the cellular immune response.

Results

9 subjects with rabies antibody titers ≤0,5 IU/ml after an rPEP with 4 doses were included in this study (=non-responders). 8/9 of these non-responders had an unremarkable medical history. 9/9 of them had normal paraclinical tests that did not

suggest an immunodeficiency. The results of the lymphocyte proliferation assay with rabies antigen showed a significant correlation between the level of the humoral and cellular response.

Conclusion

These results suggest that a 4 dose intramuscular rPEP elicits in some patients a relatively poor humoral and cellular response, even in the absence of any immunosuppression. A serology on day 21 of the rPEP seems therefore useful to identify the patients who don't respond appropriately. Those non-responders should receive additional doses until they reach an antibody titer above 0.5 IU/ml.

2. Introduction

Despite the fact that most countries in the Western Hemisphere have succeeded in eliminating rabies transmitted by terrestrial animals, the disease remains endemic in numerous developing countries.[i] Rabies remains especially widespread in most African and Asian countries and causes an estimated 60,000 human rabies-related deaths worldwide each year.[ii]

After potential rabies exposure, a rabies post-exposure prophylaxis (rPEP) is recommended. It involves active immunization and administration of rabies immunoglobulins for non-immune patients. One of the most widely used regimens for rPEP has been the Essen regimen, which includes five doses of intramuscular vaccine

on days 0, 3, 7, 14 and 28 after exposure and a serological test on day 21 to verify the adequacy of the immune response. A rabies-specific antibody titer > 0,5 IU/ml measured by the rapid fluorescent focus inhibition test (RFFIT) has usually been considered as adequate. Many years of use and many studies have merely confirmed the effectiveness of this regimen.[iii]

In 2010 the Center of Disease Control (CDC) and the World Health Organization (WHO) published new recommendations for rPEP.[iv, v] These recommendations were to reduce the number of doses for rPEP from 5 to 4, and to abandon the serological test on day 21. These recommendations were not only influenced by conclusions from research studies, but also by recurrent shortages of rabies vaccine and the fact that many developing countries were encountering difficulties in performing reliable serological testing by RFFIT.

Taking into account these new guidelines, we conducted previously an investigation about the adequacy of the humoral response in patients who consulted our institution between 2005 and 2011 for a rPEP.[vi] This study showed that 6.7% (6/90) of these patients had an anti-rabies antibody titer < 0.5 IU/ml after 4 doses. All these patients had an adequate increase of their rabies antibody titer after receiving additional vaccine doses. In an editorial, Henry Wilde suggested that those patients, who have not responded adequately to 4 doses of rPEP, suffered probably from a non-recognized immunodeficiency. [vii]

To verify this hypothesis, we performed an immunological work up in rPEP non-responders. In addition we investigated the cellular immune response to rabies antigen in these subjects.

3. Methodology

3.1 Selection of subjects

All patients who had received a rPEP at the University Hospital of Lausanne between 2005 and 2014 and who had an anti-rabies antibody titers ≤ 0,5 IU/ml after 4 doses of vaccine were contacted to be included in the study (= non-responders).

Two comparator groups were made to perform the experimental immunologic tests. The positive control group included 9 patients who had received a rPEP at the University Hospital of Lausanne between 2005 and 2014 and who had after 4 doses of vaccine an anti-rabies antibody titers > 1 IU/ml. A negative control group included 9 healthy volunteers who had never received any rabies vaccine.

3.2 Evaluation of subjects

The non-responders were seen for a consultation, which included a complete anamnesis and a physical examination. The anamnesis was focused to detect an increased susceptibility to infections.

Blood samples were taken to perform the following investigations: full blood count, measurement of IgA, IgM and IgG levels, as well as the 4 subclasses of IgG, numeration of lymphocyte subpopulations (CD4 cells, CD8 cells, B cells and NK cells), and an HIV test.

Evaluations of the humoral response to tetanus, pneumococcal, and hepatitis B vaccinations were performed. An assessment of tetanus antibody levels was performed in patients who had received a dose of tetanus vaccine in the last 10 years. A tetanus antibody level < 0.1 U/ml was considered as abnormal. An assessment of

anti-HBs was performed in patients who had been vaccinated in the last 5 years, or 4 weeks after receiving a fourth dose of hepatitis B vaccine. Undetectable anti-HBs levels were considered as abnormal. Patients who had never received pneumococcal vaccine in the past were proposed to receive one dose of the 23-valent polysaccharide anti-pneumococcal vaccine. 4 weeks later, the level of antibodies against serotypes 9N, 11A, 14, 17F, 19F, 23F were assessed. An undetectable antibody level against more than 4 serotypes was considered abnormal (<0.3 mg/l)

3.3 Assessment of cellular immune response

Following up the protocol for production, concentration, and titration of B19G- and EnvA- pseudotyped rabies virus, developed by Nicholas R. Wall, [viii] we performed a Peripheral blood mononuclear cell (PBMC) proliferation assay to our three groups of subjects. Successful PBMC assays have been made with inactivated cell culture vaccines in previous published studies, [ix, x, xi] and because of its successful use in a previous immunologic experience,[xii] we chose to perform our PBMC proliferation assay using the Flury-LEP strain Purified chick embryo cell (PCEC) rabies vaccine (Rabipur®). PBMC (250 000 cells per well in triplicates) were cultivated for 6 days in presence of rabies vaccine particles and positive controls, a mitogen (phytohemagglutinin, PHA) or recall antigens, or unstimulated. Cultures were exposed to tritiated thymidine (1 μCi per well) for 20h at the end of the culture. Cells' DNA from each well was harvested on glass fiber plates and incorporated thymidine counted using a beta counter. Results were expressed as stimulation indices (SI = mean cpm in stimulated wells / mean cpm in unstimulated wells).

A series of preliminary PBMC proliferation experiments were performed for the settings of the stimulation with Rabipur and to assess the sensibility and the

sensitivity of the assay. First, PBMC from two very good responders selected from our positive control group (PC6 and PC7), with anti-rabies antibody titers of 22.2 and 16.4 IU/ml on the 21st day of their PEP schedule were cultivated in presence of a titration of the original Rabipur® single dose 2500 mIU/ml in its freshly reconstituted or defrosted form, starting from a dilution of 1:10, then 1:3, from 250 to 0.11 mIU/ml. Both subject had similar results with a peak response at a dose of Rabipur of 1:100 (25 mIU/ml) and a decrease of their response with decreasing doses. However, even at the lowest concentration of 1:10000, their responses were still significant and the non-stimulating dose could be estimated at $1.35.10^{-4}$ mIU/ml for those two subjects.

Proliferation of PBMC from 2 non-vaccinated control subjects and 2 selected non-responders was then performed to evaluate the specificity and sensibility of the stimulation with Rabipur. The dose responses obtained permitted to determine 3 doses of Rabipur, 25, 2.5 and 0.25 mIU/ml for the further experiments. At this stage, a good correlation between proliferation and antibody levels from the 6 subjects evaluated was obtained with a Pearson factor of = 0.990. Moreover, as the response in our experimental tests were similar with the fresh and defrosted Flury-LEP strain, we conducted all subsequent experiments with the defrosted Rabipur® strain, due to its availability.

To complete our investigations and be able to set a comparative immunologic response analysis, our subjects were also tested for their memory response, using a recall antigen preparation (Memory mix, MM) containing tetanus toxoid (TT), purified protein derivative (*PPD*) from *Mycobacterium tuberculosis* and Candida mannan antigens. In addition, their non-specific cellular immunity was tested using a phytohemagglutinine preparation (PHA) at 1 µg/ml.

4. Results

18 patients were identified with anti-rabies levels ≤ 0.5 IU/ml after 4 doses of rabies vaccine given as a post-exposure prophylaxis. 12 could be contacted and 9 subjects accepted to take part in the study. Demographic details of these subjects are summarized in table 1.

4.1 Medical history and clinical examination.

All rPEP non-responders except subject NR6 had unremarkable medical histories and physical examinations. Subject NR6 had been hospitalized several times between 2004 and 2008 for diarrhea of unknown etiology and weight loss. At the time she received her rPEP in 2008, she was undernourished with a BMI of 17 kg/m^2. In addition she had an anemia and an iron deficiency. Her current clinical examination was normal and she was in a good general condition. Her BMI was 18.5 kg/m^2.

4.2 rPEP schedule details

No subject had received rabies pre-exposure prophylaxis. All subjects had received a human rabies immunoglobulin (HRIG) after exposure. The NR3 and NR8 started their rPEP schedule outside of Switzerland. All subjects except NR3 received the Essen regimen for their rPEP. The NR3 received 4 injections following the Thai Red Cross Society regimen that consists in 2-site intradermal injections on days 0, 3, and 7 and 1 intradermal injection on days 28 and 90. The NR6 with the past history of malnourishment required 5 injections to achieve an acceptable rabies antibody titer (2.5 IU/ml). On day 187, her rabies antibody titer fell to 0.3 IU/ml and she received

two additional vaccine doses. On day 353, she had a rabies antibody titer of 5.4 IU/ml.

4.3 Paraclinical investigations

All subjects had a normal full blood count and a negative HIV test. Results of the PBMC counts and immunoglobulin levels are shown in Table 2. Values are normal in all our subjects except for the CD8 T cell numeration for the NR9 and the IgG3 level for 5 subjects. These results do not suggest any immunodeficiency for any of the subjects. The humoral response to other vaccines is summarized in Table 3. The results show a good response to the tetanus vaccine (antibody titer > 0.1 IU/ml) in all subjects. Five subjects had received in the past a hepatitis B vaccination. Three subjects were tested for their response to the hepatitis B vaccine and the anti-HBs levels were detectable for 2 of them. Three subjects accepted to receive a pneumococcal vaccination. Unfortunately, further tests showed that those subjects had already an adequate antibody level to at least 4 serotypes before the vaccination. This element made the evaluation of their response to the pneumococcal vaccination non interpretable. However, we could observe a significant improvement of their antibody titers after the vaccination.

4.4 Assessment of cellular immune response

Results of the PBMC proliferation assay stimulated with MM (memorix mix antigen including TT, PPD and candida mannan) and PHA (phytohemagglutinine) showed no significant difference between the subjects and the 2 control groups.

The results of the lymphocyte proliferation assays done with the 3 different concentrations of rabies antigens are presented in figure 1. The results show the

absence of lymphocyte proliferation in the negative control group. The response of subject NC2 can be interpreted as an outlier on the basis of the Grubb test. The lymphocyte proliferation after stimulation with 2.5 and 25 mIU/ml was significantly higher in the non-responders than in the negative control group, the Z score obtained with a Mann Whitney test at the doses 2.5 and 25 were at -3.0741 (U=3, p =0.00214) and -2.5861 (U=8, p=0.0096) respectively. Finally the positive control group had a significantly better response than the non-responder group at the dose of 25 [mIU/ml] with a Z-Score obtained with a Mann Whitney test at 2.0252 (U=10, p=0.04236).

The NR6 subject mentioned above with a significant clinical history of past digestive problems and malnutrition had a particularly poor response in this lymphocyte proliferation assay. The non-responders NR8 and NR9 could be included in the study before reaching an adequate antibody response, and samples of PBMC could be obtained before and after receiving additional vaccine doses. The results of the lymphocyte proliferation assay of these 2 subjects are presented in figure 2. The results show a significant increase of the lymphocyte proliferation for the subject NR8 only after the 5^{th} vaccine dose. The rabies antibody level value measured at the second PBMC sample increased significantly also only for the subject NR9 after the 5^{th} vaccine dose (NR8 = 1.0 IU/ml, NR9 = 7.0 IU/ml).

A correlation could be established between the cellular response in the lymphocyte proliferation assay and the anti-rabies antibody levels (Figure 3) with a correlation coefficient r of 0.7899 (p = 0.000774).

5. Discussion and conclusion

In 2010 the WHO and the CDC recommended for rPEP to reduce the number of vaccine doses from 5 to 4 doses and to abandon the serological verification on day 21 after the beginning of the vaccination schedule. A previous study of our group showed however that 6,7% of potentially rabies exposed subjects did not develop an appropriate antibody level after 4 doses of rPEP.

In this study we verified if such subjects with poor humoral response to a rPEP had any evidence of an immunodeficiency. We evaluated our subjects very thoroughly by performing an in depth anamnesis, a clinical examination and the laboratory investigations which are usually recommended for the workup of a suspected immunodeficiency.[xiii] Our results show that 8/9 of our rPEP non-responders were most probably fully immunocompetent. Only one subject was possibly immunodeficient at the time of the rPEP administration, because of a history of significant malnutrition. Physical examination, numeration of immune cells and assessment of immunoglobulin levels were however normal in all subjects, except for the CD8 T-cell numeration for the NR9 and the IgG3 level for 5 subjects. In addition we didn't identify any abnormal response to other vaccines, such as the tetanus, hepatitis B and pneumococcal vaccines.

Then we investigated the cellular immune response to rabies antigen. It could indeed have been postulated that patients with poor humoral response develop possibly a strong cellular immune response, which could have been protective. Our results show however a correlation between the level of the humoral and the cellular immune response.

Our results suggest that a 4 dose intramuscular rPEP elicits in some patients a relatively poor humoral and cellular response, even in the absence of any sign of immunosuppression. Cases of rPEP failures with subsequent deaths have been reported in other publications. [xiv, xv] These facts legitimate some interrogations about the rPEP schedules currently in use. It is indeed of concern that some rabies-exposed subjects are not fully protected with the currently WHO-recommended prophylaxis, considering that rabies is an invariably letal disease.

The currently WHO-recommended prophylaxis has been adopted since 2010 and followed a complete literature review conducted by the Advisory Committee on Immunization Practices (ACIP) from the CDC.[xvi] This meta-analysis has included, inter alia, studies covering: human rPEP evaluation (11), animal rPEP evaluation (7), animal rabies pre expositional prophylaxis (8), Human pre expositional immunogenicity (12) and human post expositional immunogenicity (4). Those 4 last studies identified are the most related to our study because they documented the antibody response following a rPEP administration. Only one of them was a large-scale study that included 242 healthy veterinary students who mostly received an anti-rabies vaccine that has never been marketed.[xvii] Among other studies included, only three had been carried out in real-life conditions and using the Essen regimen.[iii xviii] The third one was a pediatric study. [xix]

For the reasons set above, further real life condition studies about rabies post expositional immunogenicity should be considered to conclude on the most appropriate rPEP schedule. It could also be interesting to evaluate in details the humoral and cellular immune responses after intradermic rPEP, which is currently in use in many developing countries and which permits to use smaller amount of vaccine for rPEP.

In conclusion, our study suggests that in addition to the 4 vaccine doses on day 0, 3, 7, 14 recommended by the WHO, it is useful to do a serologic test on day 21 to identify patients who didn't respond appropriately. Those non-responders should receive additional vaccine doses, as it seems that they might not be protected neither by their humoral response, nor the cellular immune response.

6. Tables

Table 1 : Demographic details of the subjects

Subjects	Sex	Year of Birth	Vaccination (dose, brand, lot no)				Days of vaccination	Serology		Highest AB titer measured [IU/ml], after X no of vaccine doses
			1st	2nd	3rd	4th		Day #	AB Titer IU/ml	
NR1	m	1952	Rabipur, 397011A	Rabipur, 397011A	Rabipur, 397011A	Rabipur, 397011A	D0, D3, D7, D15	22	0.2	2.7, 6 doses
NR2	f	1947	Merieux, unknown	Merieux, H1341-4	Merieux, H1287-4	Merieux, H1267-4	D0, D3, D7, D14	21	0.4	5.2, 5 doses
NR3	m	1960	Rabipur*, unknown	Rabipur*, unknown	Rabipur*, unknown	Rabipur*, unknown	D0, D0, D3, D3, D7	30	0.5	0.5, 5 doses
NR4	m	1987	Rabipur, CMA	Merieux, unknown	Merieux, B0001-9	Merieux, B0001-9	D0, D3, D7, D14	23	0.5	0.5, 4 doses
NR5	f	1984	Merieux, PMU	Merieux, G1510-4	Merieux, unknown	Merieux, H1287-4	D0, D3, D7, D129	26	0.3	1.0, 5 doses
NR6	f	1984	Rabipur, CMA	Rabipur, CVMV	Rabipur, CVMV	Rabipur, 397011A	D0, D3, D8, D15	22	0.3	5.4, 7 doses
NR7	f	1974	Rabipur, 359011C	Rabipur, 378011A	Rabipur, CMA	Rabipur, CMA	D0, D3, D7, D14	21	0.3	9.9, 5 doses
NR8	m	1948	Rabipur*, unknown	Rabipur, 533011A	Merieux, K1323-1	Merieux, K1323-1	D0, D3, D7, D14	21	0.2	7.0, 7 doses
NR9	m	1959	Rabipur CMA B	Rabipur CVMV	Rabipur CVMV	Rabipur, 541011C	D0, D3, D8, D15	22	0.2	1.0, 5 doses

***Vaccine doses received abroad; # count starting on the day of the first dose received**

Table 2 Part 1 : Numeration of Peripheral blood mononuclear cells of the subjects

	T cell		CD4 T-cell		CD8 T-cell		Monocyte		B cell		NK cell	
Normal values	780-2240		490-1640		170-880		200-800		80-490		80-690	
Subjects	cell/mm3	%	cell/mm3	%	cell/mm3	%	cell/mm3	%	cell/mm3	%	cell/mm3	**%**
NR1	915	45.5	654	32.5	285	14.2	509	25.3	101	5	432	21.5
NR2	1078	54.5	800	40.4	247	12.5	448	22.6	224	11.3	137	6.9
NR3	1601	59.9	937	35	641	24	364	13.6	290	10.9	383	14.3
NR4	1563	64.3	886	36.4	611	25.1	288	11.8	321	13.2	185	7.6
NR5	1295	63.9	951	46.9	320	15.8	323	15.9	203	10	150	7.4
NR6	1274	67.6	840	44.6	402	21.3	323	17.1	136	7.2	116	6.2
NR7	1237	56.7	674	30.9	476	21.8	461	21.1	159	7.3	271	12.4
NR8	1158	53.4	775	35.7	397	18.3	330	15.2	247	11.4	381	17.6
NR9	**617**	32.7	495	26.2	**104**	5.5	822	43.5	389	15.3	**54**	2.8

Table 2 Part 2 : Numeration of leucocyte subpopulations

	Neutrophiles		Lymphocytes		Monocytes		Eosinophiles		Basophiles		Immature granulocytes	
Normal values	1.8-7.5	40-75	1.5-4.0	25-40	0.2-0.8	2-8	0.05-0.3	1-5	0.01-0.05	0-1		
Subjects	G/l	%	G/l	%	G/l	%	G/l	%	G/l	%	G/l	%
NR1	4.26	67	1.39	22	0.58	9	0.06	1	0.07	1	0.01	0.2
NR2	2.67	54	1.56	31	0.49	10	0.19	4	0.07	1	0.01	0.2
NR3	2.82	47	2.42	40	0.55	9	0.15	3	0.04	1	0.02	0.3
NR4	2.89	50	2.18	38	0.52	9	0.14	2	0.01	0	0.01	0.2
NR5	3.45	62	1.67	30	0.33	6	0.11	2	0.02	0	0.01	0.2
NR6	3.64	65	1.49	27	0.34	6	0.07	1	0.05	1	0.03	0.5
NR7	3.25	59	1.73	31	0.52	9	0.03	1	0.01	0	0.01	0.2
NR8	3	53	1.9	34	0.46	8	0.27	5	0.02	0	0.01	0.2
NR9	6.22	78	**0.93**	**12**	0.71	9	0.1	1	0.01	0	0.01	0.1

Table 2 Part 3 : Immunoglobulin levels							
	IGG	**IGG1**	**IGG2**	**IGG3**	**IGG4**	**IGA**	**IGM**
Normal values	7-14.5	5.2-12.7	1.43-5.6	0.28-1.05	0.011-1.04	0.71-4.07	0.34-2.41
Subjects	g/l	g/l	g/l	g/l	g/l	g/l	g/l
NR1	12.2	8.78	3.46	**0.21**	0.423	1.07	1.14
NR2	10.1	5.04	4.6	**0.22**	0.941	0.87	2.66
NR3	10.3	5.27	4.54	0.55	0.784	3.2	0.79
NR4	11.4	7.25	5.82	0.66	1.04	1.43	1.59
NR5	9.05	5.19	3.38	0.35	0.04	1	0.88
NR6	8.79	5.71	2.53	**0.24**	0.144	1.26	1.76
NR7	11	5.75	4.87	0.38	0.347	2.16	1.05
NR8	10.9	8.02	2.33	**0.27**	0.056	2.08	0.89
NR9	10.9	7.24	2.54	**0.27**	0.352	2.15	0.85

Table 3 : Antibody response to other vaccines

	Hepatitis B vaccination			Tetanic vaccination		Pneumococcal #	
Subjects	**Time since baseline vaccination**	**Additional vaccine dose given at time of study**	**Anti HBs* [mIU/ml]**	**Time since baseline vaccination**	**Tetanus AB level· [U/ml]**	**Number of serotypes ¢**	
						pre vaccination	**post vaccination**
NR1	>5 years	No	NT	15 years	1.88	NT	NT
NR2	Never done	No	NT	2 years	2.58	4	6
NR3	14 years	No	NT	7 years	2.52	6	6
NR4	5 years	No	>10	3 months	1.39	NT	NT
NR5	>5 years	Yes	NT	4 years	1.45	NT	NT
NR6	14 years	No	<10	7 years	1.18	NT	NT
NR7	6 years	No	2893	11 years	2.12	NT	NT
NR8	Unknown	No	NT	Unknown	2.65	5	5
NR9	Never done	No	NT	1 month	2.14	NT	NT

*** Undetectable anti-HBs levels were considered as abnormal · A tetanus antibody level < 0.1 U/ml was considered as abnormal # in our subjects who accepted to receive a pneumocoque vaccination, a 23 valent Polysaccharide pneumococcal vaccination has been administred and 6 serotypes have been tested the day they joined the study and 4 weeks after their injection. ¢ A serotype >0.3 [mg/l] is considered as accurate NT = non tested**

7. Figures

Figure 1 : Results of the Peripheral blood mononuclear (PBMC) proliferation assays. PBMC were stimulated with of the Flury-LEP vaccine strain (Rabipur, Novartis) at concentrations of 0.25, 2.5, 25 mIU/ml.

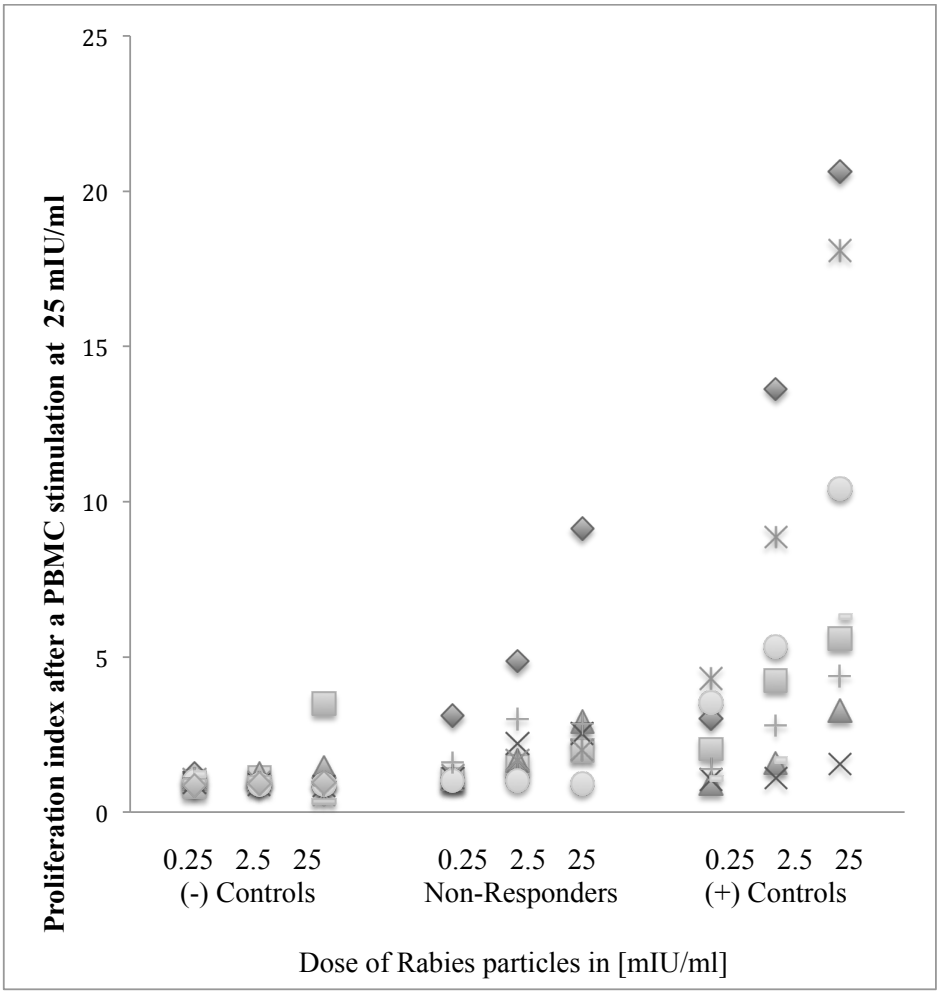


Figure 2 : Results of the Peripheral blood mononuclear (PBMC) proliferation assays of the non-responders NR8 and NR9. A PBMC proliferation assays was performed on these 2 subjects before they reached an antibody level of 0,5 IU/ml (left) and after they had received an additional dose of rabies vaccine and had reached an antibody level >0,5 IU/l (right). Results show a significant increase of the PBMC proliferation for subject NR8 only. This result can be explained by the fact that the rabies antibody level increased only for the subject NR8 : NR8 = 7.0 [IU/ml], NR9 = 1.0 [IU/ml].

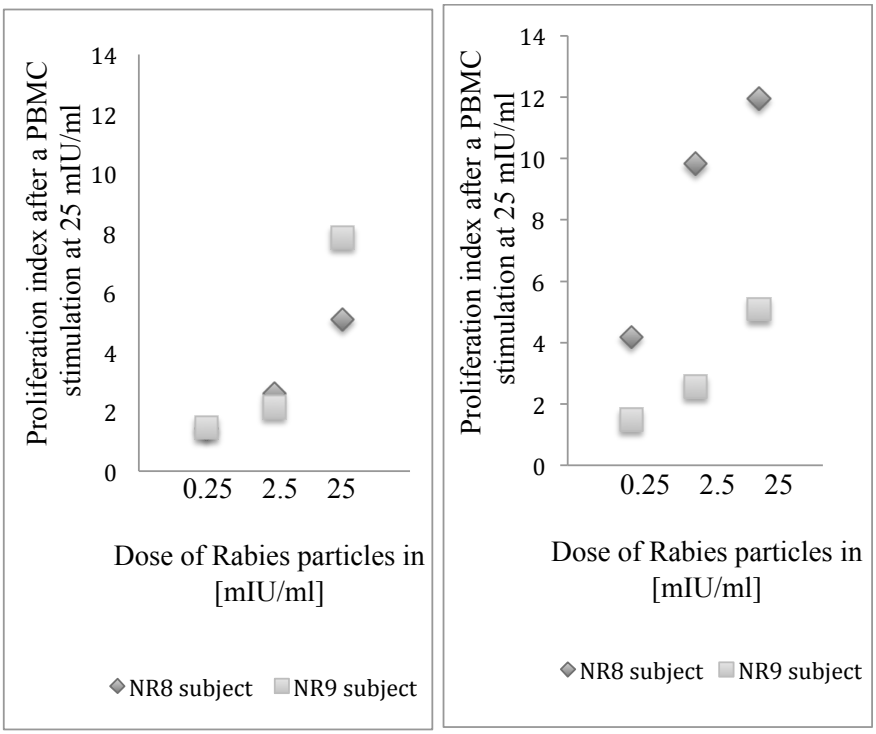
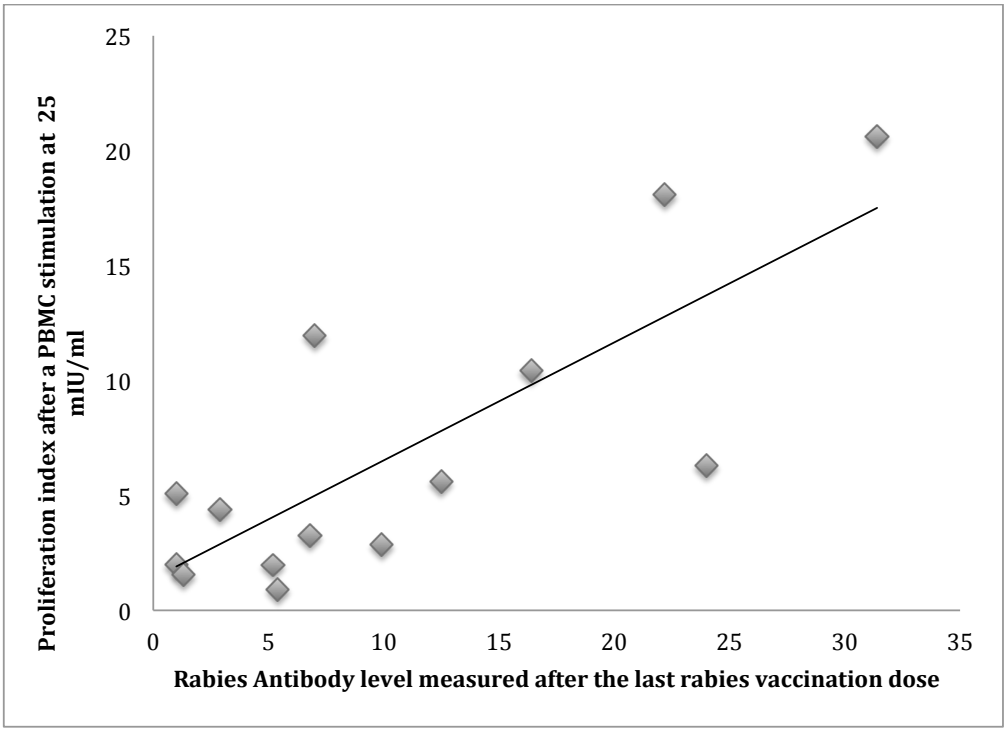


Figure 3 : Correlation between rabies antibody levels and PBMC proliferation index.

The results show a significant linear correlation (R = 0.79 by the Pearson test)-



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