Slov Vet Res 2006; 43 (3): 119-25
UDC 616.988.21:616-097:614.47

Original Research Paper

LONGEVITY OF LASTING SPECIFIC IMMUNITY AFTER PRIMARY VACCINATION AGAINST RABIES – COMPARISON OF ELISA AND FAVN TESTS

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Summary: The purpose of the study was to monitor the efficacy of primary vaccination against rabies and the need for booster doses. These studies validate at the same time the recent technological improvements in laboratory diagnostics of the level of rabies protection in human sera. Study was done at the level of antibodies considering that an antibody titer ≥ 0.5 IU/ml is protective. We used Platelia rabies ELISA kit (BIO-RAD Laboratories) for the detection of rabies anti-glycoprotein antibodies in 41 human sera of previously healthy veterinarian students. Neutralising rabies antibodies were measured simultaneously by fluorescent antibody virus neutralization (FAVN) test as well. Subjects entering the study have received 2 to 8 years prior rabies treatment with human diploid cell vaccine (HDCV, Rabivac, Chiron Germany) according to schedule: one vaccine on 1, 7, 21 and 365 day. Mean level of rabies antibody detected by ELISA was 19.6 EU/ml (SD 18.8 minimum 1 maximum 56). Results were higher in the groups vaccinated recently. Nobody had titer ≤ 0.5 IU/ml either in ELISA or in FAVN test. In the FAVN test, the average titers were higher and reached 54.4 IU/ml (SD 44.3 minimum 0.7 maximum 152.5). An immune-complex-like reaction occurring after administration of the booster doses of rabies vaccine is one of the reasons to reconsideration of the needs for administration of booster rabies vaccines. At the same time, the need for mass protection of subjects exposed to rabies virus professionally is existing worldwide . The results of these studies indicate that HDCV is highly immunogenic in both FAVN test and ELISA tests. High level of protection is lasting in human sera for at least 8 years. Average levels of detected rabies antibodies were lower in ELISA in comparison with FAVN test. Correlation between two tests was found.

Key words: rabies; rabies vaccines – drug effects – pharmacology; drug evaluation, serodiagnosis – methods; antibodies, viral – analysis; enzyme-linked immunoassay; fluorescent antibody technique; comparative study

Introduction

Published data on the longevity of lasting specific immunity after primary vaccination against rabies have approved the fact that neutralising antibody level after vaccination is protective for at least two upcoming years (1). According to the recommendation of World Health Organization and Centre for Disease Control, sufficient level of rabies neutralising antibody appointed at 0.5 IU/ml (2, 3). For persons continuously professionally exposed to rabies virus, it is recommended to have a serum sample tested for rabies antibodies between 6 months and two years. Intervals for testing depend on the level of exposure. In Slovenia, rabies researchers need to be laboratory tested every 12 months, employees for rabies diagnostics laboratory once a year, veterinarians every two years. If the titers proved by the rapid fluorescent focus inhibition test (RFFIT) are below 0.5 IU/ml, booster vaccination with one vaccination dose is indicated (2). Pre-exposure immunization for rabies is necessary for spelunkers, bat researchers, animal control and wildlife workers in rabies epiz-

Received: 30 September 2005
Accepted for publication: 15 July 2006

ootic areas, veterinary students as well as travelers visiting areas where rabies is enzootic and where immediate access to appropriate medical care, including biologicals, is limited (4).

Several serological tests for the detection of rabies virus neutralization antibodies have been described. The first test was a mouse neutralization test (MNT) developed by Webster et al. (5). The most commonly used technique for detection of protective level of rabies antibodies in sera of animals and humans is the rapid fluorescent focus inhibition test (RFFIT) developed in 1973 (6). Another cell culture based technique – the fluorescent antibody virus neutralization (FAVN) test has been shown to be more specific than the RFFIT test (7). The FAVN test is based on neutralization of rabies virus using cell culture. The reading and interpretation of results are less subjective than RFFIT, because use an "all or nothing" method of reading. Several indirect ELISA tests (Enzyme-Linked Immunosorbent Assay) that incorporate rabies glycoprotein/anti-human immunoglobulin/enzyme conjugates have been described for human (8) and animal post vaccination rabies antibodies titrations (9). The Platelia Rage kit incorporates protein A but has rabies virus glycoprotein as the coating antigen.

The aim of our study was to detect level and duration of rabies antibody in the sera of pre-exposure treated persons and to compare two laboratory tests for that purposes – FAVN and ELISA tests.

Material and methods

The immunogenity of a human diploid cell vaccine (HDCV) was evaluated using veterinary medical students. 41 healthy adults were enrolled in our trial. A person was excluded from enrolment if he/she had a previous history of additional rabies vaccination, had a history of any immunosuppresive disease or chronic disorders, oral or parenteral immunosuppressive therapy.

Mean age of subjects at the time of entering the study (year 2004) was 25.3 (SD 2.6 median 25 minimum 22 maximum 33). At the beginning of the vaccination, subjects were between 20 and 29 years (mean 21.1 SD 1.4) old. We have tested 12 men and 29 female.

Four 1.0 ml injections of human diploid cell vaccines HDCV (Rabivac, Chiron Vaccines, Germany) were administered intramuscularly in deltoid area on days 1, 8, 22 and 365 in the course of pre-exposure treatment: 16 persons have received this pre-exposure treatment against rabies 2 years before blood sampling, 9 persons 4 years before, 4 persons 5 years before, 5 persons 6 years before and 7 persons 8 years before blood sampling. The Human Research Board at Ministry of Health of Republic Slovenia approved the study protocols and informed consent forms signed by all subjects.

Blood samples were collected from subjects and sent to laboratory for testing rabies virus antibodies after one to two months after last administration of vaccine. After the collection of blood sample it was centrifuged for 10 min at 1.000 g, aliquoted and stored at –20 +/- 2°C. Rabies virus neutralizing antibody levels were measured in human sera using the Fluorescent Antibody Virus Neutralization (FAVN) (7) in Department of Virology at Veterinary Faculty of University of Ljubljana. All sera were heat inactivated (for 30 min at 56°C) and analysed for the presence of rabies virus antibodies with fluorescent antibody virus neutralisation (FAVN) test. The method was described by Cliquet and co-workers (7). Briefly, the serial three-fold dilutions of serum samples were prepared in duplicate in minimum essential medium (MEM, Gibco, Paisley, UK) and were placed on a 96-well microplate. A challenge virus strain (CVS, obtained from OIE/WHO, Nuncy, France) in titre 30-200 TCID50/0.1 ml was added in each well. After incubation for 60 min at 37°C BHK cells were added. The cells were fixed after 48 hours of incubation with cold acetone (stored at –20°C) and stained with anti-rabies fluorescent conjugate (Sanofi Diagnostic Pasteur, Marnes-la-Coquette, France). The highest neutralising antibody titre of the serum samples was considered the dilution, which completely blocked CVS propagation. The OIE/WHO reference positive serum with known neutralising antibody titre (0.5 IU/ml), negative serum controls, virus and cell controls were also included in the tests. The neutralising antibody titres obtained in sera were transformed into International Units/ml (IU/ml). Geometric mean titre (GMT) was determined by software Excel.

The same samples were tested in ELISA test in Blood Transfusion Center of Slovenia. The test assay was the PLATELIA ELISA (BIO-RAD, Marnes-la-Coquette, France). PLATELIA rabies kit is an immunoenzymatic technique for the detection of rabies virus anti-glycoprotein antibodies in human serum and plasma.

Subjects were considered protected against rabies virus infection if they achieved a FAVN test or ELISA titers of ≥ 0.5 IU/ml. FAVN test, measuring

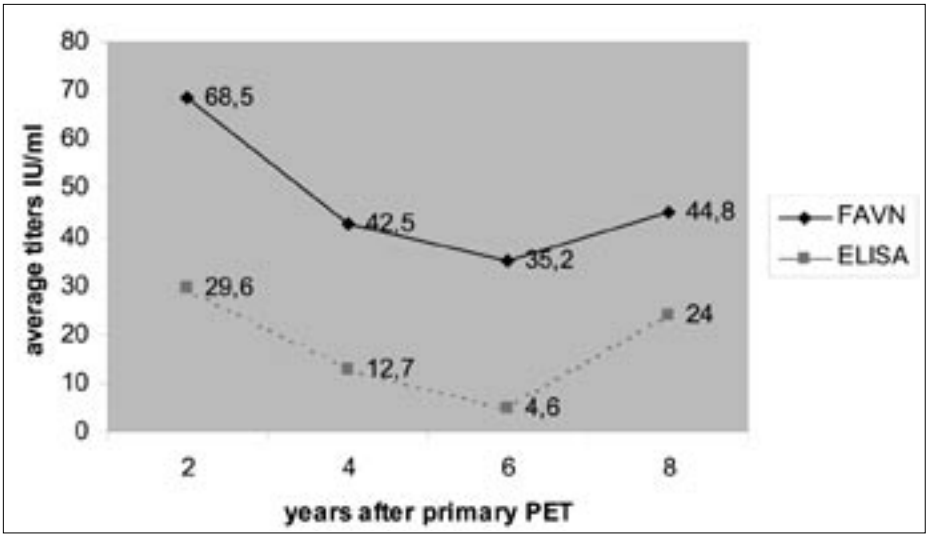

Figure 1: Average level of rabies antibodies in ELISA and FAVN tests – years after the primary pre-exposure vaccination

neutralizing antibodies, was used as the reference test.

All statistical analyses were done before the code was broken. Statistical analyses were performed using the SPSS System for Windows version.

Results

100 % of the subjects in each tested groups had post-vaccination rabies antibodies titers > 0.5 IU/ml in both FAVN and ELISA tested methods. For surveillance of exposed subjects, WHO (2) considers that high levels of rabies antibody in RFFIT protect subjects exposed to risks of rabies. Results of these studies indicate that HDCV administered intramuscularly to healthy adults previously vaccinated against rabies 2 to 8 years before the study and according to schedule 1, 7, 21 and 365 is excellently immunogenic for both glycoprotein and neutralizing antibodies synthesis. High levels of antibodies were detected in both tests (see table 1). Correlation between values of titers measured in ELISA and FAVN tests using Spearman's Correlation Coefficient was significant at the 0.01 level (2-tailed), indicating correspondence of results in both tests.

Absolute values in each group (FAVN and ELISA) were more or less different. Table 1 and Figure 1 show that average means of antibodies levels were usually higher in the groups vaccinated recently and with the FAVN test. Comparison in the average level of antibodies in years after primary pre-exposure vaccination revealed that FAVN and ELISA tests were equally able to detect decrease of level of antibodies with years after the start of vaccination. Increasing levels of rabies antibodies in both tests 8 years after primary vaccination were observed in persons who were eventually already professionally exposed as veterinarians to rabies virus.

HDCV vaccine met the immunogenicity goal of producing rabies virus neutralization and glycoprotein antibodies titers. Comparative study showed that all subjects had neutralizing as well as anti-glycoprotein antibodies levels well above the satisfying level even 8 years after the start of the pre-exposure treatment.

Discusion

We suppose that different kinds of rabies vaccines and different vaccination schedules have influence on the achieved level and the duration of protective antibody levels against rabies. The laboratory assays used for rabies antibody titration and type of rabies antibodies are important as well. Different laboratory assays are referenced for clinical decisions of many infectious diseases. Interpretation of them is sometimes a difficult task. In our study, rabies antibody response were assessed by two laboratory assays (ELISA and FAVN test) High titers were obtained with both test therefore there is any concern about protective levels of rabies pre-exposure treatment.

In our study we have commonly detected much higher protective level of anti-rabies antibodies as officially recognized as lower protective level by WHO (2) and Center for Diseases Control Atlanta (3). Tokoyama and his coworkers published similar observations several years ago (10).

Table 1: Results of rabies neutralising antibodies in 41 persons according to FAVN test and ELISA

Individuals	Number of years between the first vaccinations and blood sampling	Test FAVN		Test ELISA	
		(IU/ml)	Mean per group IU/ml)	(EU/ml)	Mean per group IU/ml)
1	2	15,5		6	
2	2	33,5		7	
3	2	19,4		3	
4	2	48,5		17	
5	2	41,3		12	
6	2	52,5		34	
7	2	79,4		56	
8	2	113,7		6	
9	2	43,1		65	
10	2	64,1		9	
11	2	113,7		15	
12	2	152,0		18	
13	2	111,5		52	
14	2	105,6		13	
15	2	121,4		2	
16	2	1,8	69,8	19	20,8
17	4	27,1		4	
18	4	123,2		52	
19	4	132,8		7	
20	4	5,4		30	
21	4	110,5		8	
22	4	115,6		44	
23	4	7,5		2	
24	4	42,4		15	
25	4	48,2	68,0	9	19,0
26	5	27,2		19	
27	5	62,1		5	
28	5	2,1		7	
29	5	64,1	38,8	9	10,0
30	6	60,8		18	
31	6	42,1		44	
32	6	22,3		15	
33	6	46,8		56	
34	6	108,5	56,1	56	37,8
35	8	4,15		5	
36	8	30,0		52	
37	8	42,3		16	
38	8	15,6		8	
39	8	13,0		7	
40	8	7,1		5	
41	8	15,9	18,3	8	14,4

Pre-exposure vaccination against rabies is usually performed with HDCV, a human diploid cell vaccine PCEC, a purified chick embryo cell vaccine; a RVA, rabies vaccine adsorbed (2). One year apart from the start of the treatment four dose HDCV rabies vaccine schedule used in our study, is recommended by vaccine producer of vaccine and some authors (11, 12, 13) as one of possible schedule for pre-exposure treatment. Existence of individual immune reactions following rabies vaccination, as for other vaccina-

tions is obvious and non-responders could be expected after rabies vaccination as well. Therefore clinical decisions should be made individually from case to case. Average level of protection is interesting more for research purposes. After accidental exposition of vaccines to the rabies virus, an additional dose of rabies vaccine is highly recommended in spite of probably high level of neutralizing antibodies.

Further studies are necessary to give evidence if high level of achieved protection with HDCV and with presented vaccination pre-exposure schedules is connected with even more than 8 years lasting immunity.

We suppose that fourth dose in pre-exposure schedule is definitely not obligated, but if a fourth dose is given as booster after one year from the start of vaccination, substantially prolongation of the protection could be expected. People at continued risk for rabies exposure should consider acceptance of the presented regimen and regular boosting with rabies vaccine as well.

Serological testing may be the useful way for reducing the number of rabies vaccine doses in the course of booster. Enhanced surveillance of the necessity for the start of pre-exposure protection and booster is advisable. In this way, the total number of professionally exposed persons to treat, who regularly need boosters, could be reduced and undesirable side effects of vaccinations as well. We predict that the use of pre-exposure rabies vaccination could in such a way even increase the demand for pre-exposure preventive treatment. In this way the total protection of professionally exposed subjects would be posed on higher level. An immune-complex-like reaction occurs after administration of the booster doses of HDCV (13). Local reactions (14) and systemic hypersensitivity reactions after booster vaccinations with HDCV (15) have been reported as well. These reactions are the additional reason so take into consideration very carefully when establishing the need for administration of booster rabies vaccines. However, rabies treatment save lives and it should be accepted as compulsory.

PLATELIA rabies kit is an immunoenzymatic technique for the detection of rabies virus antibodies in serum or plasma of human and several animal species. Some authors have used ELISA tests for detection of rabies virus antibodies in human sera (8). This way it can be used for monitoring the efficiency of vaccine testing on laboratory and field animals, and also as a research tool for monitoring the antibody titer of vaccinated subjects. The present study demonstrates that ELISA method (Platelia kit) provides very high level of detection and that correlation exists between ELISA and FAVN test results. Correlation between neutralization assay and ELISA and correlation with the FAVN test was shown in recently published study of Arai et al. (16) with higher neutralisation than ELISA titers for most samples. Results of this study are in accordance with the results revealed in our study.

Cliquet and co-workers (9) have provided another ELISA with lower sensitivity than the FAVN test. It is a useful tool for rapidly screening serum samples (retesting of ELISA negative results by a reference technique is recommended by OIE) from vaccinated companion animals and the ELISA compared favorably with data generated using the FAVN test. The major advantages of the ELISA test are that it can be completed in several hours, does not require the use of live virus and can be performed without the need for specialized laboratory containment. This is in contrasts with several days needed for conventional rabies antibody virus neutralization assays. According to the authors, ELISA assay would be a valuable screening tool for the detection of rabies antibodies in vaccinated domestic animals in combination with other prescribed serological tests. We propose that the same consideration could be accepted for human sera as well. Comparison with RFFIT or equally worthwile FAVN (17) will be very interesting.

Prevention of diseases in professionally exposed persons is one of the priorities in public health sector (18, 19). According to our opinions and opinions of some other authors (20), monitoring the titers of antibodies with consistent and validation laboratory assays could be a useful contemporary method for making decisions for the purpose to give booster or not in the professionally exposed persons to rabies. Similarly, it could be the best way to allow a decrease in the number of boosters in the cases of long lasting professional career.

Acknowledgement

The authors are indebted to all volunteers who participated in this study and staff of each site for they excellent assistance. We are grateful also to Metka Paragi from Institute of Public Health of Republic Slovenia, to Jerica Tovornik and Eva Grilc from Institute of Public Health of Ljubljana for technical assistance and support in our research.

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TRAJANJE SPECIFIČNE IMUNOSTI PO PRIMARNEM CEPLJENJU PROTI STEKLINI – PRIMERJAVA TESTOV ELISA IN FAVN

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Povzetek: Namen študije je bil opraviti kontrolo uspešnosti primarnega cepljenja proti steklini in dobiti odgovor na vprašanje ali je morebiti potrebno še dodatno cepljenje. Istočasno smo želeli preveriti laboratorijske izboljšave pri metodah določanja stopnje zaščitnih protiteles proti virusu stekline v serumih ljudi. Študija je izhajali iz podatka, da titer protiteles enak ali višji od 0,5 IU/ml, ščiti človeka pred steklino.V serumu 41 študentov veterine smo v testu ELISA, komplet Platelia (BIO-RAD Laboratories), določali protitelesa proti glikoproteinu virusa stekline. Hkrati smo nivo protiteles določali tudi v seronevtralizacijskem testu z imunofluorescenco (test FAVN). Študenti so bili cepljeni 2 do 8 let pred odvzemom krvnega vzorca s cepivom proti steklini (HDCV, Rabivac, Chiron Germany) po shemi: ena doza 1., 7., 21. in 365. dan. Povprečni titer protiteles, ugotovljen v testu ELISA, je znašal 19,6 EU/ml (SD 18,8 minimum 1 maximum 56). Višje titre smo ugotovili pri skupini, ki je bila cepljena pred kratkim. Nihče ni imel titra protiteles nižjega od 0,5 IU/ml. V testu FAVN smo dobili nekoliko višji titer protiteles, saj je znašal 54,4 IU/ml (SD 44,3, minimum 0,7, maksimum152,5). Nivo protiteles proti virusu stekline v serumu pacienta je uporaben kazalnik potrebe po izvedbi revakcinacije. Hkrati ugotavjamo, da je upravičena široka zaščita proti steklini tistih oseb, ki so poklicno izpostavljene večji možnosti okužbe z virusom stekline. Rezultati te študije kažejo, tako v testu ELISA kot v testu FAVN, da daje cepivo HDCV zadovoljivo zaščito. Ugotovili smo visok titer protiteles še 8 let po izvedbi osnovnega cepljenja. V testu ELISA smo v povprečju ugotovili nižji titer protiteles kot v testu FAVN.

Ključne besede: steklina; steklina, cepiva – učinki zdravil – farmakologija; zdravilo, ocena; serodiagnostika – metode; protitelesa, virusna – analize; ELISA; imunofluorescentna tehnika; primerjalna študija