AN ELISA SUITABLE FOR THE DETECTION OF RABIES VIRUS ANTIBODIES IN SERUM SAMPLES FROM HUMAN VACCINATED WITH EITHER CELL-CULTURE VACCINE OR SUCKLING-MOUSE-BRAIN VACCINE

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SUMMARY

An indirect ELISA for determination of post-vaccination rabies antibody was applied. Purified rabies virus was used as antigen to coat plates, and staphylococcal protein A linked with horseradish peroxidase was used for detecting IgG antibody in human sera. Sera from humans, vaccinated with cell-culture vaccine or suckling-mouse-brain vaccine, were examined. ELISA results were compared to those obtained from the virus neutralization test. The mean and standard deviation of OD were determined for 126 negative sera (pre-vaccination) and for 73 sera from vaccinated persons showing antibody titers lower than 0.5 IU/ml. Results were defined as ELISA -positive, -negative or –doubtful. Establishment of a doubtful region reduced the number of sera otherwise classified as positive (false-positive sera). In this way, the sensitivity, specificity and agreement values were respectively 87.5%, 92.4% and 88.5%. No significant differences were observed in these values when the group vaccinated with cell-culture vaccine and the group vaccinated with suckling-mouse-brain vaccine were compared. It was shown that much of the disagreement between the values obtained by neutralization test and ELISA occurred in sera obtained at the beginning of the immunization process, and was probably due to the presence of IgM in the serum samples, detected only by the former test. This ELISA method can be used as a screening test in rabies laboratories regardless of the kind of vaccine used for immunization.

KEYWORDS: Rabies; Virus antibodies; ELISA; Neutralization; Suckling mouse brain vaccine.

INTRODUCTION

The rabies vaccine most widely used in Brazil is prepared from suckling mouse brain (SMB) tissue[5]. Although it has a lower potency than cell-culture vaccines, it is less expensive. The vaccine batches for human use must have a minimum relative potency, as determined by the NIH test[12] of 1.0 IU per dose, contrasting to that of at least 2.5 IU required for cell-culture vaccines. Because of its lower antigenic content, an increased number of doses for post-exposure treatment with SMB vaccine is generally administered. It is also reported that brain tissue vaccines are not completely free of adverse neurological effects[6]. These facts stress the importance of the recommendation that people receiving anti-rabies treatment with SMB vaccine should have a serum sample tested for their VNA level, thus assuring that the minimum VNA titer is attained.

The determination of the antibody response after immunization against rabies is an accepted index of the efficacy of a vaccine and a successful treatment. The neutralization test in mice[11] is the oldest test which is still used in many countries, but it is time- consuming, and has inherent variability. Several *in vitro* tests have been developed in recent years in attempts to improve the assessment of neutralizing antibodies, including the rapid fluorescent focus inhibition test – RFFIT[9], the most widely accepted substitute for the mouse neutralization test.

In 1993, the Pasteur Institute of São Paulo, Brazil developed a simplified fluorescent inhibition micro test - SFIMT[4] based on the RFFIT[9] and the fluorescent inhibition microtest - FIMT[14] which has been used as a serological test since then. More than 9,000 serum samples, from all over the country, were tested last year. This is one of the only two laboratories in Brazil that currently determine the virus-neutralizing antibody (VNA) titers in cell-culture, located in São Paulo. The number of samples that should be tested each year exceeds the capacity of these two centers. The availability of a rapid and simple test that could be performed in other regions of the country, mainly in laboratories without cell-

culture facilities, would be of great importance.

Enzyme-linked immunosorbent assay (ELISA) has been proposed by several authors as a possible alternative to the neutralization test for determination of rabies antibodies[1,2,3,7,8].

The present study reports an indirect ELISA method for the detection of rabies antibodies in sera from humans vaccinated with cell-culture vaccine (CCV) or suckling mouse brain vaccine (SMBV), and compares the results with those obtained by the cell-culture microtest currently used in Brazil.

MATERIALS AND METHODS

Human Sera

One hundred and twenty-six human sera were collected from individuals with no previous history of anti-rabies immunization, and with negative results in the SFIMT. Three hundred and fifty three human sera were collected from individuals vaccinated with human diploid cell vaccine – HDCV (Pasteur-Mériéux - Lyon,France) or with SMBV (TECPAR, Brazil). Negative and positive pools were prepared and used as a control in every assay.

Standard Serum

The second international standard rabies serum - (WHO International Laboratory for Biological Standards - Copenhagen, Denmark), containing 30 IU/ml, was used as a reference in every assay.

Neutralization Antibody Titration

Neutralizing antibodies were measured by SFIMT[4]. Briefly, the test sera and the standard serum were distributed into series of two-fold dilutions beginning from 1/5 in a volume of 100 μl per well. Fifty μl of an optimal viral suspension previously titered was added to each well, followed by an incubation period of 60 minutes at 37°C. Fifty μl of a suspension of BHK_{21} cells, grown in minimum Eagle medium containing 10% bovine fetal serum (10^6 cells/ml), was then added to each well. The microplates were re-incubated at 37°C for 24 hours in a humidified CO_2 atmosphere. The determination of the virus infection rate was performed in each test. Briefly, after cell fixation with 80% cold acetone, cells were stained by an anti-rabies nucleocapsid fluorescent conjugate (Sanofi cod.72114). The results expressed the dilution corresponding to the well with 50% decrease of infection. The comparison of the results of unknown sera and standard serum served as a basis for obtaining the titer in IU.

ELISA

Disposable flat-bottom high-binding ELISA plates (Costar-3590) were used and all reagents were used in 200 μl volumes. Between all steps of the reaction, microplates were washed six times with PBS containing 0.05% Tween 20. For microplate

coating purified whole PV virus strain (Pasteur - Marnes la Coquette - France) diluted in 0.05 M sodium carbonate-bicarbonate buffer (pH 9.6), incubated for 1 hour at 37°C, was used. After incubation, 300 μl of blocking buffer (Tris -NaCl with 0.5% gelatin) was added to each well, and the plates were incubated for 30 minutes at 37°C. Sera and conjugate (Protein A-horseradish peroxidase - Sigma P8651) were diluted in PBS containing 0.5% gelatin and 0.05% Tween 20, being incubated for 1 hour at 37°C. Thirty minutes after the addition of the substrate (OPD 0.04% in 0.1M citrate-phosphate buffer pH 5.0 and 0.03% H_2O_2) the enzymatic reaction was stopped with 50 μl of H_2SO_4 4N. Absorbency was read at 492 nm using a microplate reader (Titertek Multiskan MCC/340). A checkerboard titration employing the positive and negative reference sera was performed to determine the best antigen protein concentration for coating the microplate and the best dilution of sera and conjugate.

Determination of Cut-off Value

The mean of the negative sera and the standard deviation "s" were determined. The mean plus 2s represented the upper limit of the negative sera with a confidence level of 98%. As a WHO working group recommended that a minimum value of 0.5IU/ml of neutralizing antibodies should be attained to demonstrate seroconversion (World Health Organization, 1992), the mean of 73 sera from vaccinated persons with concentration of antibody lower than 0.5IU/ml and the standard deviation were determined. The mean plus 2s represented the upper limit of sera from persons with unsatisfactory VNA titers.

Serum Samples From Individuals Vaccinated With Cell Culture Vaccine or With Neural Tissue Vaccine

In order to compare the results obtained by the ELISA test from sera of individuals receiving different types of vaccines (HDCV or SMBV), the sensitivity, specificity and agreement values were determined for each group. The test for differences between 2 proportions by normal approximation was used [15].

RESULTS

Determination Of Cut-off Value

After checkerboard titration, the best results for the ELISA test were obtained employing the antigen at 0.5 μg/ml (dilution 1: 20) , sera diluted at 1:400 and conjugate diluted at 1:5000.

A total of 353 serum samples from vaccinated persons were evaluated on the basis of the SFIMT.

For 126 negative sera, the mean (0.099) and the standard deviation (s=0.028) were determined. The mean plus 2s represent the upper limit of negative sera (0.155) with a confidence level of 98%.

The distributions of ELISA reading values and SFIMT are shown in Table 1. Sensitivity and specificity rates were 84.9% and 91.8% respectively.

TABLE 1

Sensitivity (Sv), Specificity (Sp) and Agreement (A)
values of the ELISA (cut-off = X + 2SD of non vaccinated
individuals) based on results obtained in the neutralization test.

	Neutralization test		
	<0.17 UI/ml	>0.17UI/ml	Total
ELISA +[1]	4	258	262
ELISA -	45	46	91
Total	49	304	353

[1]positive = OD values > 0.155
Sv = 84.9%
Sp = 91.8%
A = 85.8%

TABLE 2

Sensitivity (Sv), Specificity (Sp) and Agreement (A)
values of the ELISA based on results obtained in the
neutralization test, after the establishment of a doubtful region[1].

	Neutralization test		
	<0.5UI/ml[2]	>0.5UI/ml[3]	Total
ELISA +	6	189	195
ELISA -	64	27	91
Total	70	216	286

[1]doubtful region between OD values of 0.155 and 0.282
[2]non protected individuals
[3]protected individuals
Sv = 87.5 %
Sp = 92.4%
A = 88.5%

Taking into account 73 serum samples from vaccinated individuals whose VNA titers were below 0.5IU/ml, the mean OD readings (0.128) and the standard deviation (0.077) were determined. The mean plus 2 s (0.282) represented the upper limit of the non-protected individuals. This provided greater specificity, but lower sensitivity. In an attempt to improve the agreement between the titers obtained by both tests, it was proposed that a doubtful region should be established. This region consisted of OD values ranging from 0.155 (cut-off of the first approach) to 0.282 (cut-off of the second approach). Therefore, sera were defined as ELISA-positive, -negative or -doubtful. The distribution of ELISA reading values, SFIMT and the doubtful region is shown in Figure 1. Sera with OD values greater than 0.282 are considered as corresponding to VNA > 0.5 IU/ml.

Based on these criteria, 67 of 353 samples were classified as doubtful. The sensitivity and specificity of the ELISA test, when the values of serum samples included in the doubtful region were extracted from calculations, were 87.5% and 92.4% respectively (table 2).

ELISA Test With Serum Samples From Individuals Vaccinated With Cell Culture Vaccine Or With Neural Tissue Vaccine

There were no significant differences between the sensitivity, specificity and agreement values when sera from people vaccinated with cell-culture vaccine or neural-tissue vaccine were used. The z-values observed were respectively 0.188, 0.399 and 0.098 ($z_{0.05 (2)}$ = 1.96).

DISCUSSION

The ELISA described here offers an alternative test which, unlike neutralization assays, can be easily performed routinely. So far, such a test has not been available in many developing countries obligated to rely on testing sera by the mouse neutralization test.

The cut-off determination was made from two approaches. First, using 126 negative sera, and second, using 73 sera from vaccinated
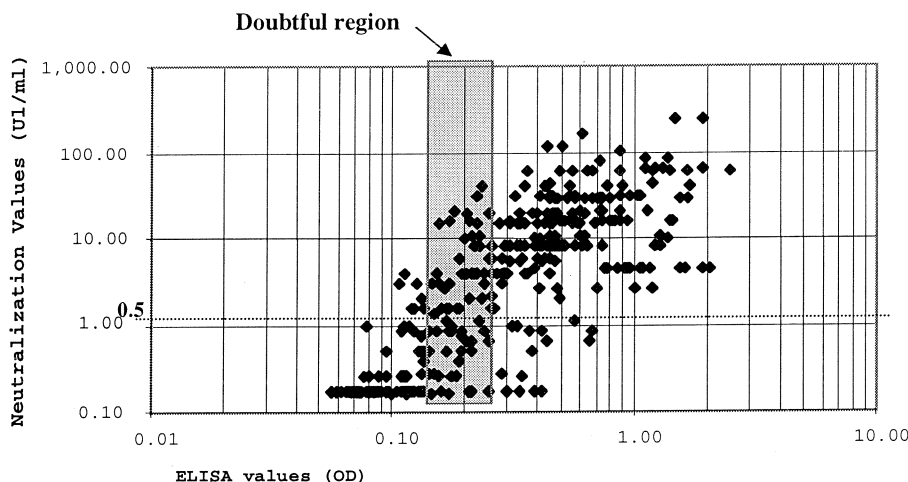

Fig. 1 - Distribution of ELISA reading values and neutralization test

individuals who did not attain titers of 0.5 IU/ml.

According to current WHO guidelines[13] as a minimum value of 0.5 IU/ml needs to be attained to demonstrate seroconversion indicative of successful vaccination, the first approach alone would be useless. Therefore, in an attempt to distinguish between titers lower and higher than 0.5 IU/ml, the second approach was used. In this way, the results show greater specificity and lower sensitivity. A doubtful region was established. The doubtful region was defined as values ranging between 0.155 and 0.282, considering the cut-off values of both approaches. The serum samples with results in this region should have their titers confirmed by other tests.

In this way, comparison of the two assays gave a sensitivity of 87.5% and specificity of 92.4%. The presence of 27 serum samples, classified as false negative, could be due to the presence of antibodies of IgM class, since the protein A from Staphylococcus is able to detect antibodies of IgG class, while the SFIMT is also able to detect IgM and IgA. This could lead to a high number of false-negative results in the ELISA test, indicating a lower sensitivity. In fact, out of the 27 serum samples with VNA >0.5IU/ml evaluated by SFIMT with negative ELISA results, 19 corresponded to samples collected at the beginning of the immunization (7 days after the first vaccine dose). Production of IgM antibodies is the initial humoral response to infection or immunization[10]. However, neither passively-transferred nor actively-induced IgM neutralizing antibodies protect mice infected[10]. Thus, IgG anti-rabies antibodies detected by ELISA may be of greater clinical importance.

Of the 70 samples with negative results in the SFIMT, 64 were negative in ELISA. The six sera classified as false-positive could be explained by the presence of non-neutralizing anti-nucleoprotein antibodies present in these serum samples, detected by ELISA but not by the neutralization test, since the whole inactivated virus particles were used as antigen in the ELISA test.

It was also observed that there were no significant differences between the values of sensitivity, specificity and agreement in the ELISA test, when the results obtained from individuals vaccinated with the cell-culture vaccine and the group vaccinated with the suckling-mouse-brain vaccine were compared, even though it is reported that neural tissue vaccines induce higher synthesis of antibodies directed to nucleocapsid proteins[8]. It is possible to conclude that this test can be used in the rabies laboratory no matter what kind of vaccine has been used for immunization.

In view of the results, the ELISA test described here could be used as an alternative test for laboratories without cell-culture facilities or even as a screening test in laboratories with high numbers of samples to be tested. It must be stressed, however, that only sera obtained after a complete treatment should be tested, since those obtained at the beginning of the immunization process may contain high IgM antirabies levels, that would not be detected by this ELISA.

In addition, the purified whole PV virus strain used could be replaced by inactivated cell-culture vaccine. We tested the human diploid-cell-culture vaccine (Pasteur & Mériéux Serums et Vaccines) at 1/40 dilution, and the results were quite similar to those obtained with purified virus (data not shown).

The ELISA test described here is simple, rapid and of limited cost. The great advantage is that the antigen is inactivated and can be used safely in the routine laboratory.

RESUMO

Adaptação do método imunoenzimático (ELISA) para detecção de anticorpos anti-rábicos em soros humanos vacinados com vacina de cultura celular ou vacina produzida em cérebro de camundongo

O método imunoenzimático (ELISA) foi adaptado para quantificar anticorpos anti-rábicos em soros de pessoas previamente imunizadas. Foi utilizado como antígeno, partículas virais purificadas inativadas, e como conjugado, Proteína A conjugada à peroxidase. Foram testados soros de pessoas vacinadas com vacina de cultura celular ou com vacina produzida em cérebro de camundongo. Os resultados foram comparados a aqueles obtidos pela prova de soroneutralização em cultura celular. A média e o desvio padrão foram calculados para 126 soros negativos e para 73 soros de pessoas vacinadas mas com título menor que 0,5 UI/ml. Foi proposta a adoção de uma região de dúvida, levando a uma diminuição de resultados falso positivos. A sensibilidade, especificidade e concordância do teste foram respectivamente : 87,5%, 92,4% e 88,5%. Não foram observadas diferenças significativas quando comparados os resultados dos indivíduos vacinados com uma ou outra vacina utilizada. Grande parte dos resultados discordantes no ELISA e soroneutralização ocorreram em soros coletados após a primeira dose de vacina, e se deveu provavelmente a presença de IgM nas amostras, detectados somente pela soroneutralização. O ELISA pode ser utilizado como um teste independentemente da vacina que foi empregada.

REFERENCES

1. ATANASIU, P.; SAVY, V. & PERRIN, P. - Épreuve immunoenzymatique pour la détection rapide des anticorps antirabiques. **Ann. Inst. Pasteur Microbiol. (Pasteur), 128A:** 489-498, 1977.

2. ELMGREN, L.D. & WANDELER, A.I. - Competitive ELISA for the detection of rabies virus neutralizing antibodies. In: MESLIN, F.X.; KAPLAN, M.M. & KOPROWSKI, H., ed. **Laboratory techniques in rabies.** 4.ed. Geneva, WHO, 1996. v. 17, p. 200-208.

3. ESTERHUYSEN, J.J.; PREHAUD, C. & THOMSON, G.R. - A liquid-phase blocking ELISA for the detection of antibodies to rabies virus. **J. virol. Meth., 51:** 31–42, 1995.

4. FAVORETTO, S.R.; CARRIERI, M.L.; TINO, M.S.; ZANETTI, C.R. & PEREIRA, O.A.C. - Simplified fluorescent inhibition microtest for the titration of rabies neutralizing antibodies. **Rev. Inst. Med. trop. S. Paulo, 35:** 171–175, 1993.

5. FUENZALIDA, L.E. & PALACIOS, V.R.H. - Un metodo mejorado en la preparación de la vacuna antirrabica. **Bol. Inst. bact. Chile, 8:** 3-10, 1955.

6. HELD, R.J. & ADAROS, L.H. - Neurological disease in man following administration of suckling-mouse brain antirabies vaccine. **Bull. Wld . Hlth. Org., 46:** 321-327, 1972.

7. NICHOLSON, K.G. & PRESTAGE, H. - Enzyme-Linked Immunosorbent Assay: a rapid reproducible test for the measurement of rabies antibody. **J. med.Virol., 9:** 43-49, 1982.

8. PERRIN, P.; VERSMISSE, P.; DELAGNEAU, J.F. et al. - The influence of the type of immunosorbent on rabies antibody EIA; advantages of purified glycoprotein over whole virus. **J. biol. Stand., 14:** 95-102, 1986.

9. SMITH, J.S.; YAGER, P.A. & BAER, G.M. - A rapid reproducible test for determining rabies neutralizing antibody. **Bull. Wld. Hlth. Org., 48:** 535-554, 1973.

10. TURNER, G.S. - Immunoglobulin (IgG) and (IgM) antibody responses to rabies vaccine. **J. gen. Virol., 40:** 595-604, 1978.

11. WEBSTER, L.T. & DAWSON, J.L. - Early diagnosis of rabies by mouse inoculation. Measurement of humoral immunity to rabies by mouse protection test. **Proc. Soc. exp. Biol. (N.Y.), 32:** 570-573, 1935.

12. WILBUR, L.A. & AUBERT, M.F.A. - The NIH test for potency. In: MESLIN, F.-X.; KAPLAN, M.M. & KOPROWSKI, H., ed. **Laboratory techniques in rabies**. 4. ed. Geneva, World Health Organization, 1996. v. 37, p. 360-368.

13. WORLD HEALTH ORGANIZATION - Prevention of rabies in humans In: WHO Expert Committee on rabies, Eighth Report. Geneva, World Health Organization, 1992. p. 21-26. (**Wld. Hlth. Org. techn. Rep. Ser.**, 824).

14. ZALAN, E.; WILSON, C. & PUKITIS, D. - A microtest for quantification of rabies virus neutralizing antibodies. **J. biol. Stand., 7:** 213-220, 1979.

15. ZAR, J. H. - **Biostatistical analysis**. 2.ed. New Jersey, Prentice-Hall, 1984. v. 22, p. 395-400.