

ENZYME-LINKED IMMUNOSORBENT ASSAY FOR QUANTIFICATION OF RABIES ANTIBODIES IN HUMAN SERA

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Summary. — A double antibody enzyme linked immunosorbent assay (ELISA) was elaborated for detection of rabies antibodies in human sera. The procedure consisted of coating polyvinylchloride plates with rabbit antirabies serum followed by attachment of partially purified fixed virus and human rabies antibodies. The rabies-specific antibodies in human antisera were quantified by means of antihuman peroxidase conjugate. Titration of antisera from human volunteers immunized with the "Fermi" vaccine revealed excellent correlation of the virus neutralization test and ELISA.

Key words: fixed rabies virus; antirabies vaccine "Fermi"; peroxidase conjugate; double antibody ELISA

Introduction

ELISA has gained wide acceptance in the diagnosis of viral diseases because of its high sensitivity, specificity and reproducibility (Voller, 1976; Atanasiu, 1977, 1978, 1979; Thraenhart, 1977; Szkudlarek, 1982; Avrameas, 1983).

The present study was based on a double antibody ELISA method for the detection of rabies antibodies in sera of human volunteers who had been vaccinated with the "Fermi" antirabies vaccine. The results indicate that ELISA agrees favourably with but is superior to the virus neutralization (VN) test in several respects.

Materials and Methods

Antigen. The antirabies vaccine was prepared from a challenge virus standard strain of rabies virus which had been grown on BHK-21 cells. The virus was pelleted from the culture fluid by ultracentrifugation at 29,000 rev/min, in a VAC-601 centrifuge, and was then suspended by sonication. The viral suspension was repeatedly sedimented over a cushion of 20% sucrose for 2 hr at 29,000 rev/min (partially purified virus). Further purification was achieved by sucrose density gradient ultracentrifugation as described previously (Kavaklova, 1983). The protein concentration was estimated by the method of Lowry (1951). The infectious titre was assayed by intracerebral inoculation of mice.

Antisera. Rabbits were injected with the purified virus (an infectious titre of $10^{5.5}$ LD₅₀/ml, protein content 250 µg/ml). The immunization schedule consisted of 1 ml subcutaneous injection

of antigen with complete Freund adjuvant, followed 30 and 45 days later by a 0.5 ml intramuscular injection of antigen without adjuvant. The animals were bled 15 days after the last injection. The antisera were tested for their virus neutralizing and complement fixing activities as previously reported (Kavaklova, 1983).

Preparation of the peroxidase conjugate. Anti-human IgG antibodies were obtained by affinity chromatography (Cambiaso, 1975) and further conjugated to horse radish peroxidase (Sigma, Type VI) by the method of Wilson and Nakane (1978).

Double antibody procedure of ELISA. The procedure was essentially the same as that proposed by Yolken and Stopa (1980). Polyvinylchloride microtitre plates (Pharmachim, Sofia) were coated with 100 μ l of rabbit antirabies serum suitably diluted in 0.05 mol/l carbonate buffer, pH 9.6. After 2 hr incubation at 37 °C the plates were kept overnight at 4 °C; then they were washed five times with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T). 100 μ l of partially purified virus dissolved in PBS-T containing 0.5% calf serum were added to the wells. Incubation was allowed to proceed for 1 hr at 37 °C. After washing as before, 100 μ l of human antisera in the same diluent were inoculated into the wells and incubation was continued for 1 hr at 37 °C. The plates were washed and peroxidase labelled anti-human IgG was added (1 hr, 37 °C). After washing, the enzyme was visualized by incubation in the dark of the substrate solution (consisting of o-phenylene diamine 0.4 mg/ml, 0.15 mol/l phosphate-citrate buffer, pH 5.0 and 0.002% H_2O_2) for 30 min at room temperature. The enzyme reaction was terminated by the addition of 50 μ l of 4 N H_2SO_4 . The optical density was monitored at 495 nm in SF-4 spectrophotometer. The specificity of the enzyme immunoassay was ascertained by including negative substrate, conjugate and serum controls. The samples were always run in duplicate. The extinction values measured were obtained from at least three experiments.

Results

Rabbit anti-rabies serum dilution

The coating of polyvinylchloride plates was carried out with different dilutions of rabbit anti-rabies serum. Since antiserum diluted 1 : 200 afforded intensity of the enzyme reaction comparable with that obtained with more concentrated antiserum, it was chosen as optimal (Fig. 1). Higher antiserum dilutions resulted in a substantially reduced colour yield.

Determination of the optimal antigen concentration

The standard virus strain in different dilutions was interacted with the antibody coated plates. As shown in Fig. 2, the increase in the extinction values paralleled the increase of antigen concentration. Peak extinction values

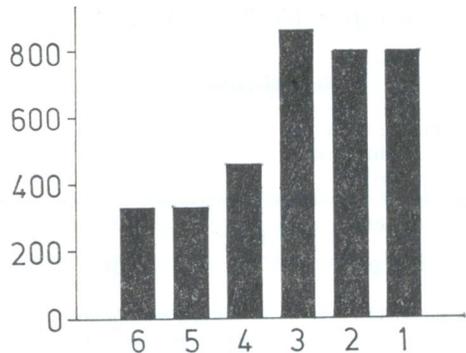


Fig. 1.

Colour intensity of ELISA as a function of antibody concentration in hyperimmune antirabies serum

Ordinate: ΔE_{495}

Serum dilutions: 1 - 1 : 50, 2 - 1 : 100;
3 - 1 : 200; 4 - 1 : 400; 5 - 1 : 800;
6 - 1 : 1,600.

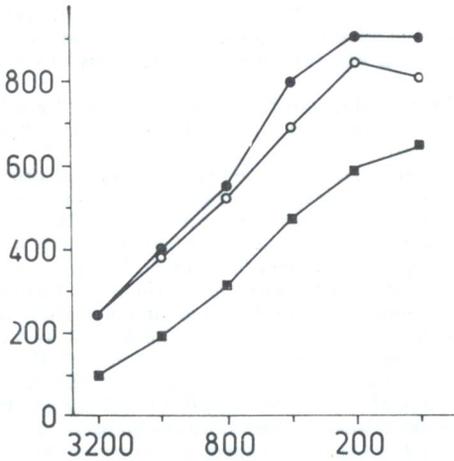


Fig. 2.
Relationship in ELISA between extinction values and the concentration of rabies virus antigen
●—● antigen diluted 1 : 20 (50 μg protein/ml);
○—○ diluted 1 : 50 (20 μg protein/ml);
■—■ diluted 1 : 100 (10 μg protein/ml).
Abscissa: serum dilutions; ordinate: ΔE₄₉₅

were reached with 50 μg of virus. Although the readings taken with 10 μg of virus were lower, this concentration was employed for performance, of the enzyme immunoassay for reasons of economy. Nevertheless, this amount was sufficient for clear distinction between the positive samples and the negative controls to be made.

Conjugate dilution

In order to establish the lowest limit of antibody detection, experiments were conducted with three dilutions of the peroxidase conjugate. The results shown in Table 1 indicate that the labelled anti-human IgG antibody diluted 1 : 1000 yielded appreciable optical density even in case of low titre human antisera.

Relationship between ELISA and virus neutralization titre

A total number of 29 serum samples obtained from volunteers who had been immunized with the "Fermi" antirabies vaccine were assayed. The results are shown in Table 2 and Fig. 3. ΔE represents the difference in the optical

Table 1. Relationship between colour intensity and conjugate dilution

Serum titre (neutralization test)	Conjugate dilution		
	1 : 500	1 : 1000	1 : 2000
1 : 800	ΔE = 1.300	ΔE = 0.900	ΔE = 0.600
1 : 400	ΔE = 1.100	ΔE = 0.600	ΔE = 0.260
1 : 150	ΔE = 0.550	ΔE = 0.350	ΔE = 0.150

For explanation see Results.

Table 2. Comparison of VN test and ELISA for assay of rabies antibodies in sera from volunteers immunized with the Fermi antirabies vaccine

Serum No.	Neutralization titre	ELISA titre	ΔE
1	800 *	1600*	0.220 **
2	800	1600	0.195
3	800	1600	0.200
4	800	800	0.200
5	800	200	0.180
6	800	200	0.150
7	400	800	0.180
8	400	800	0.160
9	400	800	0.245
10	400	400	0.200
11	400	800	0.145
12	200	200	0.195
13	170	200	0.155
14	170	200	0.215
15	150	400	0.200
16	150	400	0.160
17	90	100	0.195
18	90	100	0.240
19	50	200	0.230
20	45	200	0.225
21	30	20	0.170
22	20	200	0.140
23	10	20	0.225
24	10	20	0.210
25	10	20	0.205
26	10	400	0.155
27	10	400	0.200
28	10	200	0.230
29	10	200	0.160

* dilution reciprocals

** final dilution mixture

density between the positive and negative human serum. When the latter was diluted 1 : 100, the extinction measured was 0.040. Those ΔE values which were four times higher than the optical density of the control serum were considered to be of diagnostic importance. It may be seen that only 2 out of 29 human antisera gave low extinction values. Of seven sera with proven neutralization titre of 1 : 10, four reacted strongly positively in ELISA.

Discussion

Atanasiu *et al.* (1977, 1978, 1979) are merited for introduction of ELISA into the diagnosis of rabies virus. This method was subsequently applied by Thraenhart (1977). In these studies purified virus from strain Pasteur grown in BHK-21 cells was used.

For titration of rabies antibodies in human antisera we adapted the ELISA procedure proposed by Yolken and Stopa (1980). It was demonstrated that

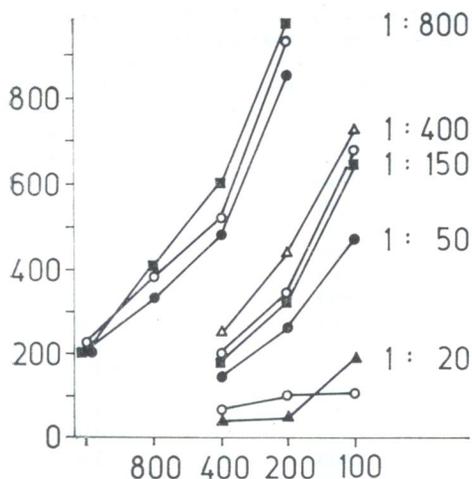


Fig. 3.
Antibodies in human sera of different neutralization titres
Four dilutions of each serum are employed in immunoenzymatic tests. The curves show a good correlation between neutralizing antibody titres and ELISA optical density values
Abscissa: serum dilutions; ordinate: ΔE_{495}

partially purified virus at 10 $\mu\text{g}/\text{ml}$ allowed the detection of rabies antibodies in sera from vaccinated individuals. The enzyme immunoassay proved to be specific as judged from the negligible extinction values of the controls. With rabbit antirabies serum used for coating the polyvinylchloride plates, even human antisera with a neutralization titre of 1 : 10 showed fairly high colour intensity in ELISA. In agreement with others (Szkudlarek, 1982; Thraenhart, 1977; Atanasiu, 1978), we were able to show the excellent correlation between ELISA and VN test. As far as low titre antisera were concerned, most of them yielded substantially higher ELISA titres than neutralizing antibody titres supporting the higher sensitivity of the enzyme immunoassay. Apart from the higher sensitivity of ELISA with respect to the neutralization test, the former method is by far more rapid and less time-consuming as the results can be obtained within hours.

Our data provide evidence that the double antibody method of ELISA is a sensitive, accurate technique for quantification of rabies antibodies in human sera.

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