Journal of Medical Virology 9:43–49 (1982)

Enzyme-Linked Immunosorbent Assay: A Rapid Reproducible Test for the Measurement of Rabies Antibody

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An enzyme-linked immunosorbent assay (ELISA) was developed for detecting IgG antibodies to the Pitman Moore strain of rabies virus in sera from subjects immunised with HDCS vaccine. End-point titres of antibody were determined using a pocket calculator preprogrammed to analyse absorbence values of test sera and negative controls. The assay was highly reproducible, and very close agreement was achieved when the results were compared with those of the mouse neutralization test. Rabies enzyme-linked immunosorbent assay is easy to perform, the results are obtained within 48 hours, and because it employs inactivated virus, the test can be used safely without special containment facilities.

Key words: rabies virus, ELISA, end-point titres, preprogrammed pocket calculator

INTRODUCTION

The determination of an antibody response after immunization against rabies is an accepted index of the efficacy of vaccine. The mouse neutralization test [Atanasiu, 1973] is the most commonly used procedure, but it is time-consuming, and its inherent variability makes it difficult to collate results between laboratories even when standard antiserum is included in each test [Fitzgerald et al, 1975; Kuwert et al, 1978]. Several in vitro tests have been developed in recent years in attempts to improve the assay of neutralizing antibody. These include fluorescent focus inhibition [Smith et al, 1973] plaque reduction [Sedwick et al, 1967], and the interference inhibition test [Nicholson et al, 1979], none of which can be accomplished without special laboratory facilities and considerable expertise. The complement fixation test, haemagglutination-inhibition test, and mixed haemadsorption test measure rabies antibodies, which are less clearly related to protection, and they have gained little acceptance.

Accepted for publication June 10, 1981.

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0146-6615/82/0901-0043$02.50

Enzyme-linked immunosorbent assay (ELISA) has been used in the serodiagnosis of many infectious diseases [Sever et al, 1977], and it has been proposed as a possible alternative to neutralization tests for rabies [Atanasiu et al, 1977, Thraenhart and Kuwert, 1977]. This paper evaluates the sensitivity and reliability of a rabies ELISA and compares the results with those obtained by the MNT.

MATERIALS AND METHODS

ELISA Technique

The technique used was a modification of the method described by Kraaijeveld et al [1980], for detection of coronavirus antibodies. The rabies antigen used for the ELISA was supplied by Behringwerke AG, D-355 Marburg (Lahn), West Germany. It was a whole virus preparation of the Pitman Moore strain of rabies virus, grown in MRC-5 cells, and concentrated and purified by continuous flow ultracentrifugation [Hilfenhaus et al 1976]. It was inactivated with β-propiolactone and had an antigenic potency of 269 IU/ml when measured by the antibody binding test [Barth et al 1981]. Electron microscopy revealed approximately 10^{10} bullet-shaped whole virions per ml with no disrupted particles or host cell material.

Wells of flat-bottomed immulon microelisa plates (Dynatech M129A; Dynatech Laboratories, Billingshurst, Sussex) were inoculated with 0.2 ml amounts of antigen diluted 1 in 200 in 0.05 M sodium carbonate-bicarbonate buffer (pH 9.6) containing 0.02% sodium azide (ELISA coating buffer; Don Whitley Scientific Ltd., Shipley, West Yorkshire). Plates were then covered with parafilm sealing tissue and incubated overnight at room temperature (21°C). After incubation, the plates were washed four times with phosphate-buffered saline containing 0.05% Tween 20 (PBST) (Don Whitley Scientific Ltd.) and shaken dry. The sera to be tested were absorbed overnight with equal volumes of foetal calf serum and serial dilutions were prepared in PBST; 0.2 ml of each dilution were added to the wells of the antigen coated plates and together with suitable controls were incubated for three hours at room temperature. After incubation, the plates were washed four times in PBST and shaken dry. Goat immunoglobulin G, directed against heavy and light chains of human IgG and conjugated with alkaline phosphatase, was obtained from Miles Laboratories, Ltd, Stoke Poges, Slough. It was diluted 1:800, added in 0.2 ml volumes to each well of the plate, and left overnight at room temperature. After four additional washes with PBST, 0.2 ml of phosphatase substrate, consisting of a 0.1% solution of p-nitrophenylphosphate disodium in 10% (W/V) diethanolamine buffer (pH 9.8) with 0.02% sodium azide and 0.01% $MgCl_2 \cdot 6H_2O$, was added to each well. Absorbence values were read after 30 minutes at 405 nm in a Flow Laboratories Titertek Multiskan spectrophotometer.

Estimation of Antibody Titre

Test sera and eight control sera from unimmunised subjects were serially diluted fivefold. End-point titres of rabies antibody were determined using a programmable TI-58 Texas Industries pocket calculator. This was programmed to determine the intercept (x) between the quadratic curve fitted to the optical density (OD) values of dilutions of test sera, and the straight line fitted to the mean plus 2.5 standard deviations of the mean of OD values of appropriate negative controls. From this intercept, the titre of antibody (X) was calculated (Fig. 1). The calculation involves two formulae:

$$\text{intercept } x = 2 + \frac{1}{B}\left[E - \sqrt{E^2 - 2B\,(Y_2 - D)}\,\right]$$

and

$$\text{end-point titre of test sample } X = d \bullet n^{x-1}$$

where

$B = Y_1 - 2Y_2 + Y_3$
$D = (S_1 + S_2 + S_3)/3$
$E = (S_3 - S_1 - Y_3 + Y_1)/2$
Y_1, Y_2, Y_3, are OD values of the test sample at dilutions d_1, d_2, d_3
S_1, S_2, S_3, are OD values (mean + 2.5 SD) for eight known negative samples at dilutions d_1, d_2, d_3
d = the denominator of the first dilution (d_1) used in the calculation (eg, 500)
n = the dilution factor.

Further details of the calculation and/or the programming of the calculator can be supplied upon request.

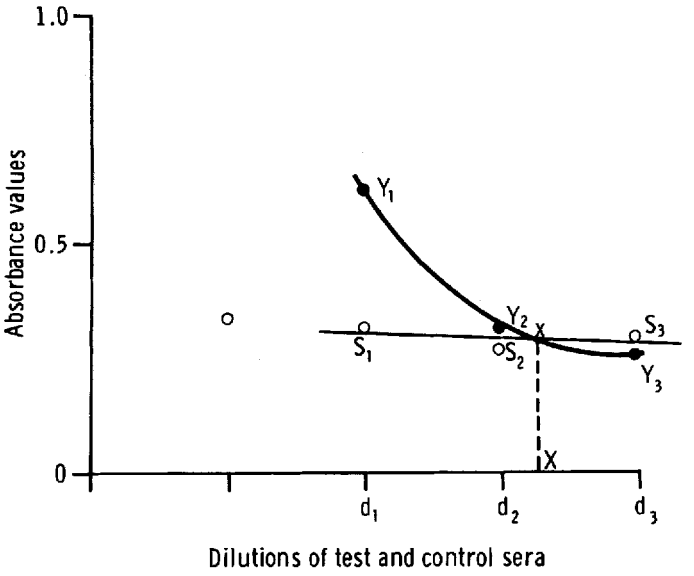

Fig. 1. Intercept of quadratic curve fitted to OD values of dilutions of test sera, and the straight line fitted to the mean plus 2.5 standard deviations of the mean of OD values of appropriate negative controls.

Rabies Neutralizing Antibody

Titres of virus neutralizing antibody were determined by Dr. G. Turner, Blood Products Laboratory, Elstree, using the mouse neutralisation test [Atanasiu 1973, Nicholson et al, 1978]. The titration end points were estimated by the Spearman-Kärber method [Lorenz and Bögel, 1973].

RESULTS

Reproducibility of the ELISA Test

The reproducibility of the assay was tested using a laboratory standard of pooled human serum with a rabies neutralizing antibody titre of 1:1,000. Eight replicate titrations of the standard were made in two plates on five occasions. Using dilutions in the range 1:500 to 1:62,500, results were obtained for 78 of 80 titrations. Analysis revealed that 71 of 78 (91%) titres showed less than a twofold difference from the geometric mean (1:15,411), and no titre differed by more than a fourfold. For individual plates, the ratio of the highest to lowest titre ranged between 1.4 and 3.4 (mean = 2.4; SD 0.8); significant variation ($P < .05$) between duplicate plates was observed on one of five occasions with a 1.9-fold difference in mean titre. When coded sera were retitrated on separate occasions, 42 of 53 (79%) differed by less than a twofold from the line of identity, and 96% differed by less than a fourfold.

Comparison of ELISA and MNT

Sixty-nine sera were titrated once by MNT and on one of more occasions by ELISA; of the sera either positive or negative by both tests, there was complete agreement in 68 (98.5%). One sample that was positive by MNT, but negative by ELISA, was obtained seven days after vaccination when the majority, if not all, of the neutralizing antibody would be expected to be IgM. To quantitate the relation between MNT and ELISA more precisely, the line of linear regression missing the origin [Snedecor and Cochran, 1967] and the correlation coefficient were determined. Analysis shows a log-log relationship between the results of the MNT and ELISA which is expressed by the formula:

$$\log_{10} \text{MNT} = 0.676 \log_{10} \text{ELISA} + 0.388$$

This shows that for relatively low concentrations of antibody ($<$ 1:50) the titres measured by ELISA and MNT were numerically similar. However, with increasing concentrations of antibody, the titres measured by ELISA became increasingly greater than those measured by the MNT. The closeness of the data to the fitted line, however, is indicated by a correlation coefficient of 0.92.

Rabies Antibody Titres Measured by ELISA After Primary and Booster Vaccination

Seventy coded sera were obtained from the Immunisation Centre, British Airways, London, for antibody titration by ELISA. No rabies antibody was found in 12 sera taken from nonvaccinated controls. By contrast, 40 of 43 (93%) samples taken three to six weeks after primary intradermal (ID) vaccination with 0.1 ml of HDCS vaccine contained antibody ranging from 1:19 to $\geq$ 1:12,500 (GMT $\geq$ 1:520). Similarly the eight samples taken from subjects 4–12 weeks after revaccination (0.1 ml ID) contained antibody ranging from 1:1,025 to $\geq$ 1:12,500 (GMT $\geq$ 1:4367). Five persons

who had received HDCS vaccine one to two years previously had titres ranging between 1:25 to 1:2855 (GMT 1:186). By contrast, two persons vaccinated recently in the Soviet Union and Bolivia had no detectable rabies antibody.

Sera from 76 vaccinees who were participating in a study of the persistence of antirabies antibody after vaccination were assayed by ELISA. Each subject had been immunised 36 months previously on days 0, 28, and 56 with 1.0 ml of HDCS vaccine intramuscularly (IM), or 0.1 ml ID. Most vaccinees received in addition a booster at 6, 12, or 24 months, either by the ID route or by the subcutaneous (SC) route. Each vaccinee had been shown previously to have neutralizing antibody 24 months after primary vaccination [Nicholson et al, 1978]. Of the 36 month sera, ELISA failed to detect antibody in 2 of 14 subjects who were not given a booster. However, rabies antibody was present in all 62 persons who had been revaccinated 12 to 30 months previously, and the titres were higher in those most recently vaccinated (Table I).

DISCUSSION

The data presented in this study demonstrate that the ELISA test and method of antibody quantitation together result in a rapid and highly reproducible assay suitable for use in serological studies of rabies.

Irregular nonspecific reactions by sera from unimmunised subjects was initially a problem not resolved by pretreatment of plates with foetal calf serum. This difficulty was overcome by including a number of nonimmune sera in each test and arbitrarily choosing the cut-off point between antibody-positive and negative sera as the mean + 2.5 standard deviations of the mean of the OD values of the negative controls. Although this prevented the assay from being more sensitive than the MNT with low titres of antibody, it reduced the likelihood of false-positive reactions to ~ 1:170 ($P = .006$). Moreover, absorbence values of test and control sera are anlaysed over a range of dilutions, which provides a more precise estimate of titres than any other method.

TABLE I. Geometric Mean Antibody Titres 36 Months After Primary Vaccination and Booster

	Primary vaccination				
	3 × 0.1 ml ID		3 × 1.0 ml IM		
Booster	GMT[a]	Range	GMT[a]	Range	GMT
None	488 (5)	101–1,544	161 (9)	(−)ve[b]–2,765	240
6 month SC	1,201 (7)	568–1,817	611 (11)	52–2,474	795
12 month ID	617 (4)	30–2,695	983 (7)	599–1,505	835
12 month SC	1,036 (5)	94–5,530	602 (6)	415–897	
24 month ID	1,337 (9)	628–3,015	1,822 (4)	1,563–2,445	1,234
24 month SC	1,124 (7)	36–3,057	1,146 (2)	671–1,957	

[a]Numbers in parenthesis refer to the number of sera in each group.

[b](−)ve results taken as being 1/10 for measuring the GMT.

The rabies antigen used in the tests was prepared in bulk using concentration and purification facilities usually employed for the production of human vaccines. Electron microscopy showed it to be free of cellular material which may result in nonspecific reactions. We found no evidence during preliminary studies that recipients of HDCS vaccine developed antibodies to human diploid cell antigens similar to egg-specific antibody, which may develop in animals or humans given killed egg-grown influenza vaccine [Harboe et al, 1961].

Investigations reported by Wiktor et al [1973] indicate that antibodies specific for the glycoprotein envelope are responsible for virus neutralization. Thus, the whole virus particles with intact glycoprotein used in our ELISA should measure true neutralizing antibodies, and the close agreement found in the present study between the MNT and ELISA supports this supposition. Antigen preparations containing disrupted virions presumably bind many clones of antibody not associated with neutralizing activity, and are much less satisfactory. Of notable value is the ability of ELISA to detect rabies antibodies of the IgG class. It is well known that IgM antibody is the initial humoral response to infection or immunisation, and that in infections with viraemia IgM is especially important. However, neither passively transferred not actively induced IgM neutralizing antibody protect mice infected experimentally [Turner, 1978]. Thus, IgG antirabies antibody detected by ELISA may be of greater importance than the early antibody response to infection or immunisation that is measured by neutralization tests.

The potential application of the assay has beem amply demonstrated in the present report. It reliably differentiated coded sera taken from unimmunized subjects and vaccinees, it showed that HDCS vaccine can be given successfully at a busy immunisation centre using the ID route, it showed the progressive fall of antibody titres after revaccination, and it failed to show antibody in two subjects who had been vaccinated overseas. The great advantage of the assay is that the antigen is inactivated and can be used safely in routine diagnostic laboratories. Moreover, a large number of samples can be handled in a short period of time with relatively little technical expertise. These factors, together with its high degree of reproducibility, suggest that the ELISA test will become an extremely useful technique for the serodiagnosis of rabies and for measuring the response to vaccination.

ACKNOWLEDGMENTS

Thanks are due to Dr. G.S. Turner, Blood Products Laboratory, Elstree, for titrating rabies neutralizing antibody, Drs. F.J. Furlong and G. Lea, British Airways Immunisation Centre, London, for providing sera, Dr. R. Barth, Behringwerke AG, Marburg, West Germany, for providing the rabies antigen, H. Davies, Clinical Research Centre, Harrow, for electron microscopy, D. Altman, Department of Computing and Statistics, Clinical Research Centre, Harrow, for mathematical assistance, and to Dr. R. Cormack, Department of Anaesthetics, Northwick Park Hospital, Harrow, for writing the programme for the calculator.

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