A Single-Radial-Immunodiffusion Technique for the Assay of Rabies Glycoprotein Antigen: Application for Potency Tests of Vaccines Against Rabies

(Accepted 4 November 1981)

SUMMARY

An assay for rabies glycoprotein antigen based on single-radial-immunodiffusion (SRD) is described. Rabies glycoprotein antigen at concentrations of 0.7 μg/ml or greater (approx. 1 international unit, IU) produced well-defined SRD reaction zones in immunoplates containing antibody to purified glycoprotein. Plots of zone area against relative antigen concentration were linear. The method was found to be of suitable sensitivity for in vitro potency assays of inactivated cell culture rabies vaccines. Qualitative differences were detected between rabies vaccines prepared by two different methods when these were analysed in sucrose gradients for glycoprotein antigen associated with intact virions or in ‘soluble’ form associated with subviral structures. In vaccines prepared by zonal ultracentrifugation the glycoprotein was totally associated with intact virus, whilst in those prepared by ultrafiltration comparable quantities of subviral antigen were also detected. The SRD test appears to have considerable potential for assays of the antigenic content of rabies vaccines and has the advantage of reducing reliance on conventional in vivo tests for immunogenicity which employ infectious virus.

The glycoprotein is the only antigen of the rabies virus particle which is important in stimulating virus-neutralizing antibodies (Cox et al., 1977). Methods to quantify this protein are therefore important for the control and standardization of inactivated rabies vaccines for use in man and animals. Currently, in vivo assays are used routinely to standardize these vaccines. The tests most commonly used are the NIH test (Seligman, 1973) and the Habel test (Habel, 1973). The former involves the immunization of mice with test and reference antigens followed by intracerebral challenge with a standard strain (CVS) of rabies virus. The latter requires the titration of a virus preparation in mice immunized with vaccine and in control mice. These tests are time consuming and have the disadvantage of employing infectious virus, necessitating the use of special containment facilities in certain countries. In addition, potency estimates derived from them show considerable variability (Wiktor et al., 1978; Barth & Jaeger, 1979). There is general agreement (Barth & Jaeger, 1979) that more satisfactory and rapid tests are required for in process control and standardization of rabies vaccines. Barth et al. (1981) have described an antibody-binding test for rabies antigen which gave more reproducible results than in vivo tests but still requires infectious virus.

SRD tests have been used for the assay of other inactivated virus vaccines, including influenza (Schild et al., 1975; Wood et al., 1977) and poliovirus (Schild et al., 1980). SRD tests have been adopted internationally for the assay of influenza vaccines enabling reproducible and accurate estimates (coefficient of variation, 5%) of their haemagglutinin antigen content to be made (Wood et al., 1981). These tests thus appeared to be worthy of evaluation for rabies antigens.

For SRD assays with rabies antigens the methods used were essentially those described by Wood et al. (1977). Briefly, potent immune rabbit or sheep serum to purified rabies
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**Dilution**

(a) 1:1 1:2 1:4 1:8

![Image of single radial immunodiffusion assay](image)

**Fig. 1.** (a) Single-radial-immunodiffusion assay of rabies virus glycoprotein. Zones were produced by ERA rabies virus glycoprotein (G) and purified ERA rabies virus (V). The undiluted antigens contained 5.6 and 68 µg/ml protein respectively. The gel contained 10 µl/ml gel rabbit anti-ERA rabies glycoprotein serum. (b) Dose-response curves when dilution is plotted against annulus area for the following: purified ERA virus (■); purified ERA glycoprotein (●); the WHO third international reference preparation for vaccine, an HDCS vaccine concentrated by continuous rate-zonal centrifugation (O); an HDCS vaccine concentrated by ultrafiltration (∆). The gel contained 5 µl/ml gel rabbit anti-ERA rabies glycoprotein serum. (c) Distribution of SRD-detectable rabies glycoprotein in sucrose gradients following equilibrium centrifugation of vaccines prepared by rate zonal centrifugation (O) and ultrafiltration (∆). The gel contained 5 µl/ml gel rabbit anti-ERA rabies glycoprotein serum.

Glycoprotein derived from the ERA strain was incorporated in agarose gels [1% agarose ME (Marine Colloids) in phosphate-buffered saline (PBS) pH 7.3 with 0.1% NaN₃] which were prepared in perspex moulds on glass plates. Wells (3 or 4 mm diam.) were punched in the gels for the addition of antigen preparations. Antigens for assay were treated with detergent [Mulgofen BC-720, GAF (Great Britain) Ltd.; 2% final concn., 30 min at room temperature] to release glycoprotein antigen and added to wells in immunoplates in 10 µl or 20 µl volumes. After allowing diffusion for 24 h the gels were washed in PBS for 48 h, dried and stained in
0.3% Coomassie Brilliant Blue to demonstrate zones of antibody–antigen reaction. The diameters of the stained zones were measured using a Transidyne calibration viewer (Transidyne General Corporation, Ann Arbor, Mich., U.S.A.) coupled to Autodata caliper equipment (Autodata, Hitchin, U.K.)

Anti-glycoprotein sera were prepared by immunization of animals with purified rabies (ERA) glycoprotein obtained by treatment of purified virus with Triton X-100 detergent and purified by isoelectric focusing (Dietzschold et al., 1978). This glycoprotein produced a single protein band of mol. wt. approx. 80000 when examined by electrophoresis on SDS–polyacrylamide gels (Laemmli, 1970). Two or three doses (approx. 20 μg glycoprotein) were given, the first dose with Freund’s complete adjuvant, and the animals were bled one week after the final injection.

An immune rabbit serum (virus neutralization titre 1:500000) was found satisfactory for use in SRD tests when incorporated in agarose gels at concentrations in the range 5 to 10 μl/ml gel. Studies carried out using an immune sheep serum (5 μl/ml) gave similar results.

Well-defined SRD zones were observed when serial dilutions of purified glycoprotein (ERA) were added to the wells of immunoplates (Fig. 1a). The minimum protein concentration at which visible stained zones were obtained was approx. 0.7 μg/ml. Similar zones were obtained with dilutions of ERA rabies virus which had been purified by gradient centrifugation (Fig. 1a) and also with the HEP, Pitman-Moore, CVS and Kelev strains of rabies virus (data not shown).

When annulus area was plotted against antigen dilution for these preparations linear dose response curves were obtained with the lines meeting at a common intercept on the d2 axis (Fig. 1b). These dose-response curves and the position of their intercepts were similar to those previously observed in SRD tests with influenza haemagglutinin (Wood et al., 1977). The relative potency of vaccines with reference to a standard may thus be calculated on the same basis as for influenza vaccines:

\[
\text{Vaccine potency} = \frac{\text{slope of vaccine curve}}{\text{slope of standard curve}} \times \text{potency of standard vaccine}
\]

Inactivated rabies vaccines for prophylactic and post-exposure treatment of humans in many countries are now derived from human diploid cell strains (HDCS) infected with the Pitman-Moore strain of rabies virus. Vaccines are prepared following concentration of tissue culture fluids by ultrafiltration (Nicolas et al., 1978) or by continuous flow rate-zonal ultracentrifugation (Majer et al., 1978). Current World Health Organization (WHO) recommendations are that vaccines should contain at least 2.5 IU of rabies antigen when compared in the NIH test to the WHO third international reference preparation of rabies vaccine to which has been assigned an arbitrary potency of 10 IU (WHO, 1979). The WHO reference antigen (a lyophilized vaccine prepared by ultracentrifugation) and vaccines prepared from tissue-culture fluid which had been concentrated by ultrafiltration were tested. Typical dose-response curves are shown in Fig. 1(b). Small but measurable zones were detected when the WHO reference antigen was diluted 1:8. The SRD test thus appeared to be capable of detecting antigen concentrations of approx. 1 IU and was therefore sufficiently sensitive to evaluate the glycoprotein content of vaccines.

In order to investigate whether the glycoprotein antigen was present on intact virus or as free glycoprotein, vaccines were centrifuged in 15 to 55% sucrose gradients for 2 h at 35 K in the SW41 rotor of the Beckman L5-50 centrifuge. Fractions (0.4 ml) were collected and examined for SRD activity in the presence and absence of detergent. The distribution of SRD activity in samples treated with detergent is shown in Fig. 1(c). In both types of vaccine a peak of SRD activity was detected at the density corresponding to that of intact rabies virus.
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Table 1. Relationship between antibody responses to rabies vaccine in human volunteers and vaccine potency detected by SRD and NIH test

<table>
<thead>
<tr>
<th>Vaccine potency</th>
<th>Antibody responses§</th>
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<tr>
<td><strong>SRD test</strong></td>
<td><strong>NIH test</strong> (IU)*</td>
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<tr>
<td></td>
<td>(IU)*</td>
</tr>
<tr>
<td>4.4</td>
<td>10.5</td>
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<tr>
<td>3.7</td>
<td>6.8</td>
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<tr>
<td>3.4</td>
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* Estimated by comparison with the third international reference preparation of rabies vaccine (10 IU).
† Glycoprotein content estimated by comparison with preparation of purified glycoprotein with known protein concentration (Bramhall et al., 1969).
‡ Adult volunteers received two doses subcutaneously of 1 ml of vaccine prepared as described by Nicolas et al. (1978). Vaccine given at day 0 and day 30, serum samples for antibody assay collected 60 days after first vaccine dose.
§ Serological data kindly provided by Dr A. Nicolas, Merieux Institute, Lyon, France. Antibody titres assessed by mouse neutralization tests in comparison with the WHO international reference preparation of rabies immunoglobulin.

Particles (1.18 g/ml). No SRD activity was detected in these fractions if detergent treatment was omitted, suggesting that the antigen was associated with intact virus which would not be expected to diffuse in agarose gels. Examination of the fractions by electron microscopy confirmed that intact rabies virus particles were present.

For vaccines concentrated by ultrafiltration but not in those prepared by ultracentrifugation, antigen activity was also detected in gradient fractions 22 to 27. This slowly sedimenting antigen was detectable even when treatment of the fractions with detergent was omitted, suggesting that it was associated with subviral particles. The presence of such 'soluble' antigen in tissue culture fluids from which rabies virus has been pelleted has been described previously (Wiktor et al., 1969), but its relevance to vaccine efficacy requires further study.

Evidence that the glycoprotein antigen was quantitatively removed from rabies virus particles by treatment with Mulgofen (2 %) was obtained from experiments in which virus particle antigen was detergent-treated and sedimented on sucrose gradients containing 1 % Mulgofen. In these studies, all antigen activity detected by SRD resided in fractions 22 to 27. No SRD activity was detected in fractions 13 to 18, corresponding to the sedimentation characteristics of intact virus.

For three batches of vaccine prepared following ultrafiltration and tested in our laboratory, SRD potencies varying from 3.5 to 10.5 IU per ml were detected. The glycoprotein content (µg/ml) of these vaccines was calculated from the SRD activity by comparison with a preparation of purified glycoprotein (Table 1). These vaccines were tested in clinical trials involving adult volunteers and antibody responses for each group are shown in Table 1. The results showed correlation between antigenic content of the vaccine detected by SRD and the titre and frequency of antibody responses in vaccines.

Our studies indicate that SRD techniques are of potential value for the assay of rabies glycoprotein antigen for research purposes and for the quantitative and qualitative testing of rabies vaccines. SRD tests are rapid and simple and have the advantage that they provide an alternative assay system reducing reliance on the use of animal protection tests employing infectious virus. However, in vitro tests of antigenic content of vaccines do not provide unequivocal evidence of immunogenicity and tests on vaccines should include confirmation of their immunogenicity in animals until considerable satisfactory experience is gained with a given product. The use of reference antigens calibrated in terms of µg/ml of rabies
glycoprotein antigen by biochemical methods would enable rabies vaccine potency to be expressed in defined terms (µg antigen activity per dose) as is the case for inactivated influenza vaccines (Schild et al., 1975), rather than in arbitrary international units. Further information is required on the correlation between antigen content of all available types of vaccines as determined in SRD tests and their immunogenicity in experimental animals and man. Such studies are in progress in our laboratory.

Part of this work was carried out when M.F. was a visiting worker at the Wistar Institute, Philadelphia, Pennsylvania, U.S.A. We would like to thank Dr H. Koprowski, Dr T. J. Wiktor and Dr W. H. Wunner of the Wistar Institute for providing the rabbit antiserum, purified ERA rabies glycoprotein and purified rabies virus preparations. We would also like to thank Dr J. Wood, NIBSC for many helpful discussions.

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REFERENCES

(Received 30 June 1981)