Antiviral Research, 1 (1981) 27–35
Elsevier/North-Holland Biomedical Press

COMPARISON OF POLIOVIRUS ANTIGEN MEASUREMENTS IN VITRO AND IN VIVO. PRELIMINARY WORK TOWARDS THE STANDARDISATION OF INACTIVATED POLIOVIRUS VACCINE POTENCY

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(Received 3 September 1980; accepted 24 November 1980)

The potency of various batches of inactivated poliovirus vaccines have been determined in vitro using an enzyme-linked immunosorbent assay (ELISA), and the results compared with in vivo potency values obtained in guinea pigs and expressed as antibody titres and antigen extinction limits, revealing three possible expressions of vaccine quality. The purpose of the study was to collect information needed to regulate the vaccine dose within a standard potency range using all three values obtained. The results expressed in D antigen units as determined by ELISA suggest that this technique is more sensitive in measuring differences in antigen concentration than either of the methods chosen to evaluate vaccine potency in vivo.

standardisation inactivated poliovirus vaccine potency

INTRODUCTION

Effective immunisation against poliomyelitis has been achieved by the use of two distinct vaccines, the live attenuated vaccine (SABIN) and the inactivated poliovirus vaccine (SALK). Increasing attention is being given in the more developed countries to the use of the inactivated poliovirus vaccine (IPV) which may carry a greater benefit to risk ratio than that offered by immunisation with the live attenuated virus alone [11].

An essentially linear relationship has been shown to exist between antibody response and quantity of IPV antigen administered to children [12], suggesting that the concentration of antigen per dose of vaccine administered is one of the critical features of effective immunisation. Producing the IPV with a standard dosage requires the use of sensitive techniques to establish and regulate antigenic mass and immunogenic capacity.

The current method for the detection of poliovirus antigens by immunogel-diffusion [2] requires an initial 50–100-fold concentration of samples before testing, resulting in an assay which is impracticable to use routinely. There have been many reports of more sensitive techniques for the detection of other virus antigens by radioimmunoassay [10] and ELISA [7, 15]. This has prompted us to develop a micro ELISA for the direct assessment of poliovirus antigens, which would obviate the need for the prior concentration of samples.

The D antigen is considered the salient part of poliovirus vaccine [1–3], although the

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conformation of viral polypeptides responsible for eliciting protective antibody in vivo is not known [4]. We were interested in the sensitivity of the ELISA in evaluating the D antigenicity of our preparations compared with the current method of measuring immunogenicity in vivo as used in this laboratory.

MATERIALS AND METHODS

Reference antigens and test samples

In order to calibrate the D antigen concentration obtained by ELISA with that observed by immunogel-diffusion, reference antigens of established D antigen content for type 1, Mahoney, type 2, MEF, and type 3, Saukett (kindly supplied by the Rijksinstituut voor de Volksgezondheid, Bilthoven, The Netherlands) were employed. These reference preparations were used to evaluate the D antigen content of our own references type 1, Brunhilde, type 2, MEF, and type 3, Saukett. All reference preparations were stored at -80° until required.

Test samples for potency testing by ELISA were taken at the termination of the processing of single batches of each IPV monovalent type prepared in this department. Potency tests in guinea pigs were carried out using mixtures of single batches of monovalent poliovaccine preparations in the proportion type 1 : type 2 : type 3, 7 : 1 : 4 by volume respectively, and the final Di-Te-Pol vaccines which were prepared by mixing 14 : 2 : 8 batches (each of equal volume) of monovalent poliovaccine preparations type 1 : 2 : 3 respectively, together with diphtheria and tetanus toxoid, adsorbing with $AL(OH)_3$ and concentrating 2–2.25 times.

Sera

Pools of hyperimmune monkey or rabbit sera for the three poliovirus types were obtained from the Enterovirus department. They were used for coating of the solid phase in ELISA after dilution (types 1 and 2) or for the preparation of IgG for coating (type 3) and the preparation of enzyme conjugates.

Preparation of conjugates

IgG was purified from hyperimmune sera by ammonium sulphate precipitation followed by DEAE-Sephadex A50 chromotography and labelled with horseradish peroxidase (HRPO) by the method of Wilson and Nakane [16]. Assessment of the HRPO-IgG conjugate fractions eluted from Ultrogel AcA 34 included separate testing of each fraction collected, pooling only those giving a low background and high specificity for the particular antigen.

ELISA procedure

Disposable polyvinyl plates were used as solid phase. Optimal dilutions of serum and conjugate were determined by checkerboard titrations. Wells of the plates were coated with 75 μl of either hyperimmune whole serum from monkeys used at a dilution of

1/10,000 (type 1 and type 2 strains) or hyperimmune IgG from rabbits used at a dilution of 1/1000 (type 3 strain), and the plates were incubated at 4°C for 4 h in a humidified box. After washing three times with phosphate-buffered saline (PBS)–Tween the wells were filled with PBS containing 1% (w/v) bovine serum albumin and left overnight at 4°C. After additional washings, dilutions of the antigen reference or samples to be tested (25 μl) were added to the wells. On each plate a boiled antigen control was included for background determination. After overnight incubation at 4°C, the plates were washed as before, conjugate was added at an appropriate dilution in foetal calf serum (50 μl) and left at room temperature for 2 h. After thorough washing, substrate was applied (100 μl of orthophenylenediamine, 0.4 mg/ml with 0.006% peroxide in citrate buffer pH 5.0), and following 30 min incubation at room temperature 2 M sulphuric acid (50 μl) was added to each well and the optical density at 493 nm was measured in a spectrophotometer with a rapid sampling microcuvette (Gilford Model 250). Unreacted substrate was used as a blank. For specificity testing and to investigate the presence of C antigen in these preparations, a blocking step was included with specific anti-D or anti-C sera before conjugate was applied. Details of this method have already been described [8].

Potency test in vivo

One ml amounts of undiluted and serial tenfold dilutions of the mixtures of monovalent vaccines (a) or Di-Te-Pol (b) vaccine were injected subcutaneously into groups of five (a) or ten (b) 250–350 g guinea pigs following an immunisation schedule described by Gard et al. [5] and Olin [9]. Two weeks later the injection was repeated. Seven days after the second injection the animals were bled by heart puncture. The collected sera were inactivated at 56°C for 30 min and stored at 4°C until required.

Determination of antibody titre

Sera from guinea pigs injected with undiluted preparations were titrated using Cooks microtitrating apparatus and tested in dilution $2^{-1}-2^{-12}$ for neutralizing antibodies against the three types of poliovirus. For each serum dilution two cups, each containing 0.05 ml of serum dilution and 0.05 ml of virus dilution ($10^{-2} \pm 0.5$ $TCID_{50}$) were incubated at 37°C for 3 h before 0.05 ml of Vero cell suspension (5×10^4 cells/ml) was added to each cup. The cups were sealed with tape and the plates incubated at 37°C for 7 days when microscopical examination of all cups for cytopathic effect (CPE) was carried out. Neutralisation was considered to have occurred if no virus degeneration of cells was found and all virus control cups had shown CPE. The neutralising titre for each guinea pig was calculated using Kärber's [6] method and the geometric mean for 5 (a) or 10 (b) animals inoculated with the same vaccine was calculated. The minimum requirement for all three types of inactivated poliovirus vaccine since 1958 was an antibody titre of log 1.5 ± 0.5. The confidence limit for the test was always included when evaluating the animals recorded response, in this case making the minimum acceptable antibody titre log 2.0.

Antigen extinction limit

Sera from guinea pigs injected with tenfold dilutions of vaccine preparation were tested in dilutions 2^{-1} or neutralising antibodies to each of the three types of poliovirus. Two cups per serum were used and neutralisation was considered to have occurred if at least one of the two cups showed no CPE. The results were recorded as extinction limit values, i.e., the log of the reciprocal of the dilution-inducing antibodies in 50% of the inoculated animals. The minimum requirements for all three types of inactivated poliovirus vaccines since 1958 was an antigen extinction limit of 2.0 ± 0.5, which after including the confidence limit for the test gave a minimum acceptable value of 2.5.

RESULTS

A good correlation has been claimed between the results obtained from D antigenicity determinations by ELISA and by gel-diffusion techniques [13]. In this report, reference preparations of known D antigen content as determined by gel-diffusion were used in the ELISA to obtain a standard curve of absorbance value against D antigen concentration (Fig. 1) everytime the test was performed.

A linear correlation between optical density and antigen concentration could normally be observed between 0.5 and 2.0 D antigen units/ml after which some tailing off was observed (Fig. 1), dependent on room temperature. This necessitated the diluting of test samples to ensure that values obtained were within the linear range of the standard curve. Our investigations have shown that ELISA can detect as little as 0.5 D units/ml of sample for type 1 and 2 strains and 1.0 D units/ml of sample for type 3 strains, as well as being specific for the poliovirus type (data not shown).

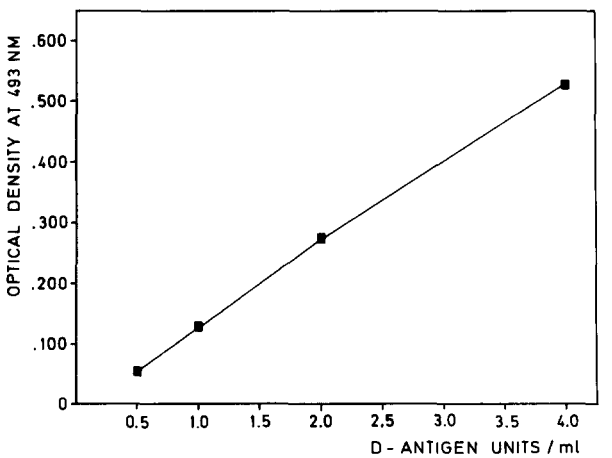

Fig. 1. Standard curve of D antigen concentration in units/ml of type 1 poliovirus Mahoney reference against optical density at 493 nm as observed in ELISA. Each point represents the average of 10 determinations on the same plate.

Using this technique, the antigenic content of some vaccine preparations have been assessed and the results compared with the estimated immunological capacity expressed in antibody titres and antigen extinction limits (Figs. 2–4) as determined in guinea pigs. It should be remembered that only antibody capable of preventing CPE in vitro is being scored in these tests.

While there appears to be reasonable agreement between good and bad vaccine preparations in terms of assigned values in both the in vivo tests and the ELISA, occasionally the D antigen value is seen to half without any reduction in potency as assessed in guinea pigs (Fig. 3, preparations 2 and 6; Fig. 4, preparations 10 and 14).

Antibody titres for Saukett preparations, when compared to Brunhilde and MEF, were lower in relation to the antigen extinction limit values observed for the same samples. A similar observation was made in the final Di-Te-Pol vaccine (Fig. 5) tested in the same manner, included here to indicate the final values reached in this test before the vaccine is released for use.

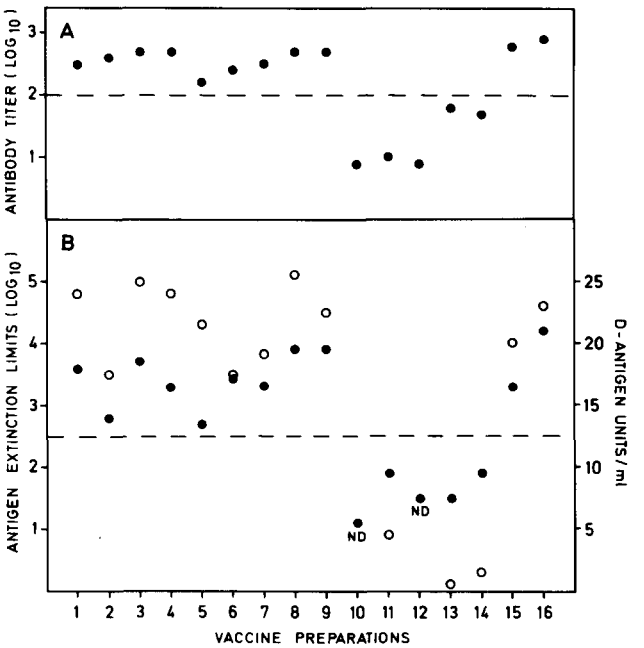

Fig. 2. Antigenic potency in vivo and in vitro of poliovaccine prepared from Brunhilde. Samples of vaccine preparations were injected subcutaneously into guinea pigs, and collected sera titred to give the end point (A,●). Serial tenfold dilutions up to 10^{-4} of the same sample were injected into guinea pigs and the collected sera tested at dilutions 2^{-1} for neutralising antibodies to the virus antigen (B,●). The same vaccines were tested in ELISA for D antigenicity and the results recorded as D units/ml of undiluted vaccine (B,○). ND = not detectable in ELISA in undiluted form. The broken lines represent the minimum requirements for antigenic potency in vivo, including the confidence limit for the test.

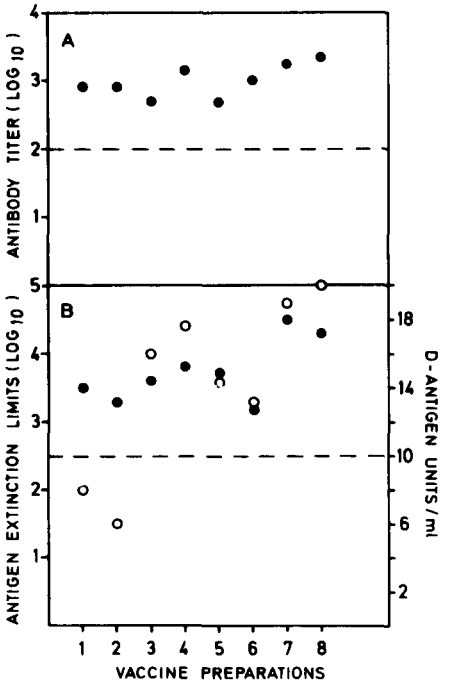

Fig. 3. Antigenic potency in vivo and in vitro of poliovaccines prepared from MEF. ○, D units/ml. For further details see legend of Fig. 2.

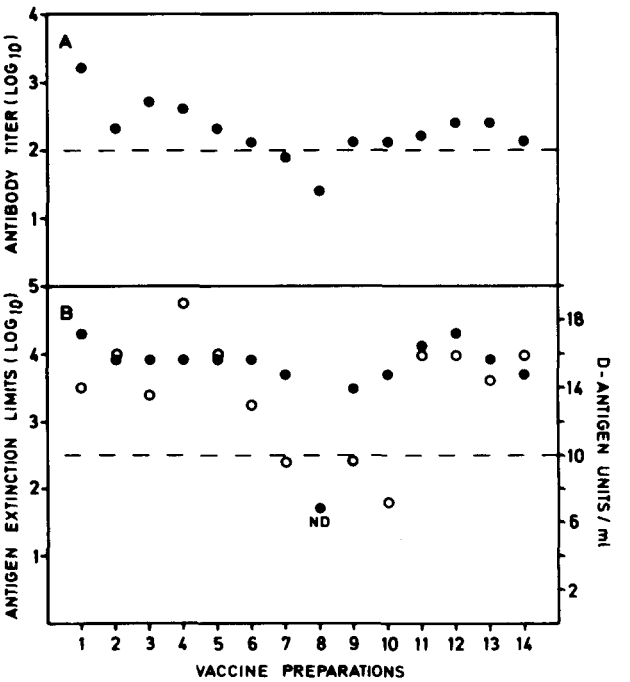

Fig. 4. Antigenic potency in vivo and in vitro of poliovaccine prepared from Saukett. ○, D units/ml. For further details see legend of Fig. 2.

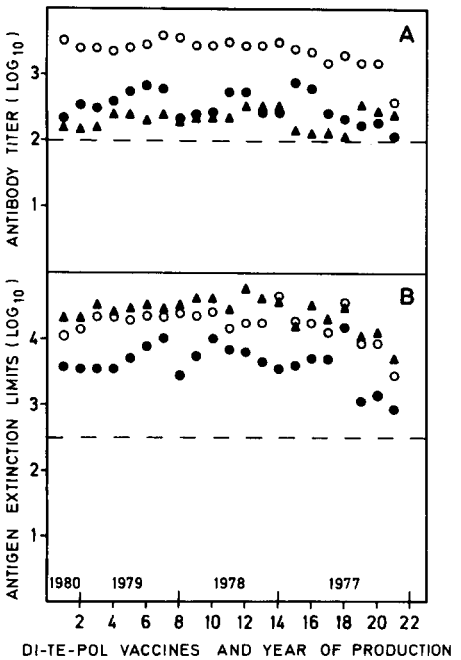

Fig. 5. Potency values for Di-Te-Pol vaccine obtained in guinea pigs. Survey of values obtained since 1977. ●, type 1; ○, type 2; ▲, type 3. Broken line represents the minimum requirement including the confidence limit for the test.

DISCUSSION

Two different techniques have been chosen to measure immunogenic capacity: a) measurement of antibody titres after administration of a fixed amount of test material, and b) titration of the test material in animals to establish the smallest amount needed to elicit detectable antibody at a fixed dilution. The antigen extinction limit has been reported as a more reliable evaluation of potency when booster injections are given [5]. In our hands, both techniques appeared equally valuable for type 1 and 2 strains with Saukett responding better in the antigen extinction test, for reasons which are not understood.

It is clear that individual batches of vaccine preparations differ in their potency as evaluated by all three methods. The sensitivity of the guinea pig in responding to low doses of vaccine is demonstrated by their ability to detect 10^{-4} dilutions of a preparation shown to contain 23 D units/ml in the ELISA (Fig. 2, sample 16). However, two-fold changes in antigen concentration gave no detectable differences in response in these animals. It is possible that the programme used for inoculation of the vaccine and subsequent bleeding time chosen post inoculation may have influenced its detectability. Recent evidence suggests that another species of animal, Wistar rats, may be more suitable

for detecting antigenic mass differences in this range if a single dose of poliovirus antigen is used [14]. The regulation of our vaccines by a fixed D antigen value will have to await the selection of a suitable animal, an inoculation and bleeding programme which permits confirmation of the ELISA test, and some knowledge of the comparative immunogenicity of the vaccines in children and the experimental animal chosen.

Evidently, the minimum requirements established for the in vivo potency test come very close to the limits for in vitro detection of antigen in the ELISA. Most of our vaccines prepared today have higher values than the local established minimum requirement used since 1958, particularly in the antigen extinction limit test. It would now be desirable to have a minimum in vivo potency value established which should also be expressed in D antigen units measurable in a sensitive in vitro test.

In the past, the use of a minimum requirement has ensured that the product met the current standard. Today, it is felt that the limit requires readjustment, together with the declaration of an accepted ceiling for antigen dose, dependent on the number of doses to be administered. Minimum potency requirements need to be established for each of the three different poliovirus strains, and in this respect it would be helpful if an international reference poliovirus vaccine standard, tested in children, were available to be used as a guide in vaccine production and in the standardisation of the final vaccine product.

ACKNOWLEDGEMENT

This work was supported by a grant from the Danish Medical Research Council (No. 512–20105).

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