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The evaluation of a physical method for the quantification of inactivated poliovirus particles and its relationship to D-antigenicity and potency testing in rats*

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The use of a density gradient procedure for the quantification of intact, inactivated poliovirus particles in vaccine preparations is described. The procedure is both sensitive and highly reproducible and the results correlate with those of potency tests in rats and with D-antigen content as measured by ELISA. Because of the occasional ambiguity observed with D-antigen assays, it is suggested that the density gradient procedure will provide valuable additional information for the *in vitro* assessment of inactivated poliovirus preparations.

INTRODUCTION

Intact, 160S poliovirus particles which carry the so-called D-antigen, induce neutralizing antibodies and are regarded generally to be essential for the production of protective immunity in vaccinated individuals.[1] Although there are reports of inconsistencies in the relationship between D-antigenicity and neutralizing antibody titre,[2,3] information on D-antigen content has proved useful in the context of the production of inactivated poliovirus vaccine and potency testing in laboratory animals. For this reason, a variety of immunoassays has been used for the quantification of D-antigens. These include conventional gel diffusion,[4] ELISA,[5] radioimmunoassay (RIA)[6] and

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single radial diffusion with zone enhancement.[7] In the Rijksinstituut voor de Volksgezondheid (RIV) gel diffusion was the method of choice until relatively recently but has now been superseded by an indirect ELISA procedure.[5]

In the case of foot-and-mouth disease virus (FMDV) there is overwhelming evidence that the intact 146S virus particle is essential for inducing protective immunity[8] although particles such as the so-called natural empty virus particles produce useful levels of immunity in vaccinates.[9] In contrast to poliovirus, however, a physical method is now widely used to quantify 146S particles in virus preparations destined for FMDV vaccines. The method, based on the initial work of Barteling & Meloen,[10] involves the centrifugation of a crude virus preparation in a density gradient and subsequent continuous fractionation of the tube contents through an ultraviolet spectrophotometer. The concentration of 146S particles in the original sample is proportional to the area of the absorbance peak above the absorbance background due to cell debris and media components.

Because of the considerable success of this physical method in the area of FMDV vaccine production and the occasional ambiguity observed between measurements of D-antigenicity of poliovirus and potency tests in laboratory animals[2,3] we decided to evaluate the potential of the physical method for quantification of intact poliovirus in preparations destined for killed poliovirus vaccine.

MATERIALS AND METHODS

Virus preparations

Virus materials used in this study were formalin-inactivated poliovirus preparations type 1 Mahoney, type 2 MEF and type 3 Saukett. Samples of the monovalent preparations were taken at the end of the processing cycle of routine production batches of killed poliovirus vaccine produced in the RIV by the microcarrier culture technique.[11]

ELISA

D-antigenicity was measured by an indirect ELISA.[5] Briefly, microtitre plates were coated with calf anti-polio IgG and the diluted, monovalent poliovirus preparation was added and incubated. This was followed by incubation with rabbit anti-polio antiserum and, finally horseradish peroxidase conjugated sheep anti-rabbit IgG. Using calibration curves constructed from ELISA data obtained from reference antigen preparations, the number of D-antigen units per millilitre of sample was determined. In this procedure there was a high specificity for D-antigen whereas C-antigen gave almost no reaction.[5]

Potency testing in rats

This was performed as described by van Steenis *et al.*[3] with minor modifications. Threefold dilution steps were employed with ten rats at each dilution. The rats were obtained from a colony which is free of known viral pathogens.[12] To restrict the number of rats, trivalent vaccine was formulated, using a ratio of 80:8:32 D-antigen units per millilitre of types 1, 2 and 3 respectively, and administered at two intramuscular sites per rat, 0·5 ml per site. Three weeks after inoculation, the animals were bled and the levels of neutralizing antibody determined in a micro-neutralization

test using Vero cells and 100 $TCID_{50}$ of virus. By reference to a standard trivalent vaccine containing a known number of D-antigen units (by ELISA) and incorporated into each potency test, rat D-antigen equivalent units were derived in a parallel line assay.

Density gradient analysis

This was performed as described by Doel, Fletton & Staple[13] with minor modifications. Continuous linear sucrose gradients from 5 to 30% sucrose w/v in 0·05 M Tris–HCl, 0·15 M NaCl, pH 7·6 were used. The volume of sample applied to each gradient was approximately 0·5 ml (determined accurately by weighing) and gradients were centrifuged at 40 000 r min^{-1}, for 55 min at 10°C in a Beckman SW40 rotor. The contents of each centrifuge tube were pumped through an 0·2 cm flow cell of a Beckman model 25 spectrophotometer set to 260 nm. Peak areas were computed from the chart record with the aid of a graphics tablet interfaced to an Apple microcomputer and the calculation of micrograms of poliovirus per millilitre of original sample made using $E^{1\%}_{1\,cm,\ 260} = 81{\cdot}6$.[14]

RESULTS

In preliminary experiments it was established that typical preparations of killed poliovirus gave discrete and quantifiable absorbance peaks by the sucrose gradient procedure.

The sensitivity of the gradient system was evaluated by preparing a twofold dilution series of each type of poliovirus and quantifying the intact poliovirus at each dilution. To minimize losses of virus in highly dilute solutions through adsorption to plastic surfaces, the Tris–HCl, NaCl diluent was supplemented with a 1% v/v final concentration of normal ox serum. The results are shown in Fig. 1 and indicate good linearity for all three types between approximately 3·5 and 0·1 μg of poliovirus per gradient. Data from the undiluted starting materials and samples of 1 ml rather than 0·5 ml applied to the gradients indicated good linearity up to approximately 14 μg of poliovirus in the peak (data not shown). The lower limit in the system was of the order 0·1 to 0·3 μg of virus per gradient, below which the peaks were too small to be easily differentiated from the background absorbance.

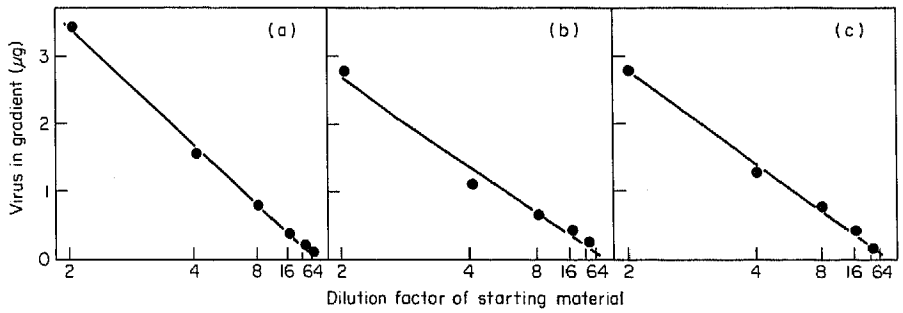

Fig. 1. The sensitivity and linearity of the density gradient procedure for the quantification of intact poliovirus particles in vaccine preparations.

The reproducibility of the gradient procedure was examined in a second series of experiments. Table 1 shows the results obtained with seven preparations of poliovirus, each preparation being analysed in triplicate. This work was extended by pooling the seven preparations and analysing the pool in four groups of six gradients over a period of approximately three weeks. Table 2 shows that the within and between group variation was low. Furthermore, the overall mean value of 12·12 μg ml^{-1} obtained from the 24 gradient experiment is in close agreement with the value of 11·94 μg ml^{-1} calculated from the data shown in Table 1.

TABLE 1. The quantification of intact poliovirus particles in seven different preparations by the density gradient procedure

Poliovirus type and batch number	Poliovirus original sample in gradient (μg ml^{-1})			
	1	2	3	Mean
1 PU80-146/147-6·1	10·20	10·61	9·30	10·04
1 PU82-164-6·1	16·70	16·19	15·93	16·27
1 PU82-165-6·1	7·69	7·68	6·39	7·25
2 PU81-224-6·1	10·78	9·94	8·67	9·80
2 PU82-225-6·1	16·05	15·68	14·21	15·31
3 PU81-328-6·1	13·71	14·64	14·33	14·23
3 PU81-329-6·1	10·30	10·34	11·60	10·65

TABLE 2. The quantification of intact poliovirus particles in a pool made from the seven preparations shown in Table 1

Day of experiment	Poliovirus original sample in gradient (μg ml^{-1})						
	1	2	3	4	5	6	Mean
0	11·65	11·80	12·32	12·26	11·83	12·56	12·07
7	12·68	11·70	12·12	12·39	12·35	11·61	12·14
13	11·57	11·62	12·41	12·62	12·16	12·77	12·19
19	11·56	12·60	12·07	11·96	11·97	12·35	12·09

Observed overall mean = 12·12 μg ml^{-1}; calculated overall mean = 11·94 μg ml^{-1} (from Table 1).

The low between-group variation shown in Table 2 is also supported by other experiments in which poliovirus type 1, 2 and 3 preparations stored at 4°C were analysed by the gradient procedure on three separate occasions over a five-month period. No significant changes in virus concentration were observed (data not shown).

In the last series of experiments we examined the potency in rats of inactivated type 1, 2 and 3 poliovirus preparations heated at 45°C for various times in relation to the D-antigen content determined by ELISA and the intact virus particle content deter-

mined by the density gradient procedure. The choice of 45°C was due largely to the fact that heating at 37°C for up to 14 days did not cause any apparent reduction in either the content of D-antigen or intact virus particles, indicating the high stability of the three monovalent vaccine preparations at 37°C (data not shown).

The results obtained with the 45°C procedure are shown in Fig. 2. The lower stability of type 1 virus relative to type 2 and 3 viruses in terms of all the parameters measured is clearly demonstrated. Because of the rapid degradation of type 1 virus, it is difficult to draw any further conclusions from this set of data.

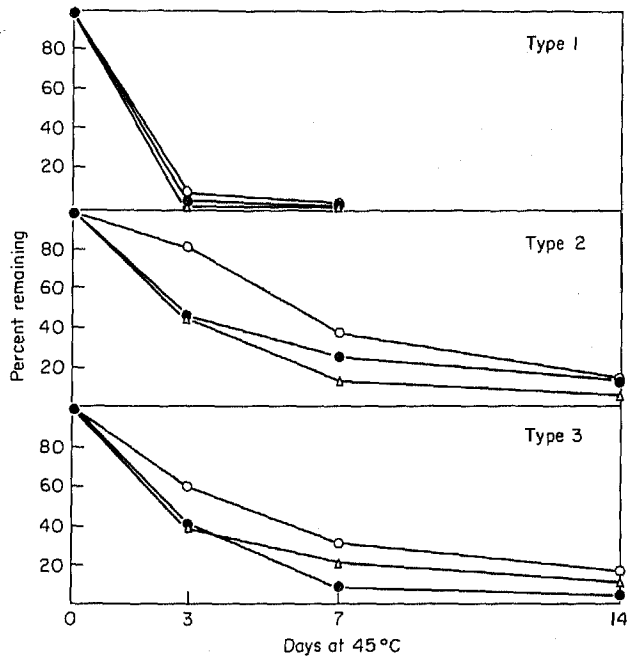

Fig. 2. The effect of heating at 45°C on three poliovirus preparations in terms of potency in rats (●), D-antigen content (○, ELISA) and intact poliovirus particle content (△, density gradient analysis). Values at three, seven and 14 days are expressed as a percentage of the value at 0 day.

The results with the type 2 and 3 viruses, however, appeared to indicate a closer relationship between the rat test and the density gradient test than between either of these and the ELISA D-antigen test. There was a tendency for the correlation between the rat test and the density gradient test to decrease after seven days at 45°C. It should be noted that the potency test was conducted with trivalent preparations to restrict the number of rats to a reasonable level whereas both the density gradient and ELISA tests were conducted with the monovalent preparations.

DISCUSSION

In this paper we have demonstrated the use and potential of a density gradient procedure for the quantification of physical, intact poliovirus particles in inactivated vaccine preparations. The procedure is simple, rapid (requiring approximately two

hours for six samples) and independent of special reagents such as immune sera. The minimum concentration of poliovirus detected was approximately 0·1 μg per gradient and good linearity and reproducibility were observed up to approximately 14 μg per gradient. From studies with FMDV,[9] virus concentrations up to at least 70 $\mu g\ ml^{-1}$ of original samples may be quantified.

Although our procedure has given good results with poliovirus concentrations of approximately 0·1 μg per gradient, we would not advocate its regular use with preparations containing such low levels of virus, for example, the original virus suspensions from which the inactivated vaccines were derived. Rather, for virus concentrations between 0 and 0·5 $\mu g\ ml^{-1}$, it would be preferable to increase the sensitivity of the density gradient procedure. This may be achieved by (a) use of 1·0 to 1·5 ml sample per gradient rather than 0·5 ml, (b) use of 0·5 cm or possibly 1·0 cm pathlength flow cells rather than 0·2 cm, and (c) use of a spectrophotometer with a higher sensitivity, e.g. 0·005 optical density (OD) full scale deflection rather than 0·05 OD.

The results obtained with virus preparations heated at 45°C for different times (Fig. 2) established clearly a correlation between the potency test in rats, the number of physical poliovirus particles and D-antigenicity as measured by ELISA. Furthermore, results with the type 2 and 3 viruses appeared to indicate generally a closer relationship between the rat test and the density gradient test than between either of these and the ELISA test. The tendency for the correlation between the rat test and the density gradient test to decrease at seven to 14 days may be due to one or more of a number of factors; for example, the variability of the rat test, the presence of immunogenic residues of degraded virus which would not be detected in the density gradients at 260 nm and, finally, the possibility of heterologous contributions to the apparent potency of each virus type. It will be recalled that the rat test was conducted with trivalent vaccine whereas the density gradient and ELISA D-antigen procedures were conducted with monovalent preparations. Although considerably more experimentation is required to resolve fully the relationships among the three test systems, there is little doubt that the density gradient procedure will prove valuable for *in vitro* testing of inactivated poliovirus vaccines. The only obvious limitation of the density gradient procedure is that it cannot be readily used to quantify each virus type in a trivalent vaccine, unlike ELISA and the potency test in rats. However, combined density gradient/ELISA procedures should circumvent this problem.

A particular advantage of the density gradient procedure is its suitability for standardization. This is due, in part, to the fact that measurement involves a physical particle with an absolute extinction coefficient and also to the lack of requirement for D-antigen specific antisera. The spectrophotometer is easily calibrated with appropriate standards, for example adenine HCl (Doel & Mowat, unpublished results) and the density gradient element may be standardized either with a reference preparation of poliovirus stored at a suitable temperature or, alternatively, a purified preparation of another virus of known concentration such as MS2 ribophage. Such a standardization exercise has already taken place among 13 laboratories with FMDV and MS2 (Doel & Mowat, unpublished results) and could be readily adapted for use with inactivated poliovirus vaccines.

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