

## Properties of Defective Interfering Particles Induced by Photodynamic Treatment on Pseudorabies Virus

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### Introduction

The term "defective virus" has been applied in a number of ways to indicate nonreplicating particles and viruses that are structurally different and functionally deficient from standard virions. Van Magnus first described such influenza virions in 1954 as being "incomplete" (24) because they were noninfectious hemagglutinating particles. That repeated passage of undiluted virus in tissue cultures would result in the production of defective interfering (DI) particles was demonstrated with herpes simplex virus (3), pseudorabies virus (1,2), and Newcastle disease virus (NDV) (2). The alteration of DNA structure in DI particles was observed in HSV and PrV only. However, the interference of DI particles with the growth of standard virus was reported for all three viruses. It would be of considerable interest to determine the mechanism of the interference. Subsequent information also suggested that the host cell plays a very critical role in the production of DI particles and the nature of their defectiveness (2,5,17,23).

In this communication we describe isolation and characterization of a population of DI PrV particles prepared with a photodynamic method.

### Materials and Methods

Pig kidney cells (PK-W2E) derived from 3-week-old pigs were used at passage 70 in the studies. The cells were grown in flasks with Eagle's medium containing 10% fetal bovine serum, 100 u of penicillin and 170 mg streptomycin per ml at pH 7.4 and maintained in a similar medium containing 2% fetal bovine serum.

The FH strain of PrV was used throughout the study. Virus infectivity was assayed in PK cells by determination of the 50% tissue culture infective doses (TCID<sub>50</sub>) or by measuring the plaque forming units per ml (pfu/ml) at 48-120 hrs. post exposure.

The photodynamic treatment of virus was that described previously in detail by Sun *et al.* (19).

Infected cell cultures were frozen and thawed three times after which the collected material was centrifuged at 1500 rpm for 15 min. The virus in the supernatant fluids was sedimented in a sucrose gradient as described previously (19). Optical density (OD) readings were taken immediately, after drop collections, at 280 m $\mu$  and 260 m $\mu$  with Gilford Instrument's spectrophotometer (model 2400-5).

DNA of purified virions as described above was extracted by the sodium dodecyl sulfate (SDS) chloroform-isoamyl alcohol method essentially according to Marmur (15).

DNA prepared from Marmur's method was centrifuged in a 5-20% sucrose gradient for 16 hr. at 13,000 rpm. OD reading was taken at 260 m $\mu$  Gilford Instrument's spectrophotometer (model 2400-5).

Crossed immunoelectrophoresis was done as described by Vestergaard (22)

with a few modifications. Glass plates (9.4 cm by 9.4 cm) were used and were covered with 1.5 mm thick 1% (wt/vol) agarose (Nutritional Biochemicals Corporation) dissolved in a buffer as described previously (20,25). The first dimensional electrophoresis was performed in 90 min. at 17 v/cm of gel and the second dimensional electrophoresis was completed in 15 hr. at 7 v/cm of gel. The plates were then dried, stained, and treated as described. (20,25). In the first-dimensional electrophoresis, the agarose gel, both a neutral detergent, Triton X-100 (0.5%) and an anionic detergent, sodium deoxycholate (0.05%) were incorporated. In the second dimensional electrophoresis, gel contained purified anti-PrV gamma-globulin prepared as described previously (21).

### Results

Samples of infected cells were withdrawn at the time intervals of visible light irradiation indicated in TABLE 1. The OD of cell suspensions were then measured at 540  $m\mu$ . After 45 min. of irradiation, the efficiency of host-cell lysing ability dropped to 13% of the control which had not been treated photodynamically. Longer incubation such as 60 min. did not make a significant difference (TABLE 1) in the protection of cells against lysis. However, the longer incubation periods have been used to reduce virus yields by  $10^6$  amounts, based on kinetic studies (19). Similarly, herpes simplex titers were reduced by  $10^5$  amounts (12).

TABLE 2 showed that the addition of photodynamically inactivated, [acridine dye plus light (ADL)] particles to a standard virus inoculum reduced the yield of infections virus by approximately 100 fold whether the cells were exposed both simultaneously or in tandem. Thus, it was demonstrated that ADL virions contained a factor which interfered with the production of infections virus. The effects of the incubation with PrV antiserum or of UV light on the interfering ability of ADL particles were also tested. Exposure of ADL particles to standard antiviral serum for 1 hr. at 37°C or UV light (20 watts) for 5 min. markedly reduced their interfering ability. It is therefore clear that the interference with the synthesis of infectious virus by ADL culture fluids resides in particles which had antigenic properties similar to that of standard PrV.

In the experiment illustrated in FIGURE 1 the sedimentation profile obtained from sucrose gradients centrifugation (15-30%) is shown. The purified DI particles had a much lower sedimentation value than that of the purified standard virions. There was 38% reduction in protein synthesis (OD 280  $m\mu$ ) and 14% reduction in nucleic acid synthesis (OD 260  $m\mu$ ) in DI particles when compared with that of standard virions. The ratio of OD 280  $m\mu$ / OD 260  $m\mu$  = 1.12 in DI particles was also different from that of infectious standard virions (OD 280  $m\mu$ /OD  $m\mu$  = 1.33). The ratio also indicated that much less protein was made in the DI particles.

TABLE 1. *Protection of host-lysing ability by photodynamic treatment.*

<i>Irradiation time (min.)</i>	<i>Percent of cells being lysed</i>
0 (control)	100.0
10	66.7
20	30.0
30	16.7
45	13.3
60	10.0

The concentration of 0.08  $\mu\text{g/ml}$  of dye (3,9-diamine-acridine dye) (A.D.) was used throughout the experiment.

TABLE 2. *Capacity of photodynamically treated PrV to interfere with the replication of standard PrV.*

Infection	Virus titer (TCID <sub>50</sub> /ml)
standard PrV control (VC)	10 <sup>8</sup>
* ADL	10 <sup>2</sup>
VC + ADL (co-infection)	10 <sup>6</sup>
VC <sup>1hr</sup> - ADL (superinfection)	10 <sup>6</sup>
** ADL + Antiserum (ADLS)	no c.p.e.
VC + ADLS (co-infection)	10 <sup>8</sup>
VC <sup>1hr</sup> - ADLS (superinfection)	10 <sup>8</sup>
***ADL + 5 min UV irradiation (ADLU)	no c.p.e.
VC + ADLU	10 <sup>8</sup>
VC <sup>1hr</sup> - ADLU	10 <sup>8</sup>

\* ADL cell cultures ( $6 \times 10^5$ ) were infected by 1 ml of photo-dynamically treated PrV (0.08  $\mu$ g/ml of AD plus 45 min. irradiation of visible light).

\*\*ADLS cell cultures were infected by 1 ml of ADL particles which had incubated for 1 hr at 37°C water bath with 1:20 dilution of standard PrV antiserum from swine.

\*\*\*ADLU cell cultures were exposed to 1 ml of ADL particles which had been treated with 20 watts of UV light for 5 min. Coinfection was performed when cultures were inoculated with both VC (m.o.i = 5) and ADL particles at the same time. Superinfection was performed when cultures were infected with VC first and ADL particles 1 hr later. The infected cultures were washed to remove unabsorbed virus as well as PrV antiserum.

Effect on coinfection of pig kidney cells with ADL defective interfering particles and standard pseudorabies virus on plaque formation was studied. Aliquots of each fraction collected after sucrose gradient centrifugation were then tested for interfering ability (pfu/ml) by comparing the progeny of cells coinfecting with standard virus and DI particles with standard virions alone as shown in FIGURE 2. Most fractions indicated about 3 log<sub>10</sub> reductions in the titer. Fractions 34, 35 and 36 had a larger measure of interference with the production of standard virions.

Demonstrations of antigenicity in the ADL particles and quantitation of total antigenic proteins in them with rocket immunoelectrophoresis has been reported (25). However, identification of individual antigenic determinants presented in the immunoelectrophoretic precipitate profile, was accomplished by crossed immunoelectrophoresis with the second dimensional gel containing anti-PrV swine sera against the purified PrV particles. Analysis of individual precipitates presented in standard PrV virions was illustrated previously (21). The precipitate pattern revealed four antigenic determinants as indicated Ag 1, Ag 2, Ag 3 and Ag 4 which have a quantitative ratio of 19%, 10%, 20% and 51% respectively. Since the area outlined by each precipitate is proportional to the amount of the corresponding antigenic determinant when the concentration of antibodies in the second-dimensional gel is kept constant, then a quantitative ratio could be determined.

Comparison of the precipitin profile between untreated standard virus and ADL particles was demonstrated in FIGURE 3A and FIGURE 3B. The untreated control showed four antigenic precipitates (FIGURE 3A) as observed in our previous study. However, the precipitation profile released from ADL particles showed only Ag 2 and Ag 4 to be present (FIGURE 3B). There were no detectable amounts of Ag 1 and Ag 3. The defectiveness was therefore not only shown in the lack of infec-

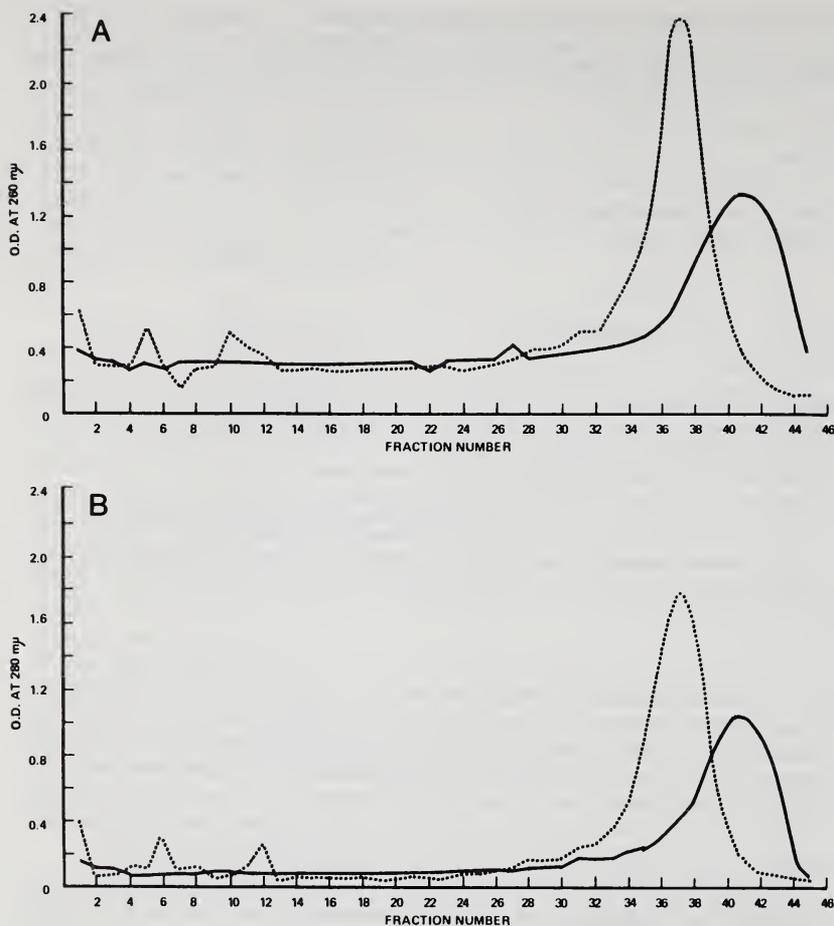


FIGURE 1. Sedimentation in sucrose gradients of defective and standard virions. Cultures were infected with standard PrV at *m.o.i.* = 10 and purified in sucrose gradients centrifugation as described in materials and methods. Each fraction collected after centrifugation was taken O.D. readings at 260  $m\mu$  (A) and 280  $m\mu$  (B). ---: untreated control; \_\_\_\_: cultures treated with acridine dye plus visible light.

tiveness but also in the antigenic components of ADL defective interfering particles.

Purified DNA was extracted by SDS—chloroform—isoamyl alcohol method as described by Marmur (15). A comparison of the sedimentation profile in a neutral sucrose gradient of purified DNA found in standard and in defective virions is shown in FIGURE 4. The study suggested that the DNA of ADL virions has a sedimentation value similar to that of standard virus.

#### Discussion

The formation of incomplete virus or DI particles is not an exclusive peculiarity of animal viruses. Similar forms have been described in DNA bacteriophage,  $T_4$

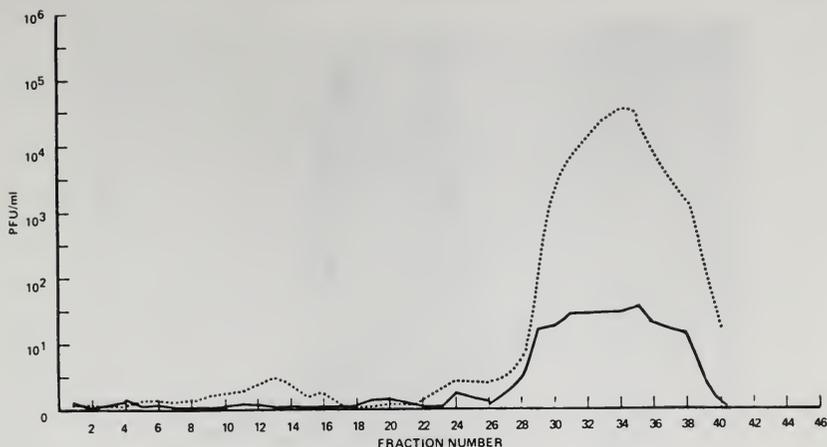


FIGURE 2. Effect of coinfection of pig kidney cells with ADL defective interfering particles and standard pseudorabies virus on plaque formation. Controlled cultures were infected with standard PrV (*m.o.i.* = 5) and coinfecting cultures were infected with standard PrV (*m.o.i.* = 5) and ADL virions (1 ml). Cultures were harvested after 24 h. of post-infection. Results are presented as the p.f.u./ml observed with each fraction collected from 15-30° sucrose gradients centrifugation. ---: cultures were infected with standard virus alone; .....: cultures were coinfecting with standard virus and ADL defective interfering particles.

(13), RNA bacteriophage,  $Q_{\beta}$  (16), and plant mosaic virus (14). All these DI particles are non-infective and have a lower sedimentation constant. However, in contrast to our ADL particles (FIGURE 4), they differ from standard virus by the absence of nucleic acid.

Huang and Baltimore (9) proposed a model of DI particles in viral disease infection in which repeated cycles of infection produce a gradually increasing proportion of DI particles ultimately expressed in natural disease as persistent or latent infections. Since our results suggest a similar phenomenon, we have considered that DI particles similar in effect to the photodynamically produced ADL particles may be important determinants of the course of acute PrV infections and of persistent, slowly progressing pseudorabies viral disease in swine.

Our results that ADL particles lost infectivity but retained antigenicity are reminiscent of an observation (24) of Von Magnus in which there was formation of noninfectious but hemagglutinating particles of influenza virus that were termed "incomplete viruses".

According to Henle's concept (7), the formation of DI particles are assumed to be the result of arrested intracellular virus development as some intermediary stage caused by factors present either in host cells or the virus itself. In the present case it is possible that DI particle formation in cell cultures is the result of host cell enzyme systems being altered under the conditions of photodynamic treatment so that certain phases of intracellular virus replication are deleted. However, the exact mechanism by which acridine dye at very low dose (0.08  $\mu\text{g}/\text{ml}$ ) prevents viral maturation is not well understood at present. Although, it is known that irradiation of protein and DNA with visible light in the presence of acridine dye leads to DNA-protein cross-linking (10, 18). It is also very possible that this cross-linking of viral

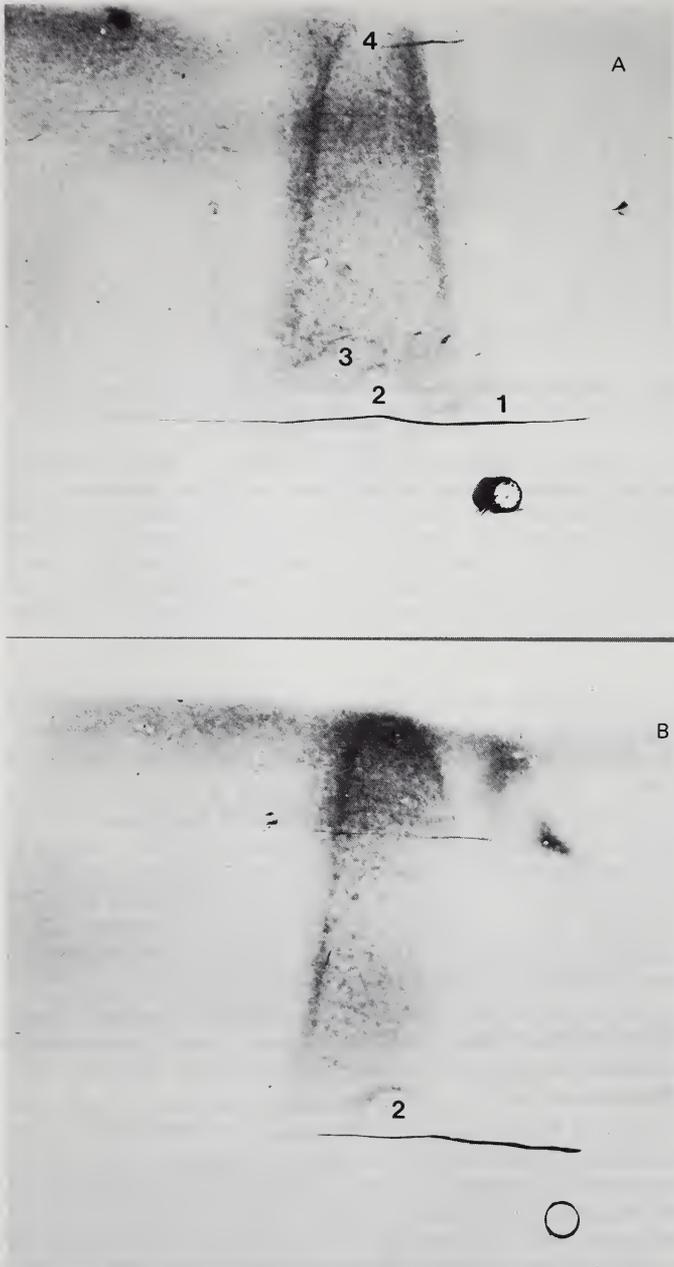


FIGURE 3. Comparison of crossed immunoelectrophoretic patterns of precipitating antigens between untreated virus control and ADL treated virions. Immunoelectrophoresis was performed as that described in materials and methods. A: untreated virus control; B: ADL treated virions.

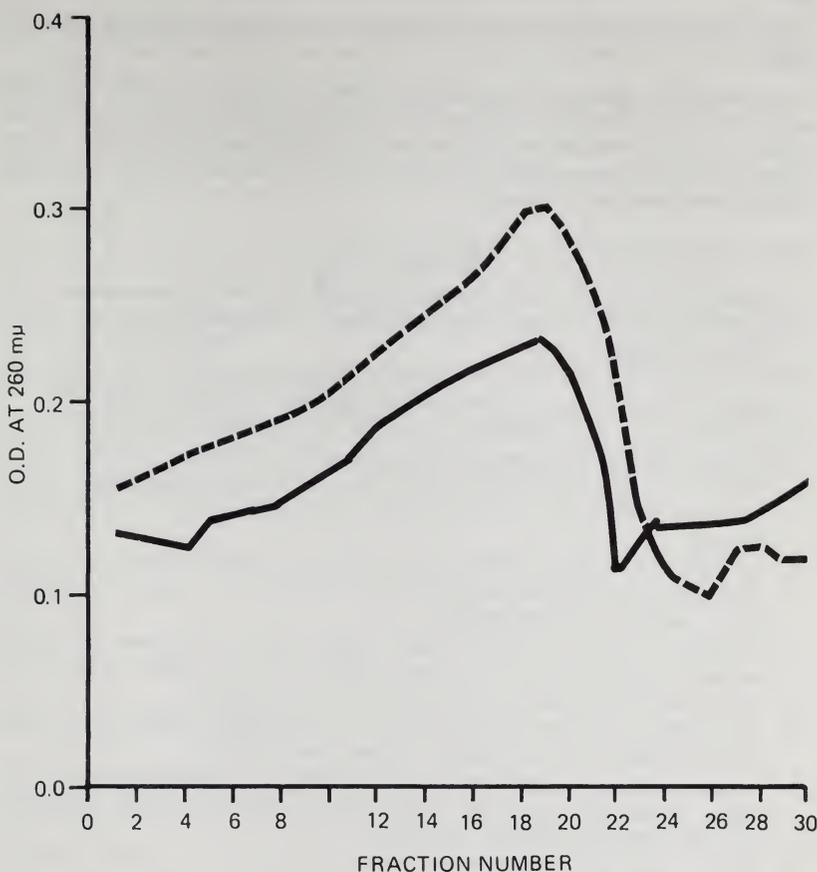


FIGURE 4. Sedimentation in a sucrose gradient of the purified DNA presented in standard and in defective virions. The virions were isolated and purified from cultures, as described in materials and methods. The DNA from these virions were extracted, as described by Marmur (13). Centrifugation was performed in a 5-20• neutral sucrose gradient for 16 hr. at 13,000 r.p.m. ----: DNA of purified standard PrV; \_\_\_\_\_: DNA of purified ADL virions.

DNA-protein complexes may interfere with the viral maturation process and thus leads to biological inactivation. This may be due to factors associated with the viruses themselves rather than to factors associated with the host cells.

We have applied both a neutral detergent, Triton X-100 (0.5%) and an anionic detergent, sodium deoxycholate (0.05%) in our two-dimensional (crossed) immunoelectrophoresis. The electrophoretic mobility of amphiphilic proteins in standard virus of DI particles can be altered by so-called "charge-shift electrophoresis" (6) due to anodic migration and resolution of antigens are greatly improved in our two-dimensional immunoelectrophoresis by the incorporation of these detergents. This type of combination of "charged-shift" electrophoresis with regular two-dimensional immunoelectrophoresis thus permitted us to have better identification

and comparison of antigenic determinants between standard virions and ADL particles.

In summary, we can say that ADL-produced DI particles differ from the fully active standard virions by the apparent lack of infectivity (25), by their capacity to interfere with the propagation of infective virus (TABLE 2 and FIGURE 2), by having a lower sedimentation constant (FIGURE 2) and by the absence of two out of four antigenic determinants (FIGURE 3).

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