Titration of African Swine Fever (ASF) Virus

By L. ENJUANES, A. L. CARRASCOSA, M. A. MORENO AND E. VIÑUELA

Centro de Biología Molecular (C.S.I.C.-U.A.M.), Velázquez 144, Madrid-6, Spain

(Accepted 28 April 1976)

SUMMARY

A haemadsorption microtest for African swine fever (ASF) virus is described. This assay is as sensitive and its response is faster than the conventional assay which uses buffy coat cultures in Leighton tubes. The method can also process a larger number of samples by using smaller amounts of swine blood and laboratory space. A plaque assay for ASF virus adapted to grow in VERO cells gives a titre similar to that obtained using the haemadsorption microtest. In both the micromethod and the plaque assay infection may be produced by a single infective particle.

INTRODUCTION

The standard method of titrating ASF virus is based on the adsorption of swine red blood cells (RBC) to infected leukocytes (Hess & DeTray, 1960; Malmquist & Hay, 1960; Malmquist, 1962; Tubiash, 1963; Haag & Larenaudie, 1965). This assay is sensitive and specific but, as conventionally done in Leighton tubes, it is time consuming and requires large quantities of swine buffy coat. We have developed a carefully standardized haemadsorption microtest based on a micromethod used by Robb & Martin (1970). A micromethod that uses cultures of swine bone marrow has been recently published by Greig (1975).

For a genetic study of ASF virus it is useful to have available a plaque assay. So far, the plaque assay described for ASF virus can be used only to titrate cell culture-adapted virus strains (Parker & Plowright, 1968). We describe here the adaptation of ASF virus to grow in VERO cells, and a plaque assay on this cell line that is as sensitive as the haemadsorption microtest carried out on swine macrophages.

METHODS

Materials and reagents. Plastic Microtest I and II plates and Petri dishes for tissue culture were from Falcon Plastics. One tenth and 0.5 ml microsyringes (Luer tip), needles and repeating dispensers were obtained from Hamilton, and sterilized as described by Robb & Martin (1970).

Heparin (preservative free), streptomycin sulphate and penicillin (potassium salt) were from Serva Feinbiochemica. DEAE-dextran (DEAE-D; average mol. wt. $2 \times 10^6$) was obtained from Pharmacia. All other chemicals were reagent grade or of the highest available purity.

Media and sera. Dulbecco’s modification of Eagle’s minimal essential medium (hereafter DME; Dulbecco & Freeman, 1959) with penicillin (100 international units (i.u.)/ml) and
streptomycin (0.1 mg/ml) was used. Leukocytes were cultured in DME containing 40% swine serum and VERO cells in DME containing 10% calf serum.

Swine and calf sera were obtained from Flow Laboratories. Swine serum was also prepared from swine blood and sterilized by filtration through a nitrocellulose membrane (Sartorius; pore size, 200 nm).

**Purification of swine leukocytes.** Swine blood (500 ml) was collected in a sterile glass bottle containing heparin (40 i.u./ml), penicillin (100 i.u./ml) and streptomycin (0.1 mg/ml). After mixing, the blood was poured into rectangular glass containers (2 cm deep x 5.5 cm wide x 10 cm high) and tilted at an angle of about 45° (Tubiash, 1963; Haag & Larenaudie, 1965). After 30 to 60 min at 37 °C, when most of the RBC had sedimented, the upper phases were pooled, mixed with 3 vol. of phosphate buffered saline (PBS) with 20 i.u. of heparin/ml, and the cells collected by low-speed centrifugation at room temperature. The cells were washed three times more with PBS-heparin, resuspended in 4 to 5 ml of ice-cold PBS with 5% swine serum, and the contaminant RBC were lysed by addition of 0.25 vol. of 0.15 M-NH₄Cl, 0.1 mM-EDTA, 0.01 M-KHCO₃, pH 7.4 (Roos & Loos, 1970), containing 20 i.u. of heparin/ml and 5% swine serum. After 3 to 4 min at 4 °C, the leukocytes were collected by centrifugation at 4 °C. The RBC ghosts were removed by centrifuging twice in DME containing 5% swine serum and 20 i.u. of heparin/ml and the sediment was resuspended in 60 ml of DME with 40% swine serum and 10 i.u. of heparin/ml. A typical preparation contained about 20 x 10⁶ leukocytes/ml, of which 6% were monocytes. These values correspond to a yield of about 40% of the leukocytes present in the original buffy coat.

**Cell culture.** Cultures of monocytes in microplates were obtained by addition of 10 μl of the purified leukocyte suspension, prepared as indicated before, to each well of a Microtest I plate, using a 500 μl Hamilton microsyringe with an automatic dispenser. The plate was closed and incubated at 37 °C in an atmosphere with 5% CO₂. After 1 to 2 days, the medium and the unattached cells (mainly lymphocytes and degenerated granulocytes) were removed with a needle and immediately 10 μl of fresh medium pre-warmed at 37 °C were added to each well as indicated before. The attached cells differentiated in 1 to 2 days to macrophage-like cells and were ready for infection with ASF virus.

To culture monocytes in Petri dishes, 1 ml of purified leukocytes (20 x 10⁶ leukocytes; 1 to 2 x 10⁶ monocytes), prepared as indicated before, was diluted with 1 ml of DME with 40% swine serum and the mixture added to a 35 mm plastic plate. After 2 days at 37 °C in 5% CO₂, the medium with unattached cells was removed and fresh medium added. One day later the attached cells had differentiated to macrophage-like cells, and they were ready for infection with ASF virus.

VERO cells were cultured in plastic plates in DME containing 10% calf serum.

**Virus.** The original ASF virus used in these studies was isolated in 1971 from the spleen of an infected animal from Badajoz (Spain). To date the virus has been passaged in swine monocytes about one hundred times, but most of the experiments reported here have been carried out with virus obtained after the fortieth passage.

Virus titres are expressed either as haemadsorption units (H.A.D.U.) or as plaque forming units (p.f.u.) per ml.

Adaptation of ASF virus (36th passage in swine monocytes) to grow on VERO cells was carried out according to the following infection schedule: 60 mm plastic plates containing 2 to 3 x 10⁶ cells were incubated with 1 x 10⁶ H.A.D.U. of ASF virus. After an adsorption period of 2 h at 37 °C, the plates received 5 ml of DME containing 2% calf serum and they were incubated for one week at 37 °C, with a change of medium after the third day of
Titration of ASF virus

incubation. At the seventh day the attached cells were removed with trypsin and re-suspended in a mixture containing equal parts of the culture media removed after the third and seventh day of incubation, at a concentration of $1 \times 10^6$ cells/ml. A sample of 1 ml of this suspension was added to each one of several plates containing $1 \times 10^6$ uninfected cells/plate. The cell monolayers were trypsinized again after an incubation of one week and the procedure repeated several times more, until a clear c.p.e. was apparent.

**Haemadsorption microtest.** Cultures of macrophage-like cells in Microtest I plates were prepared as described before. After removal of the medium, 6 to 12 wells were inoculated with 2 $\mu$l of each one of the virus dilutions in DME with 40% swine serum, using a 100 $\mu$l Hamilton microsyringe with a repeating dispenser, starting with the less concentrated dilution. After 24 h at 37 °C in 5% CO$_2$, 10 $\mu$l of swine RBC (80000 cells) in DME with 40% swine serum were added to each well with a 500 $\mu$l Hamilton syringe and a repeating dispenser, starting with the wells inoculated with the less concentrated virus dilutions. After a further incubation of 2 to 3 days, haemadsorption in each well was observed with an inverted microscope (Nikon) at a 100-fold magnification. The virus titre was determined according to Reed & Muench (1938).

**Plaque assay.** VERO cells grown to confluency in 60 mm plastic plates with DME containing 10% calf serum were washed with PBS at 37 °C and inoculated with 0.4 ml of the virus dilution, carried out in DME with 2% calf serum. After 2 h at 37 °C, tilting the plate every 15 min, each plate received 9 ml of DME containing 2% calf serum, 0.7% (w/v) Difco agar Noble and 80 $\mu$g of DEAE-D per ml, kept at 42 °C. After solidification at room temperature for 20 min, the plates were incubated at 37 °C in 10% CO$_2$. Ten days later each plate received 3 ml of neutral red at a concentration of 10 $\mu$g/ml in 0.5% (w/v) Difco agar Noble in the medium described before without DEAE-D. The plaques were scored 18 h later.

**RESULTS**

**Haemadsorption microtest of ASF virus**

Two to three days after the addition of a purified leukocyte suspension to the wells of a Microtest I plate, some cells attached to the plastic and differentiated to macrophage-like cells. By seeding 10 $\mu$l of leukocyte suspension ($2 \times 10^8$ leukocytes with $1 \times 10^4$ monocytes), the average number of attached cells differentiated to macrophage-like cells was $3 \times 10^8$ cells/well. If a culture of washed attached cells was infected with ASF virus, the cells showed a clear c.p.e. with rounding, detachment and lysis after 1 to 2 days. The addition of swine RBC to the infected cultures allowed detection of infection to be made more readily by following the appearance of haemadsorption rosettes. At 12 to 18 h post infection (p.i.) some rosettes were already present and after 48 h the number of rosettes per well was very large (more than one hundred), even in wells which had received only 5 H.A.D.U.

By using six wells per dilution and 10 $\mu$l of a virus dilution per well, the minimum dose of virus detectable by the microtest was about 40 H.A.D.U./ml. To determine the reproducibility of the assay, plates were prepared with white cells obtained from different animals and all of them infected with the same dilutions of a stock of ASF virus. The mean and the standard deviation obtained was $3.8 \pm 0.4 \times 10^8$ H.A.D.U./ml. By inoculating the wells with 2 $\mu$l and using 12 wells for each dilution of a different virus stock, the minimum dose of virus detectable and the reproducibility of the microassay were $170$ H.A.D.U./ml and $2.4 \pm 0.2 \times 10^8$ H.A.D.U./ml, respectively.

To test whether or not the response to ASF virus infection in the microtest fits a Poisson distribution, 120 wells of two Microtest I plates were infected at different m.o.i. and the
Fig. 1. Dose–response curves for the dilution count derived from the equations (a) \( P = 1 - e^s \) and (b) \( S = 1 - \sum_{i=0}^{n-1} P_i \). \( P \) and \( S \) being the frequency of infected and uninfected wells, respectively, \( s \) the average number of virus/well and \( n \) the number of particles required to produce infection (Robb & Martin, 1970).

frequency of infected wells scored. The result shown in Fig. 1(a) indicates that the experimental values do fit closely the theoretical curve derived for a Poisson distribution.

To test whether infection is produced by a single virus particle, the frequency of uninfected wells versus the average number of infective particles per well was determined. The result shown in Fig. 1(b) indicates that infection may be caused by a single infectious particle.

The microassay of ASF virus was compared with the conventional assay, which uses Leighton tubes and buffy coat (Hess & DeTray, 1960; Malmquist & Hay, 1960), employing the same stocks of ASF virus and leukocytes as before. The results, shown in Fig. 2, indicate that the final titre is the same in both cases but the maximal response of the infected cultures is reached faster (3 days p.i.) when the microtest was used.

**Adaptation of ASF virus to grow in VERO cells**

ASF virus passaged 36 times in swine macrophage-like cells was used to infect cultures of VERO cells. Subcultures of the infected cells were carried out every week, as described in
Methods. After the 8th subculture, detachment and rounding of the cells was seen and this effect was more apparent in subsequent passages. After the 11th subculture, the cell monolayers showed a strong c.p.e. with more than 90% cell destruction 4 days after infection. At this time the cells were resuspended in the culture medium, sonicated and, after clarification by low-speed centrifugation, the supernatant fluid was used to infect a fresh culture. On the first passages the virus titre decreased continuously until transfer number 4, when the titre started increasing up to passage number 8. After this, the virus titre ranged from $10^7$ to $10^8$ H.A.D.U./ml of culture medium, corresponding to an average yield of 10 to 100 H.A.D.U./cell.

ASF virus passaged 23 times in VERO cells was used to infect swine monocytes. After two passages in monocytes, the adapted virus-infected VERO giving a titre similar to that obtained with VERO cell-adapted virus not passaged through monocytes.

Plaque assay of ASF virus

ASF virus adapted to grow in VERO forms plaques on confluent cultures of that cell line. On the 10th day after inoculation of a cloned virus the average diam. of the plaque was about 1 mm. Fig. 3 shows a linear relationship between virus dose and plaque count, indicating that each plaque is produced by a single infective particle. A study of the optimal conditions for the plaque assay showed that the presence of DEAE-dextran in the inoculum does not influence the final titre value, but this value increases about threefold when DEAE-D (80 μg/ml) is present in the overlay. The same ASF virus stock titrated by the haemadsorption microtest and the plaque assay gave $1.5 \pm 0.2 \times 10^7$ H.A.D.U./ml and $1.5 \pm 0.1 \times 10^7$ p.f.u./ml, respectively. The average number of p.f.u./plaque was $3.2 \pm 0.2 \times 10^5$, obtained by sucking each plaque with a Pasteur pipette, resuspending in 1 ml of DME with 2% calf serum and freezing and thawing twice.
DISCUSSION

The usual haemadsorption test for ASF virus requires a fairly large amount of swine buffy coat. We have therefore developed a carefully standardized microtest using a purified population of adherent swine leukocytes and Microtest I plates instead of buffy coat and Leighton tubes.

The microtechnique described in this paper requires a small number of swine leukocytes and the response of the infected cells is faster than that obtained with the standard assay (Fig. 2). The dose–response curve of a virus stock closely fits a Poisson distribution and indicates that infection may be produced by a single infective particle.

Hess (1971) has reported the adaptation of two ASF virus isolates to grow in VERO cells, but no plaque assay has been described on this line. Here we show the adaptation of our virus isolate to that cell line, starting with virus passaged several times in swine leukocytes. After eight passages in VERO cells, virus titres of $10^7$ to $10^8$ H.A.D.U./ml were obtained. The adapted virus grows readily in swine leukocytes and, after being passaged twice in these cells, still produces a high virus titre in VERO cells.

Parker & Plowright (1968) showed plaque formation in pig kidney (PK) cells infected with either of two cell culture-adapted ASF virus strains. The plaque assay showed a linear dose–response curve, and plaque size heterogeneity. The assay sensitivity was about tenfold larger than that obtained by 50% end-point estimation of c.p.e. in roller cultures, but it was not compared with the sensitivity obtained by using the haemadsorption microtest. We show in this paper that our cell culture-adapted ASF virus isolate, cloned in swine monocytes, produces in VERO cells plaques with an average diam. of about 1 mm on the 10th day after inoculation. The dose–response curve is also linear and a comparison of the titre of the same virus stock showed that the plaque assay was as sensitive as the haemadsorption microtest.

Tessler et al. (1974) have detected fluorescing plaques of cell culture-adapted ASF virus
Titration of ASF virus

on VERO cells 3 days after inoculation. Although this assay has a sensitivity about fivefold smaller than the haemadsorption test, it should be very convenient for a rapid titration of cell culture-adapted ASF virus.

We are grateful to Inmaculada Cubero for skilful assistance. This research was supported by Grants from Fundación Juan March, Comisión Administradora del Descuento Complementario (I.N.P.) and Dirección General de Sanidad. L.E. is a fellow of the Fundación Científica de la Asociación Española Contra el Cancer and A.L.C. of the División de Ciencias of the Spanish Research Council.

REFERENCES


(Received 31 December 1975)