Sensitivity of Macrophages from Different Species to African Swine Fever (ASF) Virus

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SUMMARY

The swine white blood cells sensitive to African swine fever (ASF) virus are monocytes differentiated in vitro to macrophages. These cells have been characterized by their morphology, phagocytic capacity and the presence of receptors for swine immunoglobulin G in their membranes.

ASF virus does not produce any detectable effect on macrophages from humans, rabbits, guinea pigs, hamsters or rats, whereas ASF virus-infected chicken macrophages show an enhancement of cellular DNA synthesis and an intense cytopathic effect.

ASF virus, adapted to grow in VERO cells, produces a strong cytopathic effect in human macrophages leading to cell destruction. This effect is not associated with the synthesis of infectious virus, cellular or virus DNA nor with the formation of detectable virus-related structures.

INTRODUCTION

Studies on the pathogenesis of African swine fever (ASF) in domestic swine suggest that the virus mainly infects cells of the reticulo-endothelial system, the macrophages being essentially the cells allowing a productive infection (Heuschele, Coggins & Stone, 1966; Colgrove, Haelterman & Coggins, 1969). In agreement with these studies, we show in this paper that the swine white blood cells sensitive to ASF virus are monocytes, differentiated in vitro to macrophages.

Although natural infections by ASF virus seem to be confined to porcine species (Hess, 1971), the reasons for the virus resistance of the insensitive animals are unknown. A possibility is that only macrophages from swine are permissive cells for ASF virus. In agreement with this hypothesis, we show that macrophages from ASF virus-resistant animal species such as humans, rabbits, guinea-pigs, hamsters and rats, are fully resistant to the virus. However, ASF virus in chicken macrophages enhances cellular DNA synthesis and causes cell lysis without virus DNA replication.

Several laboratories have adapted ASF virus to grow in continuous cell lines derived from non-natural hosts (Hess, 1971; Enjuanes et al. 1976a). Although the mechanism of the adaptation is not known, the potential risks involved in the handling of these viruses are obvious. We show that, while human macrophages are fully resistant to non-adapted virus, VERO cell-adapted ASF virus produces cytolysis of human macrophages, in spite of the fact that neither infectious virus nor virus DNA synthesis could be detected.
METHODS

Materials and reagents. Metrizamide [2-(3-acetamido-2,4,6-triiodobenzamido)-2-deoxy-D-glucose] was purchased from Nyegaard and Co. A/S, Oslo, Norway, and Dextran 500 (average mol. wt. 500000) from Pharmacia. Zymosan (immunological reagent) and swine immunoglobulin G (IgG) were from Nutritional Biochemical Corp. Pancreatic ribonuclease (EC. 2.7.7.6) and deoxyribonuclease I (EC. 3.1.4.5) were purchased from Worthington. Proteinase K was obtained from Merck, and *Aspergillus* nuclease SI was purified from amylose powder (Sigma), according to Vogt (1973). 3H-thymidine (17 Ci/mmol) was obtained from The Radiochemical Centre, Amersham. The source of all other materials and reagents has been described (Enjuanes et al. 1976a; Enjuanes, Carrascosa & Viñuela, 1976b).

Animals. Large white pigs, 3 to 4 months old; rabbits, weighing about 2 kg; guinea pigs, weighing approx. 500 g; hamsters (*Cricetus aureus*) of about 60 g; Wistar rats of 200 g; and 2 month old male White Leghorn chickens were used. Human monocytes were prepared from blood extracted from healthy volunteers.

Media and sera. Dulbecco's modification of Eagle's minimal essential medium (DME), supplemented with penicillin (100 i.u./ml) and streptomycin (0.1 mg/ml) was used.

Animal sera were prepared from blood, and filtered through a sterile nitrocellulose membrane with an average pore size of 200 nm (Sartorius). Calf serum was obtained from Flow Laboratories, Inc.

Leukocytes were cultured in DME containing 40% autologous (swine, rabbit and human) or homologous (hamster, guinea pig, rat and chicken) serum.

Viruses and cell culture. The origin and assay of ASF virus, as well as the cell culture conditions for swine macrophage and VERO cells have been described previously (Enjuanes et al. 1976a, b). Primary and secondary cultures of chick embryos and swine kidney cells were obtained by standard techniques and grown under the same conditions.

Purification of leukocytes. Blood was collected in a sterile glass bottle in the presence of 40 i.u. of heparin/ml. Most of the red blood cells (RBC) were removed by sedimentation through 1 vol. of 9.8% (w/v) metrizamide and 4.2% (w/v) dextran 500 (density = 1.07 g/ml) in phosphate-buffered saline (PBS; Boyum, 1964). After 30 to 60 min at room temperature the upper phase, enriched in leukocytes, was collected and the cells were washed twice by centrifuging and resuspending in PBS. The final pellet was resuspended in DME with 40% serum (2 x 10^7 cells/ml) and cultured as described below. In the preparation of chicken leukocytes there was some contamination by RBC; these were eliminated by specific lysis (Enjuanes et al. 1976a).

Infection and radioactive labelling. Purified leukocytes (0.1 ml) were added to each well of a Microtest II plate and incubated at 30°C in 5% CO_2. After 2 days the non-adherent cells were removed, the medium was changed and the incubation continued for a further 2 days. The cultures were then infected with 30 μl of ASF virus in PBS at a multiplicity of 20 haemadsorption units (H.A.D.U.) per attached cell, or were mock infected. Unless stated otherwise, the ASF virus used was the virus passaged in swine macrophages. After 2 h incubation at 37°C in 5% CO_2, each well was washed twice with 0.1 ml Hank's balanced saline solution (HBSS) or DME at 37°C. Next, 0.1 ml DME containing 40% serum dialysed against PBS, and 1 μCi 3H-thymidine were added per well. At different times post-infection (p.i.), the cultures were examined for c.p.e. under the optical microscope, and the cells of three wells were resuspended in the culture medium with a rubber policeman and then mixed and frozen at -70°C. After thawing, virus titres were determined as described (Enjuanes et al. 1976a) and expressed as H.A.D.U./ml.
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Chicken and human macrophages were also cultured in 35 mm diam. plastic plates by seeding 2 ml purified leukocytes (3 × 10⁶ or 1.5 × 10⁷ cells/ml, respectively) per dish. After 2 days incubation at 37°C in 5% CO₂, the medium, with the unattached cells, was removed and fresh medium added. Two days later, the attached cells had differentiated to macrophage-like cells and they were infected with ASF virus at a m.o.i. of 20.

For determination of acid-insoluble radioactivity in the experiment shown in Table I, 50 µl of three pooled cultures were treated with ice-cold 5% (w/v) trichloroacetic acid and the precipitate filtered through a glass fibre filter (Whatman; GF/C), washed, dried and counted in a Packard TriCarb scintillation spectrometer. In the experiments shown in Fig. 1 and 3, 0.2 vol. of a solution containing 0.05 M-tris (pH 8), 0.05 M-EDTA, 0.05 M-NaCl and 2.5% (w/v) SDS was added to each well and the contents of five wells were resuspended and pooled. Protein digestion was achieved by incubation for 3 h at 37°C with 50 µg/ml proteinase K, followed by the addition of an equal dose of enzyme and overnight incubation. After digestion with 0.1 M-NaOH for 1 h, acid-insoluble radioactivity was determined as above.

Uninfected cultures of chick embryo cells in 25 cm² plastic flasks with 5 ml DME containing 5% dialysed calf serum and 2.5 µCi/ml ³H-thymidine were incubated for 3 days at 37°C in 5% CO₂.

DNA extraction. Unlabelled DNA from partially purified ASF virus, free of host DNA, was prepared as described by Enjuanes et al. (1976b). Chicken liver unlabelled DNA, ³H-DNA from ASF virus-infected chicken macrophages and ³H-DNA from primary cultures of chick embryo cells were purified as described by Enjuanes et al. (1976b).

DNA concentrations were determined by the diphenylamine method (Burton, 1956). For reassociation studies of radioactive DNA, the concentration was determined from the specific radioactivity.

DNA reassociation. The extent of the reassociation of radioactive DNA, alone or in the presence of non-radioactive DNA, was measured by determining the amount of S₁ nuclease-resistant radioactivity, as described by Enjuanes et al. (1976b).

Haemadsorption. At 4 h p.i., ASF virus-infected macrophages cultured in Microtest II plates, received 10 µl/well of a suspension of autologous RBC (5 × 10⁵ cells) and the haemadsorption rosettes were observed.

Electron microscopy. Macrophages, cultured in 35 mm diam. plastic plates, were processed for electron microscopy by adaptation of previously described techniques (Bencosme & Tsutsumi, 1970; Tsuruhara, Amano & Tsuruhara, 1971). Micrographs were obtained using a Philips EM300 electron microscope.

Phagocytosis. Phagocytosis was assayed in cell cultures of adherent swine white blood cells in DME with 40% swine serum containing 50 particles of zymosan/cell. After an incubation of 30 min at 37°C, the plates were washed twice with HBSS, fixed and processed for examination with the electron microscope as described above. Two hundred cells were observed and the number of phagocytic cells scored.

Cell membrane receptors for IgG. To estimate the proportion of adherent swine white blood cells with receptors for swine IgG, cultures in HBSS were incubated for 2 h at room temperature with autologous RBC coated with swine IgG (about 50 RBC/attached cell) prepared according to Faulk & Houba (1973). The plates were washed six times with 2 ml HBSS per washing, photographed under an inverted microscope and the proportion of rosette-forming cells from a total of 500 cells was determined. Duplicate monolayers were treated with bovine serum albumin coupled to chronic chloride-treated swine RBC. Other
controls were monolayers of primary cultures of swine kidney cells incubated with RBC coated with either swine IgG or bovine serum albumin.

RESULTS

Characterization of plastic-adherent swine mononuclear cells

To determine the identity of the plastic-adherent cells from swine buffy coat, cultured as indicated under Methods, the following criteria were considered: morphology, phagocytic capacity and the presence of membrane-bound receptors for IgG (Koller et al. 1973). More than 95% of the cells in the culture had macrophage morphology, phagocytic capacity and contained receptors for IgG in their membranes. When the RBC were coated with bovine serum albumin instead of IgG, no rosette was observed. Furthermore, when primary cultures of swine kidney cells were treated with RBC coated with either swine IgG or bovine serum albumin, no rosettes could be seen.

These characteristics of the plastic-adherent swine mononuclear cells indicate that they are essentially a homogeneous population of blood monocytes, differentiated in vitro to macrophages. As is shown below, most of these cells are productively infected by ASF virus. Therefore, we conclude that swine macrophages, derived from blood monocytes, are target cells for the replication of ASF virus. On the other hand, when mixed cultures of swine leukocytes were infected with ASF virus and observed by electron microscopy, no evidence of infection of lymphocytes or granulocytes was obtained.

ASF virus-infected swine macrophages

To ascertain that most of the cells in ASF virus-infected cultures of swine macrophages were producing virus, the intracellular events taking place in ASF virus-infected macrophages were studied by electron microscopy. Among more than 1000 infected cells examined by electron microscopy, most showed structural features characteristic of the infection of swine leukocytes (Haag et al. 1966; Larenaudie et al. 1967) or swine bone marrow cells (Nunes, Vigário & Terrinha, 1975) with ASF virus. Other features which have not been described previously are the detachment of nuclear membrane from the cytoplasm (except in the zones where cytoplasmic virus particles were close to the nucleus), and spherical vesicles with ribosome-like particles in their periphery surrounding the cytoplasmic inclusions.

Sensitivity of macrophages from various species to ASF virus

To test the susceptibility of macrophages from different animal species to ASF virus, cell cultures of plastic-adherent white blood cells were prepared and infected with ASF virus propagated in swine macrophages.

The infected cultures were observed for c.p.e. and analysed for their ability to produce infective virus and to incorporate $^3$H-thymidine into acid-insoluble material. Table 1 indicates that only swine macrophages replicate ASF virus and show a strong c.p.e. and a stimulation of DNA replication due to the synthesis of virus DNA (Enjuanes et al. 1976b). None of the other macrophages were hosts for ASF virus multiplication nor haemadsorbed RBC and none of them, except chicken macrophages, showed any c.p.e. or an increase of $^3$H-thymidine incorporation (see below).

At 4 h p.i. the cultures of rabbit, guinea pig and hamster macrophages had a virus titre of about 20 H.A.D.U./well, from a total of $4 \times 10^5$ H.A.D.U./well added at zero time, whereas the cultures of rat, human and chicken macrophages had, at the same time, a titre at least 100-fold larger. We have not explored whether this difference is due to a weak adsorption
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Table 1. Effects of ASF virus on macrophages from different vertebrates

<table>
<thead>
<tr>
<th>Animal</th>
<th>Haemadsorption</th>
<th>C.p.e.</th>
<th>Mock infected</th>
<th>ASF virus-infected</th>
<th>Virus titre (H.A.D.U./ml)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>72 h</td>
<td>4 h 72 h</td>
<td>4 h 72 h</td>
</tr>
<tr>
<td>Swine</td>
<td>+ + +</td>
<td>++</td>
<td>335</td>
<td>418 2402</td>
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</tr>
<tr>
<td>Rabbit</td>
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<td>--</td>
<td>590</td>
<td>423 600</td>
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</tr>
<tr>
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<td>NT</td>
<td>--</td>
<td>418</td>
<td>211 396</td>
<td>2.0 x 10^7 1.7 x 10^5</td>
</tr>
<tr>
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<td>NT</td>
<td>--</td>
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<td>244 177</td>
<td>2.1 x 10^7 2.2 x 10^5</td>
</tr>
<tr>
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<td>NT*</td>
<td>--</td>
<td>203</td>
<td>172 137</td>
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</tr>
<tr>
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<td>+ +</td>
<td>522</td>
<td>444 516</td>
<td>2.2 x 10^4 3.0 x 10^4</td>
</tr>
<tr>
<td>Chicken</td>
<td>--</td>
<td>+ +</td>
<td>2407</td>
<td>400 1428</td>
<td>2.4 x 10^4 7.1 x 10^2</td>
</tr>
</tbody>
</table>

*NT*, Not tested.

Fig. 1. Eclipse of infective virus in ASF virus-infected chicken macrophages (■—■); DNA synthesis in uninfected (○—○); and ASF virus-infected (●—●) chicken macrophages.

of the virus to the macrophages of the first group and subsequent loss of the virus in the washings, or to phagocytosis and further inactivation of the virus.

Chicken macrophages are peculiar because they are able to divide to some extent in vitro (Carrel & Ebeling, 1922). Table 1 shows that uninfected chicken macrophages incorporated 3H-thymidine into acid-insoluble material. When they were infected with ASF virus there was some inhibition of the 3H-thymidine incorporation at 72 h.p.i. compared with the uninfected cells. However, ASF virus-infected chicken macrophages were the only non-producing macrophages which presented a clear c.p.e. and a marked decrease of virus titre from 4 to 72 h p.i. (Table 1). These observations suggest that ASF virus produces an abortive infection in chicken macrophages.
Infection of chicken macrophages with ASF virus

Infection of chicken macrophages with ASF virus was followed by studying in more detail the fate of the cell-attached virus, electron micrographs of virus-infected cells and the nature of the DNA synthesized after virus infection. Fig. 1 shows that ASF virus-infected chicken macrophages were not capable of propagating the virus. After the adsorption period, the cell-attached viruses rapidly lost their infectivity, possibly because the particles entered into the eclipse period of an abortive infection, as shown by the strong c.p.e. produced in the infected cells.

Electron micrographs of ASF virus-infected chicken macrophages showed the presence of cytoplasmic inclusions containing partially degraded membranes of virus-related structures, probably phagocytized from the inoculum. At 9 h p.i., of about 500 cells observed, 25% showed clumping of chromatin and only one attached cell contained a virus factory, similar to that observed in the infection of swine macrophages. At 22 h p.i., when most of the cells were detached and partially destroyed, no virus-like or related particles were seen.

Figure 1 shows the time-course of 3H-thymidine incorporation by uninfected and ASF virus-infected chicken macrophages. Very soon after infection, the infected cells synthesized DNA at a faster rate than that of the uninfected cells, but that incorporation stopped at about 18 h p.i. and part of the radioactive material became acid-soluble.

To determine if the DNA synthesized in ASF virus-infected chicken monocytes was exclusively virus DNA, host DNA or a mixture of both, the reassociation kinetics of 3H-DNA isolated from either ASF virus-infected macrophages or from uninfected primary cultures of chick embryo cells were compared. Fig. 2(a) shows the initial part of a second order rate plot of the reassociation kinetics of both fragmented DNAs (Enjuanes et al. 1976b). In the $C_{ol}$ interval shown, both DNAs renature at the same rate. This result suggests
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Fig. 3. (a) 3H-Thymidine incorporation and (b) virus titre in swine and human macrophages infected with non-adapted or VERO cell-adapted ASF virus. ■—■, Non-adapted virus; ●—●, adapted virus; □—□, ○—○, mock infected.

that ASF virus DNA is not synthesized in infected macrophages. In agreement with this conclusion, Fig. 2(b) shows that the reassociation rate of 3H-DNA from infected macrophages is increased by the addition of non-radioactive chicken DNA but does not increase when ASF virus DNA is added.

Infection of human macrophages with ASF virus adapted to VERO cells

In contrast to the non-adapted virus, human macrophages infected with VERO cell-adapted ASF virus showed an intense c.p.e. which was not due to either virus multiplication or to the induction of DNA synthesis (Fig. 3).

Infected cultures were also studied by electron microscopy at 16, 36 and 72 h p.i. Contrary to the events in ASF virus-infected swine or chicken macrophages, no nuclear effect, such as chromatin condensation, was observed at any time. At 16 and 36 h p.i. there was some cytoplasmic degeneration compared with the mock-infected cells. At 36 h p.i., cytoplasmic inclusions with membrane-like material were present, but these or other cytoplasmic components did not resemble virus-related structures. At 72 h p.i., most macrophages were destroyed and the only cells seen were lymphocytes which did not seem to be affected by the infection.

DISCUSSION

Most of the studies on the pathogenesis of ASF in domestic swine suggest that ASF virus replicates primarily in macrophages and monocytes (Heuschele et al. 1966; Colgrove et al. 1969). On the other hand, it is generally agreed that the swine white blood cells susceptible in vitro to ASF virus are the monocytes differentiated to macrophages (Malmquist & Hay, 1960; Hess, 1971), a conclusion based on the capacity of the virus-sensitive cells to attach to glass, and on morphological criteria.

However, plastic- or glass-adherence and morphology are ambiguous criteria to differentiate monocytes and lymphocytes (Koller et al. 1973). Monocytes and macrophages (van Furth et al. 1972) contain receptors for IgG and complement (Berken & Benacerraf, 1966;
LoBuglio, Cotran & Jandl, 1967). This property, as well as the phagocytic capacity of macrophages, provides additional criteria to characterize these cells. We have purified swine macrophages, derived from blood monocytes, to a purity greater than 95%, as revealed by their phagocytic capacity and the presence of IgG receptors in their membranes.

Although light microscopy indicated that more than 90% of the cells were destroyed by ASF virus, virus development was studied by electron microscopy to ascertain that most of the macrophages had been infected productively. Most of the observed cells contained intracellular virus particles late after infection and showed features characteristic of the infection of swine leukocytes by ASF virus (Haag et al. 1966; Laurenaudie et al. 1967; Nunes et al. 1975). Therefore, we conclude that the macrophage is a target cell for ASF virus. This result and the observations made in infected animals suggest that the host cells for ASF virus are mainly those of the system of mononuclear phagocytes (van Furth et al. 1972). This feature and the immunological response of chronically infected swine (De Boer, Pan & Hess, 1972) classes ASF virus with a series of viruses (lymphocytic choriomeningitis, Aleutian disease, equine infectious anaemia and lactic dehydrogenase virus) which produce persistent infections. These infections are pathogenically similar with respect to their preferential replication in macrophages and the production of non-neutralizing antibodies by the chronically infected animals (Coggins, 1974; Porter, 1975).

In nature, ASF virus seems to be very specific for swine. The resistance of several animal species could be due to a failure of ASF virus to replicate in their macrophages. Our results indicate that macrophages from human, rabbit, guinea pig, hamster and rat do not propagate ASF virus, as tested by induction of DNA synthesis, c.p.e. and production of infective virus. This result supports the idea that only swine macrophages replicate ASF virus. However, ASF virus-infected chicken macrophages showed a marked c.p.e., although they were not able to produce infective virus nor synthesize virus-related structures. Very soon after infection with ASF virus, there is a stimulation of host DNA synthesis in chicken macrophages, but the mechanism and the significance of this change in relation to the infection has not been explored further.

Although several laboratories have been using ASF virus adapted to continuous cell lines derived from resistant animals (Hess, 1971), it has not been reported whether the adapted virus strains were infectious in the corresponding or related animal species. We show here that ASF virus adapted to grow in VERO cells, an established cell line of African green monkey kidney, produces cell lysis of human macrophages, although electron microscopy shows that the virus does not replicate its DNA in these cells nor induces the synthesis of any detectable virus-related structure.

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