Immunology 1982 **46** 75

Complement-mediated lysis of African swine fever virus-infected cells

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Accepted for publication 15 October 1981

Summary. Using an homologous pig system, the lysis of African swine fever virus-infected cells by antibody and complement was investigated. The optimal conditions necessary for lysis are described, and it was found that the system was unique amongst reported virus infections in that infected cells were lysed by the classical complement pathway and not the alternative pathway. Development of antibody capable of initiating complement-mediated lysis was relatively late in the infected pig, although functional *in vitro* assays suggested that it might act as a significant effector mechanism. Investigations of sera taken from pigs infected with varying African swine fever isolates indicate that the assay may provide a means of discriminating between strains.

INTRODUCTION

African swine fever (ASF) virus is a large (175–215 μm diameter) enveloped cytoplasmic DNA virus. Some isolates cause a peracute syndrome in pigs with 100% mortality, whereas others produce a less severe form of the disease with some animals surviving as chronically infected carriers (Hess, 1971). There is no effective vaccine and slaughter is the control measure normally employed. In Malta, ASF resulted in the loss of the total pig population of more than 80,000 animals (Wilkinson, Lawman & Johnston, 1980).

In infected pigs antibody is readily detectable by complement fixation, precipitin, fluorescent and other immunoassays (Hess, 1971), although, like Aleutian disease in mink (Porter, Larsen & Porter, 1981) no neutralizing antibody is produced. There are no published descriptions of any mechanism capable of preventing virus dissemination or of eliminating virus antigen, so how some pigs recover from ASF virus infections remains to be determined. It is apparent that before this disease can be successfully controlled more needs to be known about the virus–host interactions and in this paper we report on the action of complement against ASF virus-infected cells.

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0019-2805/82/0500-0075$02.00

MATERIALS AND METHODS

Virus

The tissue culture attenuated Uganda African isolate of African swine fever virus (Hess, Cox, Heuschele & Stone, 1965) was propagated in IB-RS-2 cells grown in Eagle's medium supplemented with 10% ox serum and antibiotics (110 i.u./ml penicillin and 0.2 mg/ml streptomycin). Stock virus was prepared by subjecting 24-hr postinfected (hPI) cells to three cycles of freeze-thawing, removing cell debris by centrifugation and storing supernatant virus at $-70°$.

Complement

Normal pig serum, stored at $-70°$, was used as a complement source.

Antisera

Unless otherwise stated, serum from a pig which had received six inoculations of attenuated Uganda ASFV over a period of 6 months was used. It was heat inactivated (56°, 20 min) and stored at −70°. All other test sera from pigs infected with various ASFV isolates were treated in a similar manner.

Target cells

Confluent IB-RS-2 monolayers grown on Petri dishes were infected at a multiplicity of infection (MOI) of 2 and labelled with 200 μCi $Na[^{51}Cr]O_4$ (The Radiochemical Centre, Amersham), as described previously (Rouse, Wardley & Babiuk, 1976). Uninfected cells were similarly labelled as a control. After 24 hr at 37°, cells were resuspended in medium by vigorous agitation with a pipette. After three washings the viable cell concentration was adjusted to 2×10^5/ml in Eagle's medium.

Cytotoxicity assay

Assays were performed in microtitre plates (Titertek, No. 76-001-05, Flow Laboratories, Irvine, Scotland). Aliquots (100 μl) of target cell suspension and hyperimmune serum were then added, followed by 50 μl of complement at a dilution of 1:4. All tests were duplicated using uninfected targets to ensure specificity. After incubation at 37° for 90 min, the plates were centrifuged at 200 *g* for 5 min and 150 μl of supernatant was removed from each well for the measurement of radioactivity with a Kontron MR1032 Gamma Counter. The percentage specific lysis (SL) was measured using the following formula: SL = [(mean c.p.m. test − mean c.p.m. control)/(total releasable c.p.m. − mean c.p.m. control)] × 100.

Total releasable ^{51}Cr was determined by measuring the amount of radioactivity released from target cells suspended in 1% Triton X-100. Control release was determined using cells in the presence of complement alone.

Time course of infection

IB-RS-2 cells were incubated with virus (MOI = 2) and ^{51}Cr. After 90 min cells were washed three times in Eagle's medium, trypsinized and resuspended to 8×10^4 cells/ml and aliquoted (250 μl per well) into microtritre plates. The plates were centrifuged at 200 *g* for 5 min and incubated at 37°. At 2-hr intervals 150 μl of supernatant was removed from each of four wells to be replaced by 100 μl of hyperimmune serum at a dilution of 1:50 and 50 μl of complement at a dilution of 1:4. After a further 90 min at 37°, 150 μl of supernatant was removed for the measurement of radioactivity. All controls plus the use of uninfected targets were included.

ASFV cell surface antigens

The kinetics of the appearance of ASFV cell surface antigen was studied by taking cells at different times post-infection, washing them twice in phosphate-buffered saline (PBS) and resuspending them in 200 μl of ASF Uganda immune serum at a dilution of 1:50. After 30 min at 37°, the cells were again washed three times in PBS, resuspended in 200 μl of ^{125}I protein A (appropriately diluted in phosphate-buffered saline plus 2% bovine serum, PBS-ox) and incubated for a further 30 min. Finally, cells were washed three times before associated ^{125}I counts were measured. At each stage, infected cells with non-immune sera and uninfected cells with immune sera were included as controls.

Kinetics of complement-mediated lysis

Microtitre plates were set up as described under cytotoxicity assay except that plates were immediately centrifuged and supernatant aliquots removed thereafter at 10-min intervals for ^{51}Cr measurement.

Determination of the pathway involved in the lysis of ASFV-infected cells.

Complement was heated at 50° for 20 min. A series of dilutions of the heated complement and of normal complement was then used in the cytotoxicity assay to assess their relative activity.

The effect of chelation with ethylenediamine tetracetic acid (EDTA) and EGTA, with subsequent replenishment with Ca^{2+} and Mg^{2+} ions was investigated as described previously (Joseph, Cooper & Oldstone, 1975).

Yield reduction assay

Confluent monolayers of IB-RS-2 cells in 24-well Linbro tissue culture plates (Catalogue Number 76-033-05, Flow Laboratories) were infected at a MOI of 0·01. At 8 hPI, the medium was removed and replaced with hyperimmune serum and 0·5 ml of complement at a dilution of 1:4 in each of six wells. Infected control wells contained complement alone, serum alone or medium alone. The plate was incubated at 37°. At 2-hr intervals the supernatant from each well was removed and stored at −70°. Cells were then re-fed with freshly thawed complement and/or serum as required. The virus titre in the supernatant

samples was determined by titration in pig PBLs as described previously (Wardley & Wilkinson, 1980).

Suppression of immune injury

Target cells were incubated with or without hyper-immune serum at a final dilution of 1:2 for 18 hr at 37°. Uninfected ^{51}Cr-labelled targets were similarly treated. Cells were then washed three times in cold Eagle's medium before being used in the manner described under cytotoxicity assay. All samples were tested in triplicate with the normal controls.

Fixed cell radioimmunoassay (RIA)

The wells of a soft polyvinyl microtitre plate (Dynatech) were coated with glutaraldehyde-fixed cells by a modification of the technique described by Stocker & Heusser (1979). Briefly, ASFV-Uganda infected cells (24 hPI) and uninfected cells were washed three times in PBS (pH 7·2), resuspended at 4×10^5 cells/ml and dispensed in 50 μl amounts into polyvinyl plates. After centrifugation at 200 ***g*** for 5 min, plates were gently immersed in a litre beaker containing a freshly prepared 0·25% glutaraldehyde solution in PBS cooled to 4°. After 15 min the glutaraldehyde was tipped from the wells and the plates were washed by immersion in three successive changes of PBS. Finally, the plates were immersed in a beaker containing PBS-ox and incubated in this solution at room temperature for 1 hr to saturate non-specific protein binding sites. The plates were then removed from the PBS-ox, sealed and stored at 4° until use.

To test whether sera contained antibody capable of binding to these virus-infected cells, 50 μl aliquots of 1:10 dilutions of test serum in PBS-ox were added to each of four wells and the plates incubated at 37° for 1 hr. The plates were then washed three times in PBS and 50 μl aliquots of ^{125}I-labelled protein A appropriately diluted in PBS-ox were added to each well. After a further 1 hr incubation the plates were washed three times in PBS and allowed to dry before the counts associated with individual wells were measured. Results were expressed as c.p.m. in infected cells minus c.p.m. in unifected cells.

RESULTS

Antibody binding and complement lysis

Using a RIA, anti-ASFV antibody was first shown to bind to infected IB-RS-2 cells at 8 hPI. However, it was not until 11-12 hPI that the density of this antibody binding was sufficient to elicit complement-mediated

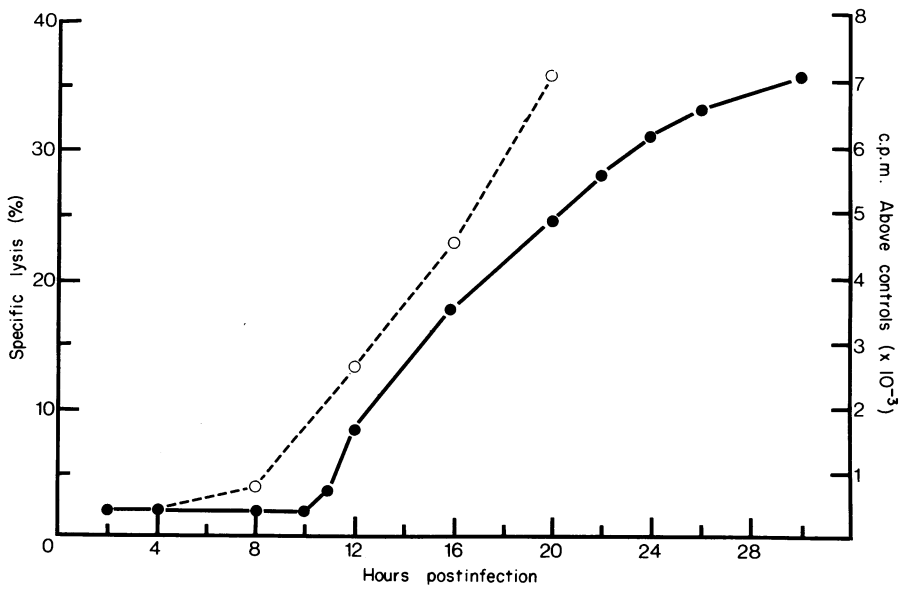

Figure 1. The development of cell surface viral determinants and the susceptibility of cells to complement-mediated lysis. (O- - - -O) Anti-ASFV-bound antibody measured by the binding of ^{125}I-labelled Protein A. (●- - - -●) Complement-mediated lysis measured by ^{51}Cr release assay.

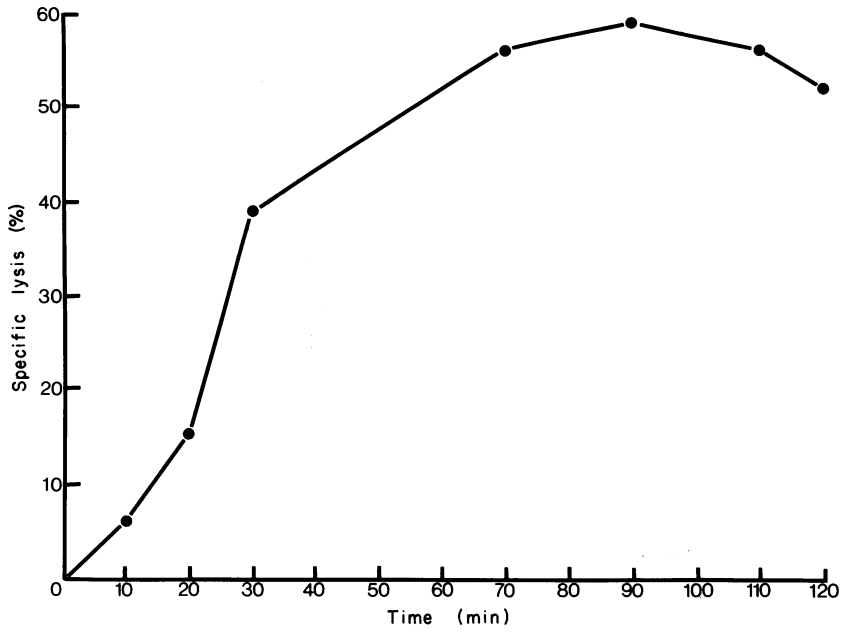

Figure 2. Kinetics of complement-mediated lysis. Twenty-four hour post-ASFV infection IB-RS-2 cells, immune serum and pig complement.

lysis (Fig. 1). Such infected cells first showed lysis 10 min after the addition of complement, rising to a plateau of activity by 70–90 min (Fig. 2).

Complement pathway determination

It has previously been demonstrated that, because of the low concentration of alternative pathway components, a 1:6 dilution of a complement source can result in 90% loss of alternative pathway activation (Sissons & Oldstone, 1980). In our system, however, 50% of activity remained after dilution to 1:50 (Table 1). Similarly, heating the complement to 50° for 20 min caused only a small reduction in activity at low dilutions (Table 1), whereas it is known that such treatment completely abrogates the alternative pathway by inactivation of factor B (Joseph *et al.*, 1975). Finally, treatment of the pig complement source with 0·01 M EDTA (a chelator of Ca^{2+} and Mg^{2+} ions) prevented lysis of infected cells. Replenishment of physiological levels of Ca^{2+} ions restored cytolytic activity, whereas the addition of Mg^{2+} had no effect (Table 2). Ca^{2+} ions are an essential requirement in the classical pathway and Mg^{2+} ions are necessary in the

Table 1. The effect of heating and dilution on the ability of pig complement to cause lysis of ASFV infected cells.

	Dilution of complement						
Treatment	1:5	1:10	1:20	1:40	1:60	1:80	1:100
None	67·0*	66·0	61·0	44·0	25·0	14·0	5·0
50° 20 min	62·0	54·0	40·0	17·0	2·0	0	0

* SL (%) = [(mean c.p.m. − mean c.p.m. control)/(total releasable c.p.m. − mean c.p.m. control)] × 100.

Table 2. The effect on EDTA and EGTA on complement lysis

Treatment	SL (%)
None	54
EDTA 0·01 M	0
EDTA + $6·4 \times 10^{-3}$ M Ca^{2+}	35
EDTA + $6·4 \times 10^{-3}$ M Mg^{2+}	0
EGTA 0·01 M	0
EGTA + $6·4 \times 10^{-3}$ M Mg^{2+}	2
EGTA + $6·4 \times 10^{-3}$ M Ca^{2+}	25

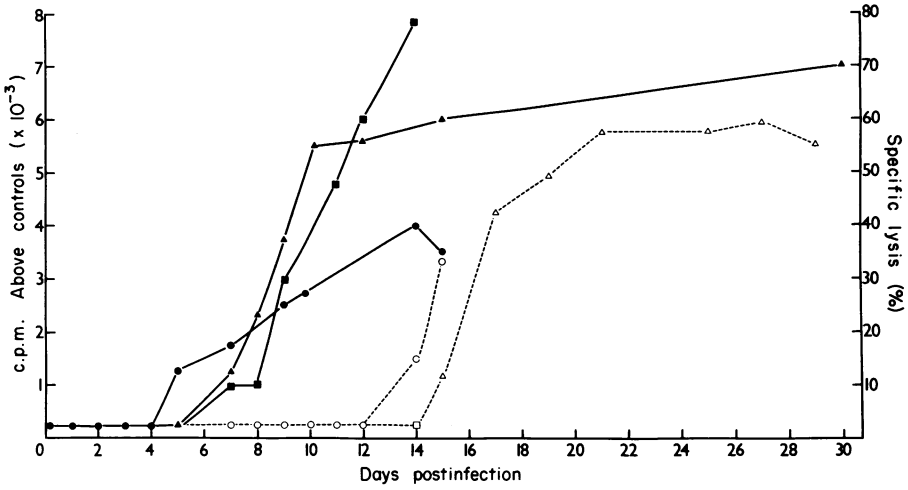

Figure 3. Development of antibody in three pigs infected with virulent Uganda virus. Solid lines denote development of antibody capable of binding to 24-hr Uganda post-infected IB-RS-2 cells. Broken lines denote development of antibody capable of mediating lysis in the presence of complement. Animals died at terminal points.

alternative pathway (Joseph *et al.*, 1975). Similarly, treatment with EGTA, a relatively specific chelator for Ca^{2+} ions, prevented lysis of ASFV-infected cells, with activity being restored on the addition of Ca^{2+} ions (Table 2). These experiments indicate that ASFV-infected cells are lysed by classical pathway activation. It should be remembered, however, that the above comparisons have been made with an homologous human system, whereas we are using an homologous pig system.

Development of antibody in ASFV-infected pigs

From Fig. 1 it can be seen that specific antibody to cell surface determinants of ASFV appears by 5–7 days post-infection (dPI), whereas antibody effective in fixing complement to a degree sufficient for cell lysis did not appear until 14–15 dPI. It is of interest to note that by this time RIA-measured antibody appeared to have reached a plateau, suggesting that the onset of complement-mediated lysis is not simply the result of increasing levels of specific antibody in the serum initiating lysis but rather that there exist two distinct populations of antibody with only the latter having the ability to initiate complement lysis.

Yield reduction assays

The fact that ASFV-infected cells are susceptible to complement-mediated lysis by 11–12 hPI and that infected pigs produce antibody capable of lysing virus-infected cells suggested that this mechanism might act to prevent virus dissemination. To test this hypothesis *in vitro*, yield reduction assays were set up and the results of a typical virus titration of 48-hr supernatants are shown in Fig. 4. Controls included untreated cells, antibody alone and complement alone and, using this system, no virus was detected in cells treated with antibody and complement, whereas control treatments contained between 10^3 and 10^4 $TCID_{50}$ of ASFV.

Suppression of immune injury

Table 3 shows that there is no suppression of immune injury through antigenic modulation in ASFV-

Table 3. The effect of preincubation of ASFV-infected cells with immune serum on complement-mediated lysis

	SL (%)		
Target cells	Incubated with Ab	Incubated without Ab	Difference
Infected	100	26	74
Uninfected	25	6	19

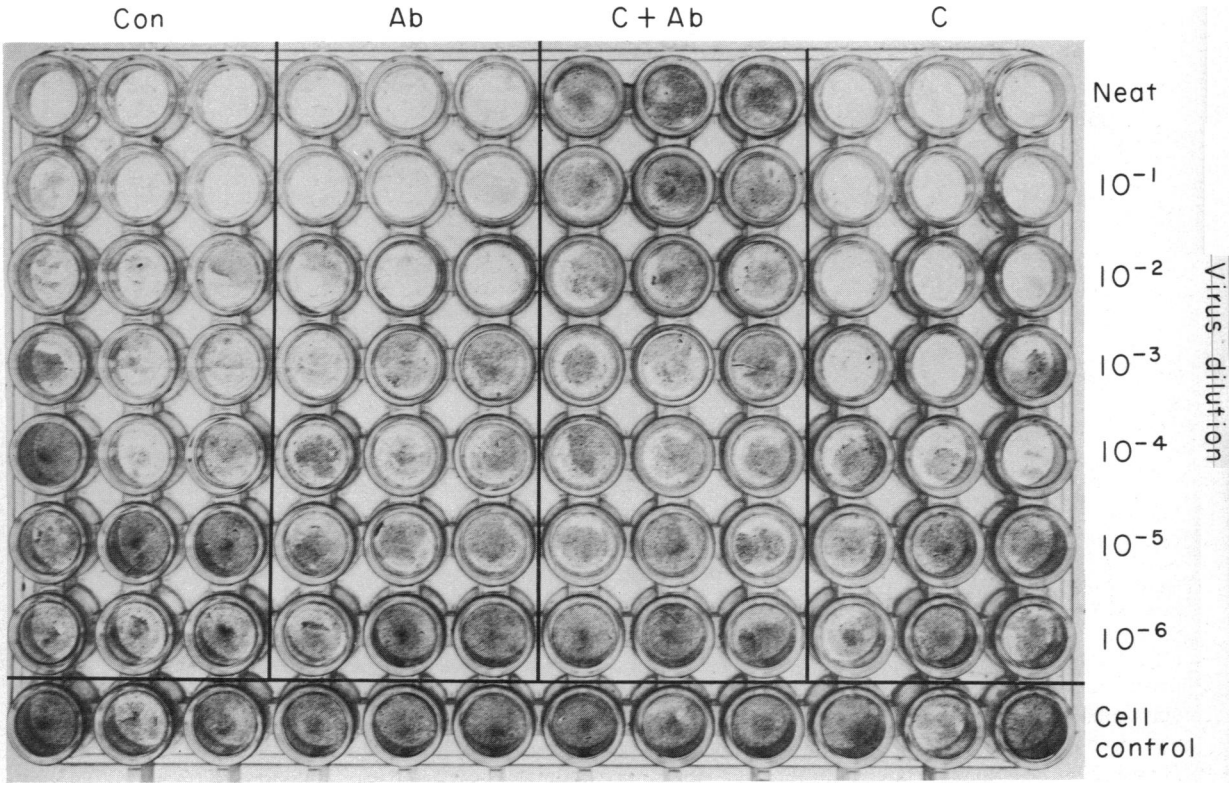

Figure 4. Titration of 48-hr samples from yield reduction experiment. Samples titrated in PBL cultures and plate stained and fixed in methanol/crystal violet for 10 min after 5 days of incubation at 37°. Presence of virus indicated by loss of cell sheet and thus no staining. Con, virus grown in normal medium; Ab, virus grown in presence of 1:50 dilution anti-Uganda antibody; C+Ab, virus grown in presence of 1:50 dilution anti-Uganda antibody + 1:4 dilution of pig complement; C, virus grown in presence of 1:4 dilution of pig complement. All supernatants changed every 2 hr.

infected cells. Rather, prolonged incubation with anti-ASFV antibody enhances the cytolytic effect of complement.

Cross reactivity of sera

Figure 5 shows that antibody to all the isolates of ASFV tested bound specifically to cells infected with the Uganda isolate. Preinoculation sera did not react. However, only certain sera contain antibody effective in fixing complement. All sera were taken between 24 and 35 dPI and from the results in Fig. 3 one would expect detectable levels of complement-fixing antibody by this time. Although not all sera produced by infection with isolates from Africa caused complement-mediated lysis of Uganda-infected cells, the majority did. However, none of the sera produced by non-African isolates caused complement-mediated lysis. This indicates that the European and South American isolates may be antigenically distinct from the majority of the African isolates.

DISCUSSION

The objectives of this investigation were to determine whether complement in the presence of anti-ASFV antibody had cytolytic activity against ASFV-infected cells and to evaluate whether this might play a part in protection against ASFV infection. It is clear that using an homologous system ASFV-infected pig cells bearing viral antigen at the cell surface are rapidly lysed by the action of complement and specific antibody (Fig. 2). The characteristics of this lysis are

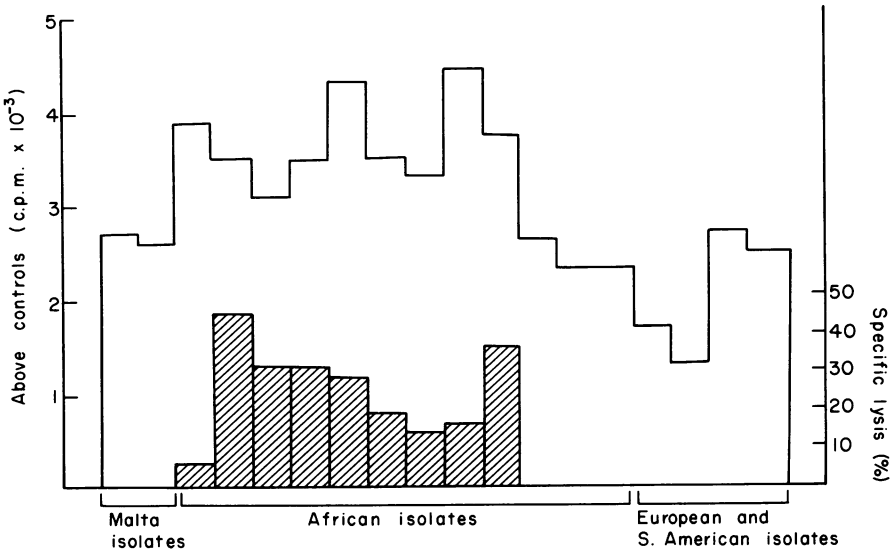

Figure 5. Binding of antibody and antibody-mediated complement lysis by sera taken 25–35 dPI with various ASFV isolates. Bound antibody (□) and complement-mediated lytic activity in same serum (■).

comparable in a number of respects to other homologous virus-cell systems, in particular the kinetics of ASFV-infected cell lysis and the measles virus system (Sissons, Cooper & Oldstone, 1979). However, the ASFV system differs in one major respect as preliminary experiments strongly suggest that the classical pathway alone is involved in the lysis of ASFV infected cells. All reported cases of complement-mediated lysis of infected cells in other homologous systems indicate the involvement of the alternative pathway. Such systems include measles, influenza, mumps and herpes simplex virus type 2 with a range of host cells (Perrin, Joseph, Cooper & Oldstone, 1976). Activity of complement against these virus-infected cells is invariably abrogated by heating at 50°, a procedure which is known to inactivate the heat-labile component factors of the alternative pathway. This does not occur with ASFV (Table 1). Also with the alternative pathway a 1:4 dilution of the complement source reduces lysis by 50% or more (Oldstone, Sissons & Fujinami, 1980) whereas in the ASFV system a 1:50 dilution is needed to cause a similar effect. The third piece of evidence indicating classical pathway involvement comes from using chelating agents. The physiological addition of Ca^{2+} ions then restored complement activity whereas Mg^{2+} did not. Although further work involving the use of serum specifically depleted of critical complement components is required to confirm these findings, it seems likely that ASFV is peculiar amongst reported viral infections in nucleated cells in its activation of the classical pathway in preference to the alternative pathway. The implications of this in relation to the immunology of ASFV infection remains to be resolved although there may be a possible link between the inability of bound antibody to neutralise ASFV and bound antibody to act as a necessary precursor for C3 alternative pathway activation.

To try to ascertain whether complement-mediated lysis might be important in eliminating virus two sets of experiments were performed. Firstly, sera taken from pigs infected with the original virulent Uganda isolate were examined and in such animals antibody to cell surface antigen developed at 5–7 dPI (Fig. 3). However, this population of antibody was ineffective in binding complement, and it was not until 14–15 dPI that complement activating antibody appeared. In infections with virulent isolates the majority of animals die before 14 dPI and thus it appears that its formation is too late to prevent the extensive pathological damage induced by virulent isolates (Hess, 1971).

Secondly, an *in vitro* yield reduction assay was performed to determine whether the onset of complement-mediated lysis at 11–12 hPI was sufficiently early

to prevent virus maturation. The results presented in Fig. 4 showed that such lysis appeared to occur before virus maturation *in vitro* and substantially reduced virus yields. In surviving animals infected with less virulent isolates, however, virus can still be isolated for, at least, 200 days (Wilkinson, Wardley, Williams, manuscript in preparation) and the possibility existed that virus was escaping elimination by inducing some form of antigenic modulation. Our results, however, would not support this idea. The situation in the infected animal is likely to be a far more complex one, and the relevance of the complement action *in vivo* is uncertain, particularly as the majority of pigs infected with the virulent isolates would die before complement fixing antibody is produced. Antibody transfer experiments need to be done to see if clinical signs and/or the level of viraemias in infected pigs respond to complement-mediated antibody lysis.

Apart from the apparent uniqueness of ASFV using the classical complement pathway and the possibility of this being one of the effector mechanisms important in recovery, complement-fixing antibody also appeared to offer itself as a method for discriminating between isolates. Figure 5 shows that although sera against the ASFV isolates tested bind to the surface of Uganda-infected cells, only sera from isolates from Africa, the origin of the Uganda isolate, induce the cytolytic activity of complement. However, not every African isolate had this capability. It has been shown that, with one exception, all ASF viruses isolated outside Africa after 1960 belong to the same haemadsorbing group (Vigario, Terrinha & Nunes, 1974) and thus it is believed that all ASFV taken outside Africa after this time originate from the outbreak in Portugal in 1960, whereas the different areas in Africa could contain distinct types of ASFV. Thus, it is possible that complement-mediated lysis may provide a functional test for differentiating between ASFV strains in a manner which has previously been impossible. It seems likely that the specificity of the lytic complement assay is due to the fact that it only measures antibody to certain antigens displayed at the cell surface, whereas other tests may detect antibody to many viral antigens, including the internal components of ASFV, which are probably common to all strains.

ACKNOWLEDGMENT

We thank Dr P. Sissons for helpful discussions.

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