CHAPTER III

REPLICATION OF ASF VIRUS IN LLC MK_2 CELL CULTURES

INTRODUCTION

Studies on the replication of ASF virus in cell culture lines are scarce and probably this is because of the difficulties encountered in adapting this virus to various cell systems.

Plowright et al., (1966) reported on the growth curve of ASF virus in pig kidney cell lines and on the inhibitory effects of 5-iodo and 5-bromo-2' deoxyvridine and Actinomycin-D. Coggins (1966) studied the growth of three isolates in PK-2a and BC cell cultures. Cpe were observed on about the 5th day post-inoculation when the infective titres were in excess of 10^5 $TCID_{50}$. Virus release was slower in pig kidney than in BC cells and the level of cell associated virus exceeded that of the free virus through out the replicative cycle. Pig kidney cells were less sensitive than BC cells in determing infective titres. This author also established that ASF virus was unaffected by trypsin, freezing and thawing and ultrasonic treatments.

Because the results of the previous experiments indicated that MK cells could be used as a complementary system to the BC cell cultures, it was decided to study the replication and the maximum yield of harvested virus in MK cell monolayers.

MATERIALS AND METHODS

Virus

Two strains of virus, Lillie-148 HAd^- and 2055 HAd^+, both at the 12th passage in MK cells, were used. The arabic number after the type of cell culture, indicates the passage level at which the strain was used.

Viral adsorption

Replicate titrations were carried out by infecting MK cell monolayers with serial dilutions of both virus preparations ranging from 10^{-1} to 10^{-7}. Adsorption was allowed to take place for fixed intervals at $37^{o}C$ in a rotating apparatus. At the end of each interval, one set of titrations was rapidly removed from the incubator, the inoculum discarded and each culture tube washed in 4 changes of PBS. After the addition of maintenance medium the cultures were returned to the incubator.

Assessment of the amount of virus adsorbed to cells after 120 min incubation

MK cell monolayers in tissue culture tubes were infected with 0,2ml of the two undiluted virus preparations and rotated while incubating at $37^{o}C$ for 120 min. A control aliquot of the virus suspension was maintained at $37^{o}C$ for 2 h. After completion of the incubation period the inoculum was removed and the monolayers washed in 4 changes of PBS; the cells were then scraped and harvested in Eagle's medium and centrifuged. Cells recovered from five tubes were considered equivalent to 1ml of inoculum. They were disrupted by freezing and thawing and titrated together with the control aliquot.

Growth curves

Monolayers in culture tubes were infected as for the previous experiment. At the end of the 120 min adsorption period they were washed in 4 changes of PBS and the maintenance medium added.

Five culture tubes were taken at random, immediately after the addition of the medium and at intervals of 24 h. They were subsequently frozen and thawed on 3 successive occasions, pooled and titrated.

Virus cell-association and the effect of freezing and thawing

The 10^{-1} dilutions of the two virus preparations were used to infect flasks containing monolayers of MK cells.

Three flasks were removed from the incubator at intervals corresponding to the four stages of the Cpe. The cells were scraped with the aid of a rubber policeman after which the contents of the three bottles were pooled, homogenized and an aliquot taken. The remaining culture fluid was centrifuged at 3 200 r p m (x g 1717) for 30 min, the supernate was decanted and stored at 4^{o}C. The sedimented cells were washed three times in PBS. After the last centrifugation the cells were resuspended in a volume of maintenance medium equal to that of the original. Infectivity titrations were carried out with each preparation obtained.

Samples of medium and cell mixtures, obtained after generalization of Cpe from the experiment above, were frozen and thawed three times. Titrations were carried out before and after centrifugation at 3 200 r p m for 30 min.

RESULTS

Each result reported is the mean of three experiments conducted under identical conditions.

Viral adsorption

The results of the infectivity titrations conducted to establish the rate of adsorption with the two strains of ASF

virus are given in Fig. 9. It was concluded that adsorption was complete after an incubation period of 120 min.

Assessment of the amount of virus adsorbed to cells after 120 min incubation

While the mean infectivity of the inoculum of strain Lillie-148 before adsorption was $10^{5,35}$ that of cell adsorbed virus was $10^{4,1}$ $TCID_{50}$. With strain 2055 the viral titres were $10^{5,5}$ and $10^{4,5}$ $TCID_{50}$ respectively. This suggested that with both preparations approximately 90% of the virus was adsorbed within the 120 min period.

Growth curves

Results are given in Fig. 10. Using a mean virus input of $10^{5,3}$ $TCID_{50}$, peak virus titres were obtained for both preparations, on the 6th day post-inoculation and coincided with the complete destruction of the monolayers. Peak infective titres persisted for at least 24 h before thermal inactivation occurred.

Virus cell-association and the effect of freezing and thawing

The results of the experiments given in Table 11 indicate that ASF virus is not readily separated from infected cells. Freezing and thawing of the virus preparations did not have any detrimental effect on infectivity, however, when it is followed by centrifugation, the infectivity of virus stocks is lowered.

FIG. 9 Adsorption of ASF virus in MK cells

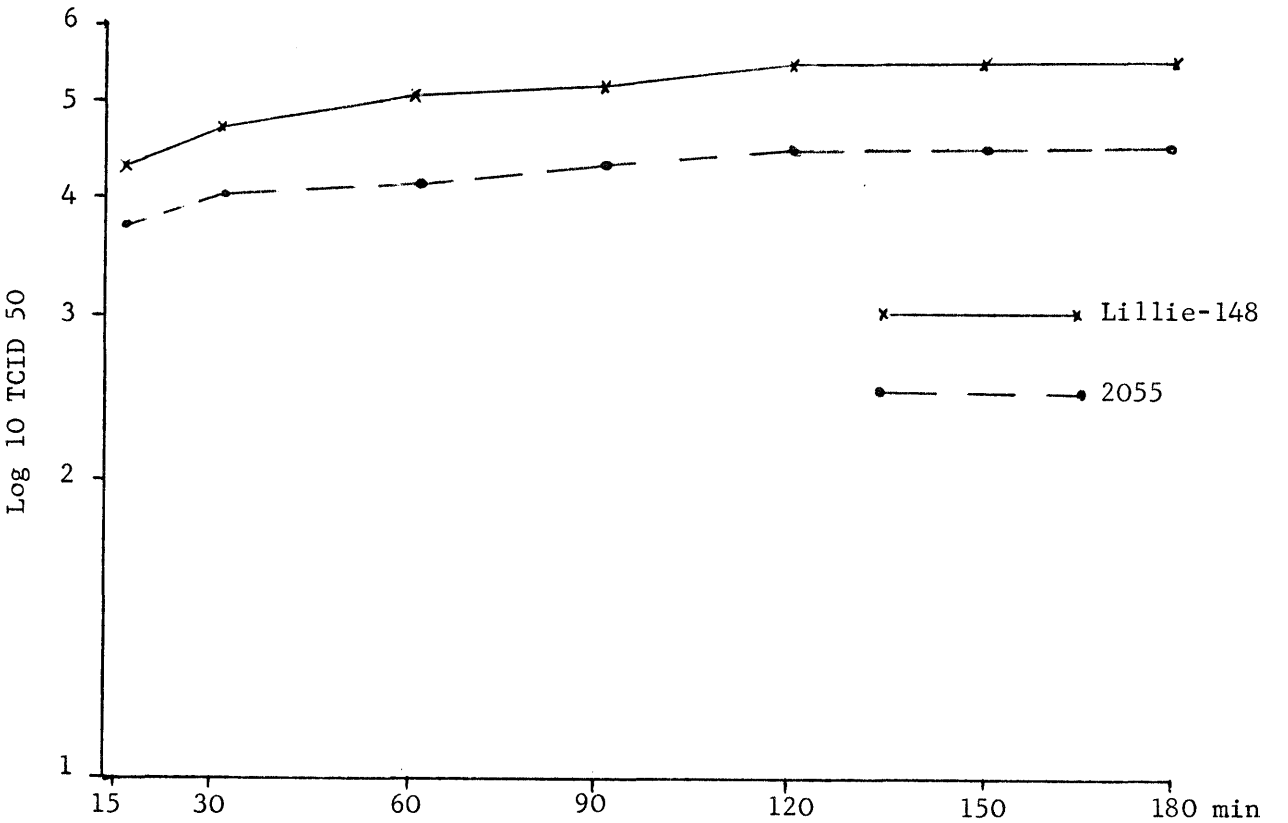

FIG. 10 Growth curve of ASF virus in MK cells

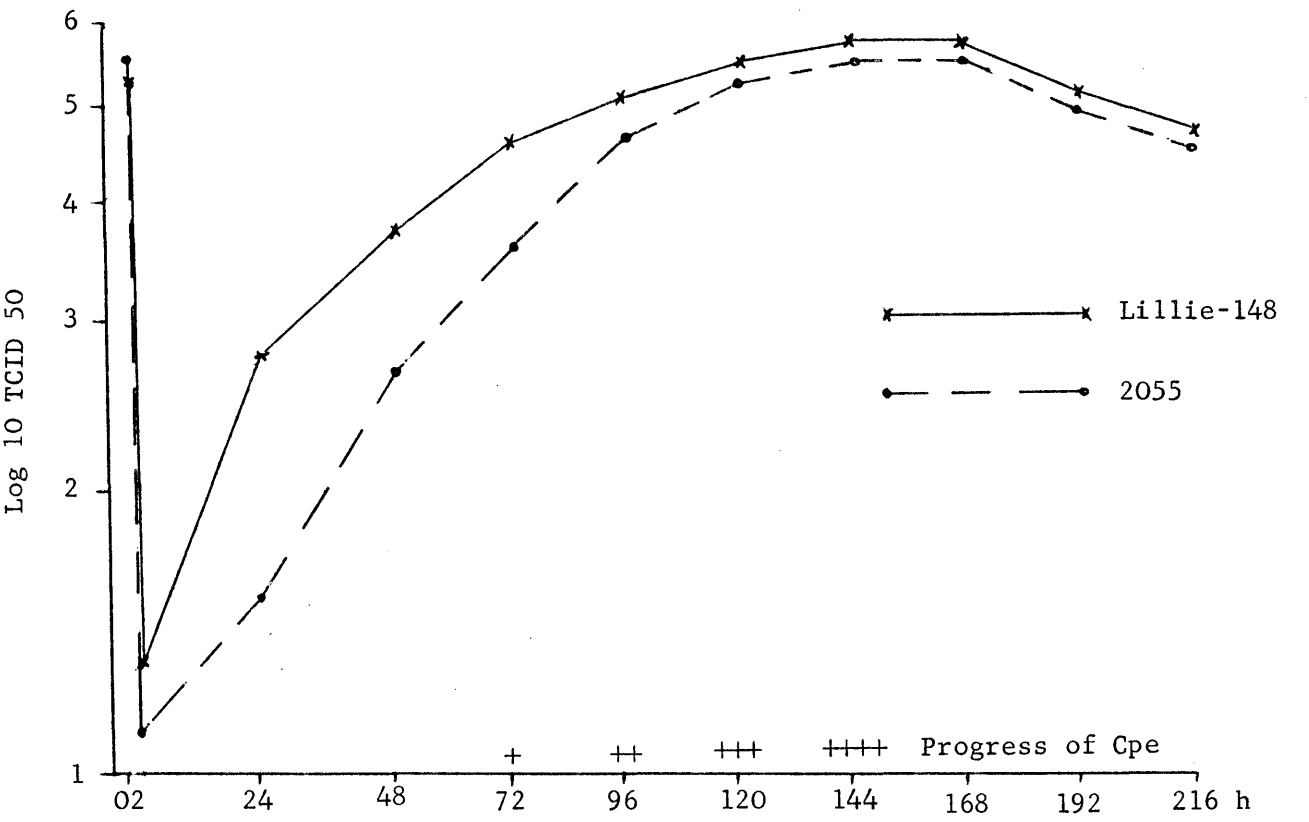

TABLE 11 Infectivity of free and cell-associated virus during replication of strains Lillie-148 and 2055 MK12

Strain	Progression of Cpe	Medium	Cells	Cells + Medium
Lillie-148	+	3,3*	4,5	4,8
	++	4,5	5,0	5,2
	+++	5,3	5,5	5,8
	++++	5,6	5,6	5,8**
	Freezing & thawing			5,8
	& centrifugation			5,6
2055	+	2,5	3,6	3,6
	++	3,5	4,5	4,6
	+++	5,0	5,5	5,5
	++++	5,2	5,5	5,6**
	Freezing & thawing			5,6
	& centrifugation			5,0

*Log 10 $TCID_{50}$/0,2 ml. Mean of 3 infectivity titrations

**Preparation used to study effect of freezing & thawing followed by centrifugation.

CONCLUSIONS

These results suggest that ASF virus replicating in MK cell monolayers has an optimal adsorption time of 120 min, during which approximately 90% of the virus population is adsorbed on to the cells. With the two preparations used, Cpe were observed when the viral concentration had reached mean values of $10^{4,0}$ $TCID_{50}$. The peak infective titres coincided with the complete destruction of the monolayers and thermal inactivation started 24 h later. At least 50% of the virus remains cell associated when Cpe is generalized and three cycles of freezing and thawing did not seem sufficient to break up this association.

CHAPTER IV

INFLUENCE OF SERIAL PASSAGE IN CELL CULTURES ON THE HAEMADSORBING CHARACTERISTICS OF ASF VIRUS

INTRODUCTION

Adsorption of erythrocytes is an effect which has been observed with cell cultures infected with a variety of viruses and it is apparently due to the incorporation of a virus protein into the plasma membranes of the infected cells (Shelokov, Vogel & Chy, 1958; Laurenaudie, Toulier, Santucci & Carnero, 1967).

Cell cultures infected with ASF virus haemadsorb porcine erythrocytes (Malmquist & Hay, 1960; Malmquist, 1963; Greig et al., 1967). The mechanism of the reaction has been studied by Breese & Hess (1966) and Larenaudie et al. (1967) with inconclusive results, though the effect apparently occurs at the level of the intact cell membrane. Haemadsorbing characteristics are not essential for infectivity (C. Sanchez Botija & R. Sanchez Botija, 1965; Coggins, 1968b; Pini & Wagenaar, 1974 and Pini 1976) and according to Vigàrio et al. (1974) the envelope of the virus may be of importance in haemadsorption since non-enveloped virions in his laboratory failed to produce this effect.

To assess whether maintenance in cell cultures had any influence on the haemadsorbing characteristics of the isolates of ASF virus, serial passages were carried out in parallel in MK and BC cells.

MATERIALS AND METHODS

Virus

Strains of ASF virus 2055 HAd^+, CV HAd^+, Lillie-148 HAd^- and TS 237 were used. From the results of previous experiments the latter strain was an heterogeneous population of HAd^+ and HAd^- virus. The serial passages of strains 2055 and Lillie-148 were initiated from the original spleen suspensions stored in the interim at $-70^{o}C$ whereas for strains CV and TS 237 they were initiated from the BC stocks at the 6th and 3rd passage respectively. Identity of viruses was confirmed at regular intervals by immunofluoresence.

Assay method

Cell monolayers in plastic tissue culture flasks were infected with 0,2 ml of the 10^{-1} virus dilution. When Cpe or HAd or cell lysis had generalized, cultures were frozen and thawed three times and used for the next passage. Infectivity titrations were carried out at intervals for each series in MK and BC cell cultures simultaneously, according to the methods described.

RESULTS

An average of two subcultures per week were made with the various strains of virus in the BC series while an average of 1,5 passages per week were undertaken in the MK series. No significant differences were observed with regard to the duration of incubation at the various passage levels. Unless otherwise stated, no significant abnormalities were seen, in the appearance and intensity of HAd in BC cells whenever this effect was present.

Strain 2055 HAd^+

MK series. Forty six serial passages were carried out in MK cell cultures. When comparative titrations were carried out in MK and BC cell cultures at the 7th, 15th, 26th, 41st and 46th subculture, the infective titres were found to be comparable in both systems. As shown in Table 12 strain 2055 lost its haemadsorbing characteristic between

the 41st and 46th MK passage and the infective titre in BC cells at the 46th subculture was estimated on the presence of cell lysis.

TABLE 12 Assessment by infectivity titrations in BC cells of the haemadsorbing characteristics of strain 2055 of ASF virus following serial passages in BC or MK cell cultures

Serial passages Strain 2055	Infectivity titres in BC cell cultures HAd and/or	Lysis	Difference
MK 7	4,0*	4,0	0
15	3,5	3,5	0
26	6,5	6,5	0
41	5,5	5,5	0
46	0,0	6,5	6,5
BC 11	4,5	4,5	0
21	4,5	4,5	0
41	5,5	5,5	0
70	4,5	4,5	0
75	4,5	5,5	1,0
76	3,5	6,5	3,0

*Log 10 $BCHAd_{50}$ or $BCID_{50}$ / 0,2 ml

BC series. Seventy six serial passages were carried out. Comparative titrations in the MK and BC cell cultures at the 11th, 21st, 41st, 70th, 75th and 76th passages showed that MK cells were less sensitive in establishing the infectivity of strain 2055 adapted to BC cell cultures. The mean virus titres in the former system were 1000 times lower than in the latter. From the results given in Table 12 the presence of an HAd^- virus population became demonstrable in the limiting virus dilutions between the 70th and 75th passage.

Strain CV HAd$^+$

MK series. Forty one passages were carried out. Comparative titrations in MK and BC cell cultures at the 10th, 19th, 37th and 41st passage showed that both systems were equally sensitive for estimating virus infectivity. The results are given in Table 13 Strain CV lost its haemadsorbing characteristic between passage 37 and 41.

TABLE 13 Assessment by infectivity titrations in BC cells of the haemadsorbing characteristics of strain CV of ASF virus following serial passages in BC or MK cell cultures

Serial passages Strain CV	Infectivity titres in BC cell cultures HAd and/or Lysis	Difference
MK 10	4,5*	0
19	5,5	0
37	4,5	0
41	0,0	5,0
BC 9	4,0	0
18	4,5	0
43	6,0	0
56	5,5	0
61	5,5	1,0
75	4,5	2,0
76	3,5	3,0

*Log 10 $BCHAd_{50}$ of $BCID_{50}$ / 0,2 ml

BC series. Seventy six passages were made. Comparative titrations carried out in MK and BC cell cultures at the 9th, 18th, 43rd, 56th, 61st and 75th and 76th passage confirmed that the former system was 1000 times less sensitive in estimating the infectivity of the strain adapted to BC cells. As shown in Table 13 a HAd$^-$ virus population became demonstrable at the terminal virus dilution by the 61st BC passage.

Strain TS 237

MK series. Only 10 subcultures were made. The sensitivity of BC and MK cell cultures was found comparable when infectivity titrations were made in both systems at the 2nd, 5th, 7th and 10th passage. As shown in Table 14 the HAd^- virus population was already predominant at the 2nd passage and haemadsorbing virus could not be detected by the 7th passage.

TABLE 14 Assessment by infectivity titrations in BC cells of the haemadsorbing characteristics of strain TS 237 of ASF virus following serial passages in BC or MK cell cultures

Serial passages Strain TS 237	Infectivity titres in BC cell cultures HAd and/or Lysis		Difference
MK 2	4,5*	5,5	1,0
5	2,5*	5,5	3,0
7	0,0	5,5	5,5
10	0,0	5,5	5,5
BC 5	7,5	7,5	0,0
15	5,5	5,5	0,0
25	5,0	5,0	0,0
37	6,5	6,5	0,0
45	4,5	5,5	1,0
50	5,5	6,5	1,0
55	5,0	5,0	0,0
60	6,0	6,0	0,0

*Log 10 $BCHAd_{50}$ or $BCID_{50}$ / 0,2 ml

BC series. Sixty subcultures were made and comparative titrations in BC and MK cell cultures carried out at passage 5, 15, 25, 37, 45, 50, 55 and 60. The BC system was again 1000 times more sensitive than the MK system for estimating infectivity.