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Factors affecting the production of foot-and-mouth disease virus in deep suspension cultures of BHK21 Clone 13 cells

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It was shown by Capstick, Telling, Chapman & Stewart (1962) that BHK21 Clone 13 cells would multiply in suspension culture and remain susceptible to foot-and-mouth disease (FMD) virus. Recently Telling & Elsworth (1965) and Telling & Stone (1964) have demonstrated that these cells can be grown in a 30 l. vessel equipped with automatic pH and temperature control apparatus, thus opening the possibility of producing FMD inactivated vaccines on a commercial scale.

The experiments described here were undertaken to determine the optimum conditions for viral infection in deep suspension culture to release maximum FMD viral antigen for use in inactivated vaccines.

MATERIALS AND METHODS

Cells

BHK21 Clone 13 cells were used after passage for between 100 and 150 days in suspension culture. They were then grown for 3 days in 2 l. glass vessels as described by Telling & Elsworth (1965). After a growth period of 3 days the cells were infected with virus. The exhausted medium was removed by centrifugation and unless otherwise stated the cells were resuspended for virus infection in Eagle's medium with 10% tryptose phosphate broth (T.P.B.) and 5% horse serum. Antibiotics were incorporated at final concentrations of penicillin and streptomycin 100 units, neomycin 70 units, polymixin 20 units and mycostatin 25 units/ml. The bicarbonate content of the complete medium was adjusted to a final concentration of 3·0 g./l. Antifoam (Emulsion RD)* was added at 0·02%. The cells used in these experiments were at least 90% viable at the time of infection.

Virus strains

All virus used originated from infected cattle epithelium. The virus was passaged in BHK monolayers, and the supernatant fluid frozen as stock seed virus in 60 ml. volumes at $-70°$ C. One stock of seed virus was used for a complete series of experiments involving one variable. No deliberate attempt was made to adapt

* Midland Silicones Ltd., London.

the viruses to BHK cells, and only sufficient passages were carried out to provide enough virus for stock seed. The viruses used were:

Type O	Strain Israel 1/63	6th BHK passage
Type A	Strain A 119	7th BHK passage
Type A	Strain Iraq 24/64	7th BHK passage.

Virus assays

Infective virus titres were assayed by the plaque technique on 2-day monolayers of BHK 21 Clone 13 cells. Plaques were counted 2 days after infection.

Complement-fixing antigen titres were estimated using Arklone P extracted supernatant culture fluids as described by Brown & Cartwright (1960). In preliminary experiments it was determined that the use of Arklone P in the presence of serum does not remove the 7 mμ virus component, but it does, however, remove most of the anticomplementary activity present in some cultures. Culture supernatant fluids were extracted by manual shaking three times with an equal volume of Arklone P. Complement-fixing antigen titres were determined by a modification of the method of Bradish, Jowett & Kirkham (1964). Two-fold dilutions of antigen, one 70% dose of complement and the optimal antiserum dilution were used in the test. After fixation for $1\frac{1}{2}$ hr. at 37° C. and addition of the haemolytic indicator system, haemolysis was allowed to proceed for 1 hr. at 37° C. The r.b.c. were then deposited by centrifugation and the degree of haemolysis estimated in an absorptiometer. The antigen titre of a sample was expressed as complement fixing units (cfu) and was the reciprocal of the antigen dilution causing 30% reduction in haemolysis compared with the controls.

In our hands this test has a 'between test' standard error of 14% ($P = 0{\cdot}05$) and a 'within test' standard error of 5% ($P = 0{\cdot}05$).

Culture vessels

Two identical 800 ml. culture vessels were constructed. These vessels were of similar proportions and construction to the bacterial culture vessel described by Elsworth, Capel & Telling (1958) but without baffles.

The vessels were equipped with automatic pH and temperature recording controlling apparatus as described by Telling & Stone (1964), and Telling & Elsworth (1965). The pH was controlled at the desired value $\pm$ 0·05 pH unit and the temperature at the desired value $\pm$ 0·5° C. The vessels were sterilized *in situ* by direct steam injection at 4 lb./in.^{2} for 4 hr. Effluent air from virus infected vessels was sterilized by heating in a tube furnace at 400° C.

Virus–cell cultures

Both vessels were used for virus–cell experiments. One vessel was used to determine the effect of a variable and the other maintained as a control. The medium and cells were prepared in bulk in glass containers and a measured volume transferred to the vessels under slight positive pressure of filtered air. The temperature, pH control and stirring mechanisms were then put into operation and

when the desired pH and temperature values were reached the culture was infected with virus. The thawed stock seed virus was added as 10 % of the culture volume. Where necessary the stock seed was diluted in medium to give the required infective titre in the appropriate volume.

Samples of the cultures were removed at frequent intervals after infection and the cell concentration was determined after trypan blue staining. The cells were then removed from the sample by centrifugation and the supernatant stored at 4° C. to await infectivity and complement-fixing titrations.

In the later stages of an infected culture it was difficult to obtain accurate estimates of dead cell numbers as the cell count was obscured by increasing amounts of cell debris. It was found that a useful estimate of the progress of viral infection could be obtained by counting only the surviving viable cells and expressing these as a percentage of the starting viable cell concentration.

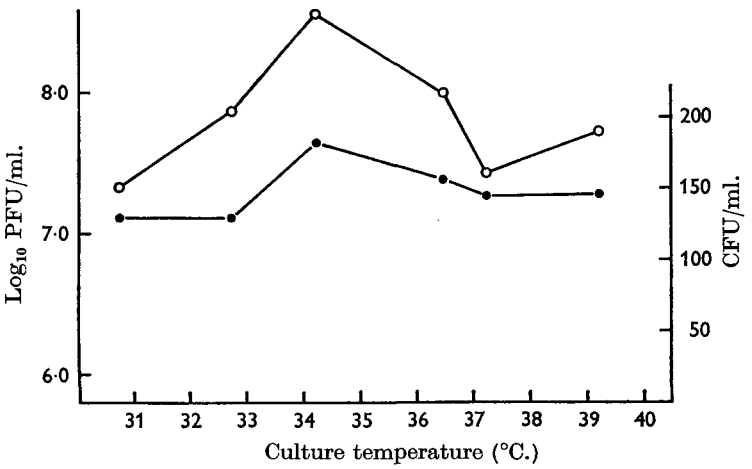

Fig. 1. The effect of culture temperatures on peak extracellular FMD A/Iraq virus titres. ○—○, Open circles pfu/ml. ●—●, Closed circles cfu/ml.

RESULTS

Effect of temperature

The effect of temperature on peak extracellular A/Iraq virus titres is shown in Fig. 1. At least two observations were made for each point. Virus titres declined on either side of 34·25° C., but estimating from the shape of the graphs the optimal temperature is probably nearer to 35° C.

The culture temperature had a profound effect upon cell survival following infection. This is shown in Table 1 in terms of the 30 % cell survival time at 34·25° C. At temperatures below 34·25° C. cell survival increased markedly, apparently due to reduced virus replication. This was reflected by lowered peak virus titres.

Table 1. *Effect of culture temperature on 30 % cell survival time of cells infected with A/Iraq FMD virus*

Culture temperature (°C.)	30 % Cell survival time (hr.)
30·75	70·5
32·75	51·6
34·25	25·5
36·5	22·2
37·25	16·5
39·25	26·2

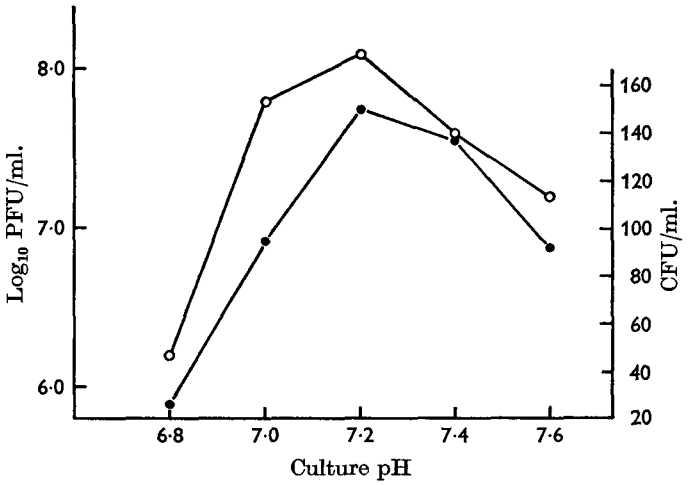

Fig. 2. The effect of culture pH on peak extracellular FMD A119 virus titres. ○—○, Open circles pfu/ml. ●—●, Closed circles cfu/ml.

Effect of culture pH

The peak extracellular virus titres were examined over the pH range 6·8–7·6 at 0·2 pH intervals. The results using A119 virus are shown in Fig. 2. Maximum extracellular virus titres were reached at pH 7·2 and declined above and below this value. The 30 % cell survival time decreased from 46 hr. at pH 6·8 to approximately 22 hr. at pH 7·2 and above.

The pattern of infective extracellular virus release during the period of an infected culture at pH 7·2 and 7·6 is shown in Fig. 3. Although at high pH levels cell infection and virus release appeared to take place normally during the first 4 hr. of culture, virus reproduction was much reduced in the later stages.

Cell concentration

In this series of experiments 'O' Israel seed virus was appropriately diluted before infection of the culture to maintain an approximate input virus/cell ratio of one plaque-forming unit (pfu) per 30 cells. The effect of cell concentration on the extracellular virus titres and on number of pfu per cell and cfu per $10^{6\cdot0}$ cells is shown in Table 2, for the range $1 \times 10^{6\cdot0}$ to $9\cdot6 \times 10^{6\cdot0}$ cells/ml. Infective antigen

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titres per ml. showed no increase over this range. Complement-fixing antigen titres per ml. increased as the cell concentration increased, but not in direct proportion. As the cell concentration was increased the yield per cell either of complement fixing or infective antigen decreased.

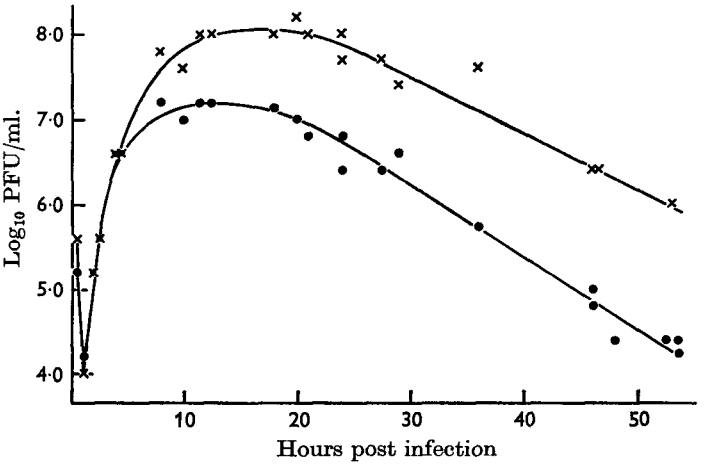

Fig. 3. The extracellular infective virus released from BHK 21 Clone 13 cells during cultures held at pH 7·2 and pH 7·6. ×—×, Crosses pH 7·2. ●—●, Closed circles pH 7·6.

Table 2. *Effect of cell concentration on extracellular virus titres and antigen released per cell*

Cell concentration per ml. × $10^{6\cdot0}$	Infective antigen		Complement-fixing antigen	
	pfu./ml.* log_{10}	pfu/cell*	cfu/ml.*	cfu/10^6 cells
1·1	7·8	56·5	79·4	72·5
1·9	8·0	53·4	93·6	50·5
2·5	7·9	31·9	75·3	30·6
3·0	7·7	15·2	124	41·0
4·6	8·2	35·5	155·5	33·8
7·5	8·0	12·6	196·5	26·1
8·0	7·9	9·2	198·0	24·7
9·6	7·8	7·0	N.D.	—

* Measured at the time of peak titre.
N.D. = not determined.

Multiplicity of infection

The volume of seed virus required to infect a culture is of considerable importance on a large scale and experiments to determine the effect of varying the input virus/cell ratio were carried out. During these experiments cell concentrations were kept in the range 2·4–3·2 × $10^{6\cdot0}$ cells/ml. The results expressed in yield of virus antigen per cell at the time of peak titre are shown in Table 3 for A/Iraq virus. The yield of complement-fixing antigen per cell was independent of the input virus/cell ratio. The infective yield per cell was significantly reduced at virus/cell ratios below 1:320.

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The effect of serum

The inclusion of 5% filtered horse serum in the medium results in increased infective and complement-fixing antigen titres. A typical experiment is shown in Table 4. In the culture in which serum was included there was an approximate threefold increase in complement-fixing antigen. We have been unable to demonstrate a similar effect following infection of monolayer cultures of BHK cells. The addition of ox serum produced similar results, but some serum batches were found to contain non-specific neutralizing activity which considerably reduced the antigen titre of some virus strains.

Table 3. *Effect of input virus/cell ratio on the virus released per cell*

Log no. of cells to one pfu	Infective units/ cell* (pfu)	Complement-fixing units* per $10^{6\cdot0}$ cells
0·59	51·5	57·1
1·12	51·6	42·8
2·50	52·1	36·6
3·56	26·8	67·4
3·66	36·8	45·2
4·75	21·1	44·1
5·32	36·1	49·8
5·88	18·6	46·9

* Measured at time of peak titre.

Table 4. *The effect of addition of 5% horse serum to cultures infected with A 119 FMD virus*

Time post inoculation (hr.)	No serum		5% horse serum	
	pfu/ml. $\log_{10}$	cfu/ml.	pfu/ml. $\log_{10}$	cfu/ml.
3	5·4	N.D.*	5·2	N.D.
20·5	7·25	25	7·81	N.D.
22·5	7·34	30	7·90	45
28	7·29	39	7·88	100
45	6·48	41·5	7·20	108
52	N.D.	49·0	N.D.	124
69	N.D.	44	N.D.	150

* N.D. = not determined.

DISCUSSION

The results obtained here must be interpreted in the light of the desired end-product, and the processes following the virus culture. Thus, if only high titres of infective virus are required the culture can be terminated at a much earlier stage than if maximum antigen (as expressed by complement-fixing activity) is the required end-product. With all the virus strains used here the time of peak complement-fixing activity occurred approximately 24 hr. or longer after the time of peak infective virus titre.

Temperature and pH control during virus infection were found to be necessary if maximum titres are required. However, in the case of pH control, it is not known

whether pH was the only factor contributing to the observed variations in peak titre. For example, the method of pH control used calls for sparged air beneath the impeller if the pH shifts to the acid side of the controlled value. As cultures continually produce acid during the first 48 hr. of infection, those cultures controlled at a high pH value receive more air, and therefore more oxygen than cultures controlled at low pH values. It is thus possible that dissolved oxygen or the effect of sparging on the cells may have been contributing factors to the results. The time of peak infectivity in the cultures was not affected by changes in the input virus/cell ratio over the range described in Table 3. During this series of experiments the maximum infectivity titre occurred at 23 ± 3 hr. after virus infection.

Assessment of the best cell concentration for use during vaccine production is difficult as the system becomes increasingly inefficient with increasing cell concentrations. For inactivated FMD vaccines a high complement-fixing antigen titre is required (Brown & Newman, 1963). However, it is difficult to fix a minimum acceptable CF antigen titre applicable to all strains of FMD virus, as the amount of complement-fixing antigen produced varies with the virus strain used. Similarly, vaccines prepared from a range of virus strains have varying immunogenic potential when measured *in vitro* (authors–unpublished information). Each virus strain and the level of CF activity required must be assessed separately, bearing in mind the vaccine dose rate, composition, etc. We have found that vaccines containing 1 ml. of antigen prepared from cultures with cell concentration of $2{\cdot}0$–$2{\cdot}5 \times 10^{6{\cdot}0}$ cells/ml. confer satisfactory protective levels in cattle; but this can only be used as a general guide.

If an efficient virus concentration procedure was envisaged in the later stages of production, it would be more productive to infect cells at a concentration of $1 \times 10^{6{\cdot}0}$/ml.

In the series of experiments dealing with input virus/cell ratios, peak infective titres remained the same, and occurred at the same time with input virus/cell ratios ranging from 1:1 to 1:320. This is of considerable interest from a large-scale production viewpoint, as theoretically 1 l. of monolayer seed virus (approximate titre $10^{7{\cdot}0}$ pfu/ml.) could be used to seed a 400 l. culture vessel without a reduction in the titre of peak virus. It is of interest that Telling, Radlett & Mowat (1967), using the same cell system, found that Semliki Forest virus yield increased as the input virus decreased.

The use of serum in the medium during the infective process, while giving a valuable increase in antigen titre, may well be inconvenient in many cases where virus is produced for biochemical or immunochemical studies. Bovine serum frequently contains specific neutralizing activity in those countries where FMD is endemic and horse serum is not always available in quantity. However, in our opinion the increases in antigen titres are sufficient to warrant the inclusion of serum in the virus culture medium at present. The ideal solution would be the development of a strain of cells which was not serum-dependent during the cell growth and virus infection processes and further work is being carried out on these lines.

SUMMARY

For maximum utilization of deep cultures to produce FMD virus it was important to have adequate control of culture temperature and pH. Culture temperature should be controlled within the range 34·25°–35° C. and culture pH at 7·2. The culture system became less efficient as the cell concentration was increased from $1 \times 10^{6 \cdot 0}$ to $9 \times 10^{6 \cdot 0}$ cells/ml. A cell concentration of $2 \cdot 5 \times 10^{6 \cdot 0}$ cells/ml. represented a working compromise between efficiency and antigen titre/ml. for inactivated FMD vaccine production.

The input virus/cell ratio had no effect on the time or titre of peak virus yield in the range 1:1 to 1:320. This makes the production of seed virus from small numbers of monolayer cultures feasible and economical.

Virus yield was improved by the addition of 5% serum. It would be more satisfactory if a serum-free cell strain could be developed.

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