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A method for preparing smallpox vaccine on a large scale in cultured cells*

BY C. KAPLAN AND LISEL R. MICKLEM
Lister Institute of Preventive Medicine, Elstree, Hertfordshire

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INTRODUCTION

Traditional methods of preparing smallpox vaccine entail passage of vaccinia virus through living mammalian hosts. These methods have several disadvantages, chief amongst which is the difficulty of obtaining a vaccine free of bacterial contaminants. Vaccinia virus has been propagated in the chorioallantoic membrane of developing chick embryos for vaccine production (Goodpasture & Buddingh, 1933, 1935; Buddingh, 1943; Stevenson & Butler, 1933, 1934, 1935, 1939). It has been successfully used in India (Pandit, 1941–46) and for many years in Texas (Irons & Cook, 1957), but there is some doubt about its stability (Cook, Crain & Irons, 1948; Buddingh & Randall, 1951; Nagler, 1944). Recently, attempts to find an alternative method of culture which would rapidly yield a bacteria-free suspension of virus have focused attention on the possibilities of various forms of tissue culture.

Many workers were successful in propagating the virus in various tissues growing in plasma clots. Their methods were primarily suited to the study of the virus and its relationships with the cells, although Carrell & Rivers (1927) and Eagles & McClean (1929) realized the possibilities of developing this kind of culture for large-scale vaccine production.

Maitland & Maitland (1928) made their classical experiments with this virus in minced hen kidney in a medium containing hen serum and a balanced saline. Li & Rivers (1930) adapted their method for large-scale vaccine production, and vaccine so produced during the next few years was immunogenic in man, although the immunity was not always lasting. Rivers, Ward & Baird (1939) recommended primary vaccination with tissue culture vaccine which they regarded as a means of avoiding the 'dangers and inconvenience associated with primary vaccinations with calf lymph virus', followed by revaccination with calf-lymph 6 months later.

The simplification of the problems of tissue culture by antibiotics was followed by fruitful work on vaccine production in plasma-clot and Maitland-type cultures (Wesslén, 1953, 1956; Ramon, Richon, Thiery, Salomon & Salomon, 1953, 1954*a*; Ramon, Richon, Thiery, Salomon & Doucet, 1954*b*; Kapsenberg, 1955; Cutchins & Warren, 1957). We tried Wesslén's technique in this laboratory but failed to obtain yields of virus of sufficient potency; and our attempts to improve the yield by

* Much of this work was incorporated in a Thesis presented to The University of London by L. R. M. for the degree of Ph.D.

altering the composition of the nutrient medium were unsuccessful. The technique, moreover, has the drawback that the requisite bovine embryos are difficult to obtain in this country and are difficult to prove free of pathogens dangerous to man.

The chick embryo cell culture method now to be reported is a modification of that developed by Dulbecco & Vogt (1954). We are indebted to Dr P. D. Cooper for demonstrating the technique. Cells are dissociated by exposure to trypsin, and cultivated in Petri dishes under a constant flow of CO_2/air mixture. In these cultures we can produce vaccine potent enough to satisfy the requirements of the Therapeutic Substances Regulations of this country. Preliminary clinical trials of the vaccine have been satisfactory.

MATERIALS AND METHODS

Trypsinization of chick embryo tissues

About three dozen 12-day-old chick embryos were decapitated, eviscerated and cut into pieces of about 1 cm.^{3}. The pieces were washed in phosphate buffered saline (P.B.S.) of Dulbecco & Vogt (1954), part (*a*) (NaCl, 16·0 g.; KCl, 0·4 g.; Na_2HPO_4, 2·3 g.; KH_2PO_4, 0·4 g.; H_2O, 1600 ml.), and digested with 0·0125 % (w/v) crystalline trypsin (Armour and Co.), approximately equal to 0·09 Anson unit per 100 ml. dissolved in the Ca^{++} and Mg^{++} free P.B.S. The trypsinization was done in a M.R.C. pattern blood-transfusion bottle with slow magnetic stirring, in two stages; the first for 10 min. at 37° C in about 100 ml. of fluid, after which the supernatant liquid was discarded, the second for 22 hr. at 4° C in 200–300 ml. of fluid (Bodian, 1956). The dissociated cells were washed free of trypsin by three centrifugations, the first two resuspensions being in P.B.S. and the third in culture medium. Centrifugation at 510 *g* for 6 min. was sufficient to sediment the cells without excessively tight packing. Resuspension was done gently with a pipette of wide bore to avoid cell damage.

Nutrient medium

The nutrient medium adopted for routine use was:

Earle's saline (Earle, 1943)	85 %
Hartley's digest broth	5 %
Native horse serum (Seitz filtered)	10 %
	100 %

To this was added:

L-Cysteine hydrochloride	0·02 % (w/v)
Lactalbumen hydrolysate	0·5 % (w/v)
Penicillin	100 units per ml.
Streptomycin	100 units per ml.

This nutrient was a modification of one recommended by Dr J. C. N. Westwood (Westwood, MacPherson & Titmuss, 1957).

The Earle's saline Part A (NaCl, 6·8 g.; KCl, 0·4 g.; $MgSO_4.7H_2O$, 0·2 g.; $NaH_2PO_4.H_2O$, 0·125 g.; dextrose, 1·0 g.; 0·4 % phenol red, 2·5 ml.; H_2O,

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697·5 ml.; $CaCl_2.2H_2O$, 0·2 g. in 100 ml.) was made up with the lactalbumen hydrolysate, bottled in measured volumes and autoclaved. Earle's saline Part B ($NaHCO_3$, 2·2 g.; H_2O, 200 ml.) incorporated the L-cysteine hydrochloride and was sterilized by filtration. Hartley's digest broth was prepared in the usual way (Mackie & McCartney, 1948), except that it was sterilized by Seitz filtration. Parts A and B of the Earle's saline were mixed, and the digest broth, serum and antibiotics added immediately before the medium was used.

The seed virus

The Lister Institute Vaccine Strain at the eightieth dermal passage in rabbits was purified by differential centrifugation, freeze-dried for storage and reconstituted as required.

Titrations

Titrations were done by the pock counting method in the chorioallantoic membranes of 12-day-old chick embryos described by Westwood, Phipps & Boulter (1957). Virus titres were expressed as infectious units per millilitre (i.u./ml.).

Preparation of the cultures

The cell suspension was diluted to contain $2{\cdot}75 \times 10^6$ cells/ml. counted by the methods of Rappaport (1956) or Kaltenbach, Kaltenbach & Lyons (1958). The suspension was inoculated with virus 0·1 i.u. per cell, well mixed, and distributed in 13·5 cm. diameter Petri dishes with an automatic dispenser. Incubation was at 37° C in a humid chamber under a constant flow of CO_2/air mixture adjusted to maintain a pH of about 7·2 as indicated by the colour of the phenol red in the nutrient.

Harvesting the virus

We confirmed that most of the recoverable vaccinia virus remains attached to the cells or cell debris (Eagles & McClean, 1929; Feller, Enders & Weller, 1940; Noyes & Watson, 1955). It was necessary, therefore, to harvest the cells and disrupt them to release the virus. The cells were broken by homogenization with a Servall omnimixer at 4° C for 30 sec. at 14,000 r.p.m. Homogenization for longer periods, or subsequent ultrasonic vibration at 300 kcycles for 20 sec. resulted in no further release of virus.

Routinely the pooled harvest from two Petri dishes was sampled for titration. The values cited are the averages of the results obtained from several experiments.

RESULTS

Preparation of vaccine

Virus yield

The fall in titre after the fifth day (Fig. 1) probably results from the cumulative effect of heat inactivation (Fig. 2) and slowing or cessation of virus production from widespread destruction of cells.

Varying of the inoculation ratio (i.e. the average number of i.u. per cell initially introduced) between 0·001 and 1·0 made little difference to the harvest ratio

(i.e. the average number of i.u. harvested per cell initially introduced). Neither did modifications of the nutrient, such as increasing the glucose concentration or periodic renewal of nutrient, have much influence. It seemed that the cells' capacity for supporting virus multiplication was limited, and that the only way to increase the yield would be to increase the cell concentration.

Many workers with a wide variety of virus and tissue culture systems have pointed out the importance of maintaining an optimum proportion of tissue and fluid, and of optimum culture volume in relation to the size of the vessel in which it is contained (Robbins & Enders, 1950; Periera, 1954). Increase of cell concentration might upset this balance; e.g. by reducing the quantity of nutrient avail-

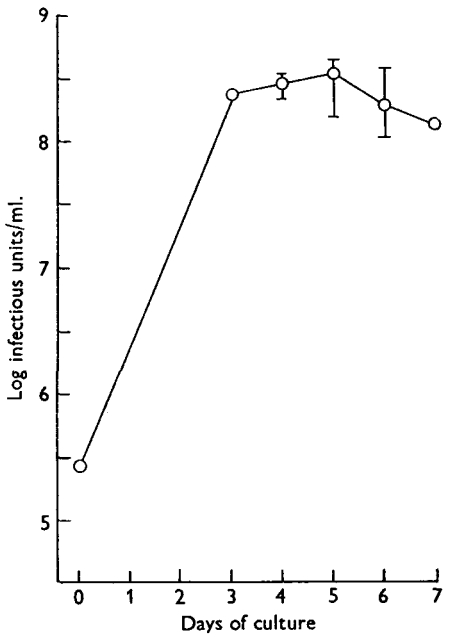

Fig. 1

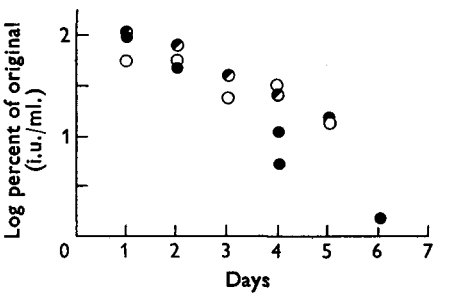

Fig. 2

Fig. 1. The rate of virus multiplication (average of 8 experiments) in tissue culture. 25 ml. of cell suspension ($2{\cdot}75 \times 10^6$ cells/ml.) inoculated at a ratio of 0·1 i.u./cell. The vertical lines indicate the range of titres obtained.

Fig. 2. The inactivation of vaccinia virus at 37° C (average of 5 experiments). ○, Nutrient without lactalbumen hydrolysate and L(−)-cysteine hydrochloride; ◐, nutrient at pH 6·8; ●, nutrient at pH 7·2–7·4.

able per cell, and increasing the concentration of waste products. In static cell culture, moreover, the cells settling on the floor of the Petri dish would be much closer together. There would be less room for their multiplication, but the beneficial 'alteration' of the nutrient by the cells envisaged by Fischer & Jenson (1946), Sanford, Earle & Likely (1948) and Earle, Sanford, Evans, Waltz & Shannon (1951) would be facilitated; and the spread of infection by cytoplasmic contacts between the cells would be increased (Bland & Robinow, 1939; Robinow, 1950; Noyes & Watson, 1955).

There was some indication that both the cell concentration and the degree of crowding of the cells on the floor of the Petri dish influenced the virus yield. The results of eight tests of cell number and nutrient volume relationships are summarized in Table 1 and Figs. 3 and 4. Fig. 3 records the titres (i.u./ml.) and Fig. 4 the harvest ratios (i.u./cell) obtained on successive days after inoculation with virus. The initial number of cells, n, used for the greater part of the work was about $6{\cdot}8 \times 10^7$ per Petri dish. It can be seen that n cells in 25 ml. gave harvest ratios of 90–125 i.u./cell. n cells in 50 ml. gave similar harvest ratios, but being distributed in the double volume the titres were halved. When $1{\cdot}5n$ cells were cultured in this double volume the titres were raised accordingly. When $2n$ cells were cultured in 50 ml. the titres were not only restored to the level for 25 ml., but

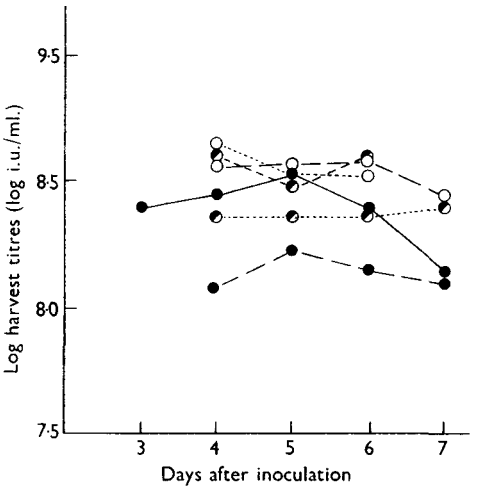

Fig. 3

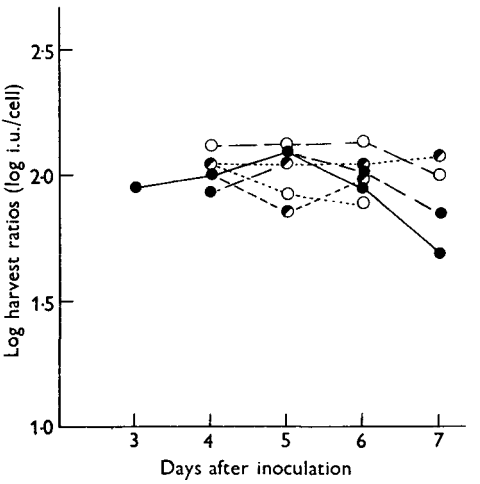

Fig. 4

Fig. 3. The influence on virus yield (harvest titres) of varying the cell concentration and volume of cell suspension per Petri dish.

Fig. 4. The influence on virus yield (harvest ratios) of varying the cell concentration and volume of cell suspensions per Petri dish. ●—●, n cells in 25 ml.; ●– –●, n cells in 50 ml.; ○– –○, $2n$ cells in 50 ml.; ◐– – –◐, $1{\cdot}5n$ cells in 25 ml.; ◐- - -◐, $1{\cdot}5n$ cells in 50 ml.; ○- - -○, $3n$ cells in 50 ml.

Table 1. *The influence on titre and harvest ratio, of varying the cell concentration and the volume of cell suspension introduced into the Petri dish*

		Average titre $\times 10^{-8}$ i.u./ml. Days after inoculation					Average harvest ratio (i.u./cell). Days after inoculation				
Cells/Petri dish	ml./dish	3	4	5	6	7	3	4	5	6	7
$n = 6{\cdot}875 \times 10^7$	25	2·5	2·8	3·4	2·5	1·4	90·9	101·8	126·6	90·9	50·91
$n = 6{\cdot}875 \times 10^7$	50	—	1·2	1·7	1·4	1·0	—	87·28	123·6	104·2	72·73
$2n = 1{\cdot}375 \times 10^8$	50	—	3·6	3·7	3·8	2·8	—	130·9	134·6	138·2	107·8
$1{\cdot}5n = 1{\cdot}03 \times 10^8$	25	—	4·0	3·0	4·0	—	—	96·99	72·73	96·99	—
$1{\cdot}5n = 1{\cdot}03 \times 10^8$	50	—	2·3	2·3	2·3	2·5	—	111·5	111·5	111·5	121·3
$2n = 2{\cdot}06 \times 10^8$	50	—	2·5	3·5	3·3	—	—	109·1	84·86	80·0	—

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increased slightly and consistently, giving harvest ratios of 130–138 i.u./cell. Since the cell concentration was the same as when n cells were cultured in 25 ml., it seemed that the increased proximity of the cells and perhaps the greater depth of fluid were important. Further cell increase to $3n$ cells in 50 ml. did not increase the titres; the harvest ratios fell to 85–110 i.u./cell. $3n$ cells may have been overcrowded on the floor of the dish. However, it was equally likely that the volume of nutrient per cell was too small; because $1{\cdot}5n$ cells in 25 ml. similarly gave harvest ratios of only 70–100 i.u./cell, whereas $1{\cdot}5n$ cells in 50 ml. gave 110–120 i.u. per cell.

It is possible that the cells would withstand the effects of higher concentration if they were maintained freely suspended throughout the nutrient by stirring in a large bottle. Spread of virus by cytoplasmic contacts between the cells would be impeded so that a higher inoculation ratio would probably be necessary. Culture in these concentrations would have an additional advantage of considerably reducing the number of Petri dish cultures, and consequently the risks of contamination. Experiments with this kind of culture are now in progress.

Preparation of virus suspension

Immediately after harvest the virus was centrifuged from the nutrient at 10,000 *g* for 20 min., and resuspended in McIlvaine's buffer (McIlvaine, 1921) 0·1M disodium phosphate, pH 7·2. This enabled the virus to be concentrated if necessary, and suspended in a fluid more favourable to stability; and the 10% horse serum which, on vaccination, might have affected serum-sensitive subjects was removed in the supernatant fluid.

Properties of vaccine

Sterility

Our aim was to keep the method of production as simple as possible. Apart from a careful bench technique and the use of antibiotics in the culture, no elaborate precautions were taken to maintain sterility. Samples (about 0·5 ml.) of the first twelve batches of tissue culture vaccine were spread on blood agar. One was contaminated (Table 2).

Immunogenicity

Five normal rabbits were vaccinated with tissue culture vaccine. Four weeks later they were challenged by scarification with a potent sheep lymph. All animals had a high degree of immunity (Table 3).

Stability

The potency of glycerolated vaccine stored in bulk at $-10°$ C was maintained for many months. During the initial clinical trials, however, potency deteriorated rapidly in transit, so that the percentage of takes was lower than that obtained with sheep lymph. The concentration of various ions in the suspending fluid influences the rate of inactivation of vaccinia virus by heat (Kaplan, unpublished). When 0·1M instead of the usual 0·004M disodium phosphate was present in the buffer both the loss of infectivity during storage at 22° C (Table 4) and the deterioration during clinical trial were considerably retarded (Table 5).

Table 2. *Virus content and bacterial contamination of the first twelve batches of tissue culture vaccine produced under routine conditions*

Batch number	Virus titres $\times 10^{-8}$ i.u./ml.	Growth on blood agar
T1/58	4·4	0
T2/58	4·9	0
T3/58	5·0	0
T4/58	5·4	Scanty Gram +ve coccus, coagulase −ve*
T5/58	2·0	0
T6/58	3·2	0
T7/58	4·4	0
T8/58	5·4	0
T9/58	5·4	0
T10/58	2·5	0
T11/58	4·2	0
T12/58	1·8	0

* Saphrophytic micrococcus.

Table 3. *The response of rabbits vaccinated 4 weeks previously with tissue culture vaccine, to challenge by a potent sheep lymph inoculated by superficial scarification*

Rabbit no.	Days after inoculation	Reciprocal of dilution of test lymph 1000	2000	4000	8000	16,000
903	3	?2*	0	0	0	0
	5	1*ab*	1*ab*	0	0	0
904	3	?	?1	?	?	?
	5	0	1*ab*	0	0	0
905	3	0	0	0	1	0
	5	0	0	0	1*ab*	0
906	3	?1	0	0	0	0
	5	1*ab*	0	0	0	0
907	3	?2	1	0	0	1
	5	1*ab*	1*ab*	0	0	1*ab*

* 1, 2 = number of lesions on scarified areas. *ab* = abortive lesion: fades rapidly or develops a black, necrotic centre.

Table 4. *The stability on storage of tissue culture virus in McIlvaine buffer containing different concentrations of disodium phosphate*

Na_2HPO_4 concentration	Storage temperature (° C) for 10 days	Virus titre (i.u./ml.)
0·004 M	−10	$2·9 \times 10^8$
	22	$1·6 \times 10^7$
0·1 M	−10	$2·0 \times 10^8$
	22	$7·9 \times 10^7$

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Clinical trials

Group Captain R. M. Cross, of the R.A.F. Institute of Pathology, Halton, Bucks, conducted a series of clinical trials. We are indebted to him for permission to quote his results.

Table 5. *The stability during clinical trial of tissue culture virus suspended in McIlvaine buffer containing different concentrations of disodium phosphate*

Expt. no.	Virus titres (i.u./ml.) On issue	Virus titres (i.u./ml.) On return after 1 week	Na_2HPO_4 conc. (M)	Revaccination results (%) 'Takes'	Revaccination results (%) 'No takes'
1	$1{\cdot}2 \times 10^8$	$4{\cdot}0 \times 10^6$	0·004	80	20
	$2{\cdot}5 \times 10^8$	$1{\cdot}3 \times 10^8$	0·1	91	9
2	$1{\cdot}4 \times 10^8$	$6{\cdot}0 \times 10^6$	0·004	80	20
	$2{\cdot}3 \times 10^8$	$1{\cdot}8 \times 10^8$	0·1	96	4

Table 6. *Primary and revaccination success rates* (%) *with stabilized tissue culture vaccine in* 0·1 M *McIlvaine buffer and sheep lymph vaccine*

Vaccination history	No. of takes/number inoculated with: Tissue culture vaccine	No. of takes/number inoculated with: Sheep lymph vaccine
Primary	275/275 (100%)	145/145 (100%)
Revaccination	242/261 (93%)	180/186 (97%)

Stabilized tissue culture vaccine in 0·1 M buffer gave the same high 'take' rate as sheep lymph in both primary vaccinations and revaccinations (Table 6). One year after primary vaccination with tissue culture vaccine, 50 subjects were challenged by revaccination with potent sheep lymph. 'Only one out of 50 could be regarded as a definite take. 22 showed induration but no vesiculation. These could either be immediate or weak accelerated reactions.'

DISCUSSION

Production of vaccinia virus in tissue culture has several advantages over traditional methods. The virus suspension produced is free from contaminating bacteria and moulds; the method does not entail the use of animals. Vaccines may be prepared much more rapidly than is possible by traditional methods. The host cells are easily and cheaply obtained. The use of fresh cells for each culture, trypsinized directly from the embryos reduces any danger of transformation of cells. The use of the same rabbit seed virus for each culture should prevent any progressive modification of immunogenicity or of the tissue affinity of the virus. The final virus suspension does not need to be purified by differential centrifugation because it consists only of liquid nutrient medium containing virus particles and cell fragments which do not need to be removed. The vaccine has proved

successful in pilot clinical trials, and large-scale trials are planned. If these are successful there is no reason why this method should not be adopted for routine vaccine production.

SUMMARY

A tissue culture system using chick embryo cells gave bacteria-free vaccinia virus suspensions of sufficient potency to use as a vaccine. Clinical trials with vaccines prepared by this method gave similar results to those with sheep lymph vaccine.

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