The Use of Suspensions of Isolated Cells for the Study of the factors affecting the Multiplication of Fowl-plague Virus

By H. G. PEREIRA

Common Cold Research Unit, Harvard Hospital, Salisbury, Wiltshire

SUMMARY: The multiplication of fowl-plague virus was studied in cultures prepared with suspensions of isolated cells of chick embryo tissues. Under favourable conditions, virus infectivity increased exponentially for 20 hr., after a latent period lasting 4-6 hr. Virus haemagglutinin production varied linearly with the number of cells added to the cultures, the regression line of this relationship showing a slope of approximately 1 when the cell concentration was kept constant and a slope higher than 1 when the number of cells was varied together with the cell concentration. Haemagglutinin production was decreased by increasing the size of the virus inoculum. A similar decrease was observed in cultures in which the depth of the fluid layer was increased. Cultures incubated at 32° for 2 days showed no haemagglutinin titres. The titres reached in cultures incubated at 35, 37 and 39° did not differ significantly. Cultures kept at 37° maintained their capacity to support virus multiplication at an approximately constant level for at least 3 days. At 22° and at 4° this capacity decreased progressively with time of storage, the decrease being more pronounced at the lower temperature. Virus multiplication was inhibited by horse serum.

Suspensions of isolated cells were used by Rous, McMaster & Hudack (1935) for the study of cell-virus interactions, and more recently by Dulbecco & Vogt (1953) for investigations on the growth of equine encephalomyelitis and poliomyelitis viruses. The multiplication of fowl-plague virus in cultures prepared with chick embryo cell suspensions was previously reported (Pereira, 1953). These cultures consist of a known number of washed cells suspended in a saline solution of known chemical composition and constitute a system in which some of the factors involved in virus production may be controlled. The object of the present paper is to report the results of the study of some of these factors.

MATERIALS AND METHODS

Preparation of cell suspensions. The method used for the preparation of chick embryo cell suspensions was based on the technique devised by Shannon, Earle & Waltz (1952) and modified by Dulbecco (1952). Ten-day chick embryos were collected, decapitated, washed in Gey's (Gey & Gey, 1936) balanced salt solution (BSS) and minced by means of Craigie's pressure mincer (Craigie, 1949) with a coarse plunger (1 mm. wide grooves). The minced tissue was collected in BSS (3 ml./embryo) and the suspension was left to stand for 10 min. The supernatant was removed and discarded and a 0.5% (w/v) solution of trypsin (L. Light and Co. Ltd., Colnbrook, Bucks) in BSS was added to the deposit in the proportion of 3 ml./embryo. The tube was placed

in a water bath at 37° for 10 min. following which the tissue fragments were broken up by repeated sucking in and out of a syringe fitted with a lumbar-puncture needle. The suspension was then composed almost entirely of isolated cells and the remaining clumps were eliminated by straining through 100-mesh Monel wire gauze. The suspension was diluted 1/3 in BSS and then centrifuged for 5 min. at about 1000 r.p.m. The supernatant was discarded and the deposit was washed twice by re-suspension in BSS and centrifugation. The final suspension was again strained through 100-mesh wire gauze. Cell counts were made in a haemocytometer using white cell counting pipette and diluting fluid. Suspensions made in the proportion of 4 ml. BSS/10-day embryo contained about 10^7 cells/ml.

Virus. The 'Dutch' strain of fowl-plague virus was maintained by allantoic passage in 10-day embryonated eggs and the virus-containing allantoic fluids were preserved in sealed capillaries at -76°. Two batches of capillaries were used throughout these studies, both containing $10^{9.7}$ egg infectivity doses/ml.

Virus titrations. (a) *Haemagglutination tests* were performed by a pattern technique using 0.2 ml. of twofold dilutions of the virus preparation to which were added equal volumes of a 0.5 % (v/v) suspension of washed chicken red cells. The results were expressed as the log of the number of agglutinating doses (AD) present in the total volume of each culture, one AD corresponding to the amount of virus capable of causing 50 % agglutination of the red cells contained in 0.2 ml. of the suspension.

(b) *Egg infectivity tests* were performed by the inoculation of 0.05 ml. of tenfold dilutions of the virus preparations in broth saline into the allantoic cavity of 10-day embryonated eggs. Each dilution was inoculated into four eggs and the end-point was calculated by the method of Reed & Muench (1938). The results were expressed as the log of the number of 50 % egg infectivity doses (EID₅₀) present in the total volume of each culture.

Preparation of cultures. The cultures were set up in $3 \times \frac{5}{8}$ in. tubes, stoppered with rubber bungs, sloped at an angle of about 5° from the horizontal and incubated at 37°. The total volume was adjusted to 1 ml. These conditions were kept constant unless otherwise specified. Penicillin and streptomycin were added to all the cultures to give a final concentration of 50 units and 50 µg./ml., respectively. In the growth-curve experiment the cultures were set up in 2 ml. ampoules which were sealed, sloped and incubated. At appropriate intervals, groups of 3 ampoules were frozen and preserved in solid CO₂. At the time of titration each culture was thawed in a water bath at 37°, its contents were lightly centrifuged and the supernatant was titrated by egg infectivity.

RESULTS

The growth curve of fowl-plague virus

The curve of infectivity production in cultures started with $10^{8.8}$ cells and $10^{4.7}$ EID₅₀ of fowl-plague virus is shown in Fig. 1. The number of EID₅₀ detected at time 0 corresponded to about 5 % of the amount inoculated. Additional experiments revealed that 80 % of the infectivity of the inoculum

was lost as a result of the process of freezing and thawing and an additional 15 % was retained in the deposit of the cultures. The infectivity was further decreased to about 1 % of the inoculum after 4 hr. of incubation and then increased exponentially for 20 hr. At 40 and 48 hr. the infectivity was only slightly higher than at 24 hr. In another experiment in which cultures were tested after 2, 4 and 6 hr. of incubation the period of low infectivity was shown to last between 4 and 6 hr.

Fig. 1. Growth curve of fowl-plague virus in cell suspension cultures.

Number of cells

Two series of experiments were performed to study the relationship between the number of cells present in the cultures and the amount of virus produced. In the first series the number of cells in successive groups of tubes was varied, together with the total volume of fluid, the concentration being constant in all the tubes ($10^{6.7}$ cells/ml.). In the second series the number of cells was varied, together with the cell concentration, the volume of fluid being constant in all the cultures (1 ml.). In all these experiments the total number of cells was varied in the region between 10^6 and 10^7 . The virus inoculum was added to all the cultures in a final dilution of 10^{-5} ($10^{4.7}$ EID₅₀/ml.). The tubes were slanted, incubated at 37° for 2 days, following which the supernatants were titrated by haemagglutination. The results are shown in Figs. 2 and 3, in which each point corresponds to the average of four determinations. In both series a linear relationship was observed between the number of cells present in the cultures and the amount of haemagglutinin produced. The slopes of the regression lines gave values of 0.998 for the series with constant cell concentration and 1.709 for the series with varying cell concentrations. A *t*-test between the two slopes showed that they are significantly different ($t=4.29$ with 51 D.F., $P<0.001$). The possibility that the increased virus production in high cell concentrations might be due to a stimulation by substances derived

from the embryo and improperly washed away in the process of preparation of the suspension was considered. A suspension was prepared by the usual technique except that the cells were washed by four cycles of centrifugation and resuspension instead of three. Cultures prepared with constant numbers of cells suspended in the three last supernatants obtained in the washing process and in BSS revealed no difference in virus-producing capacity.

Fig. 2. Relation between number of cells and haemagglutinin production. Constant cell concentration. Varying volumes.

Fig. 3. Relation between number of cells and haemagglutinin production. Varying cell concentrations. Constant volume.

Size of the virus inoculum

Two groups of sixteen tubes, each containing $10^{6.7}$ cells, were inoculated respectively with $10^{6.7}$ and $10^{3.7}$ EID₅₀ of fowl-plague virus. The volume was adjusted to 1 ml. in all the tubes. After 2 days of incubation under standard conditions the supernatant fluid of each culture was titrated by haemagglutination. The average log of the number of AD produced per tube was calculated for each series. For the series receiving the larger virus inoculum this value was 1.83 with a standard deviation of 0.08 and for the series receiving the smaller virus inoculum it was 2.21 with a standard deviation of 0.11. A *t*-test between these two averages showed a significant difference ($t=10.21$ with 30 D.F., $P<0.001$).

Temperature of incubation

Four groups of twelve cultures were prepared, each tube containing $10^{6.7}$ cells and $10^{4.7}$ EID₅₀ of fowl-plague virus. The different groups were incubated respectively at temperatures of 32, 35, 37 and 39°. After 2 days the supernatant fluid of each culture was titrated by haemagglutination and the results obtained for each group were averaged. Table 1 summarises the results. In the cultures incubated at 32° no haemagglutinating activity was detected after 2 days of incubation. The average log AD of the groups of cultures incubated at 35, 37 and 39° were not significantly different.

Table 1. *Influence of temperature of incubation on virus haemagglutinin production*

Temperature of incubation (°)	Average log AD	Standard deviation
32	<0.67	—
35	2.31	0.24
37	2.24	0.14
39	2.37	0.12

Depth of fluid layer

Two groups of sixteen cultures were prepared, each tube containing $10^{6.7}$ cells and $10^{3.7}$ EID50 of fowl-plague virus. The first group was set up in $4 \times \frac{1}{2}$ in. tubes which were incubated upright, the fluid forming a column about 15 mm. high. The second group was set up in $3 \times \frac{5}{8}$ in. tubes which were incubated at an angle of 5° from the horizontal, the fluid column at its deepest part showing a height of about 3 mm. After 2 days incubation at 37° the supernatant fluid of each culture was titrated by haemagglutination. The average log of the number of AD produced per tube gave values of 1.59 with a standard deviation of 0.19 for the cultures with deep fluid layer and 2.21 with a standard deviation of 0.11 for the cultures with shallow fluid layer. A *t*-test between these two averages showed a significant difference ($t = 11.06$ with 30 D.F., $P < 0.001$).

Storage of cell suspension

The effect of storage at different temperatures on the capacity of the cell suspension to support virus multiplication was investigated. Thirty cultures were prepared containing $10^{6.8}$ cells in a volume of 0.9 ml. Three tubes were immediately inoculated with $10^{4.7}$ EID50 of fowl-plague virus in a volume of 0.1 ml. and incubated at 37° . The remaining tubes were divided into three groups which were sloped and kept at 4, 22 and 37° respectively. After 24 hr. three tubes of each group were inoculated with fowl-plague virus as above and incubated at 37° . At 48 and 72 hr. further groups of tubes were treated similarly. After addition of the virus the tubes were incubated at 37° for 2 days and then preserved at 4° until the end of the experiment, when the supernatant fluid of each culture was titrated by haemagglutination. The result is shown in Fig. 4 in which each point represents the average log AD of the three tubes of each group. The virus-producing capacity of the cultures kept at 37° remained approximately constant up to 72 hr. At 22 and at 4° the virus-producing capacity decreased progressively with the time of storage, this decrease being more pronounced at the lower temperature. In the cultures kept at 37° the cells were observed to adhere to the glass forming a rather irregular sheet containing many fibroblast-like elements.

Inhibitory action of horse serum

Six groups of four cultures were prepared containing $10^{6.8}$ cells and $10^{4.7}$ EID50 of fowl-plague virus. To five of the six groups unheated normal horse serum was added at concentrations varying from 2 to 10% and to the remaining group no horse serum was added. After 2 days of incubation under

standard conditions the supernatant fluid of each culture was titrated by haemagglutination. The result is shown in Fig. 5 in which each point represents the average log AD of each group. These values decrease linearly with increasing concentrations of horse serum. At the end of the incubation period all the cultures showed a layer of cells adhering to the wall of the tube. In

Fig. 4. Effect of storage at different temperatures on the virus-producing capacity of cell suspension cultures. x, cultures stored at 37° before addition of virus; O, cultures stored at 22° before addition of virus; ∇, cultures stored at 4° before addition of virus.

Fig. 5. Inhibition of haemagglutinin production by normal horse serum.

the cultures without serum this layer was very irregular and revealed on microscopic examination very few intact cells. As the serum concentration increased the cellular layer showed increasing numbers of fibroblast-like elements. Further investigation demonstrated that the factor in horse serum responsible for this inhibition resisted heating at 56° for 30 min. and was inactivated by trypsin.

COMMENTS

The study of the factors influencing the growth of viruses in tissue cultures was reviewed by Robbins & Enders (1950). The interpretation of the results obtained has often been complicated by the imperfect standardization of some of the factors studied, because of technical difficulties. With the object of achieving this standardization the cultures used in the present study were started with suspensions of isolated cells. The virus of fowl-plague multiplies readily in this system reaching relatively high haemagglutination titres which proved of great value for the simultaneous titration of relatively large numbers of culture materials.

The growth of fowl-plague virus in cell suspension cultures revealed several features in common with other viruses studied under similar conditions (Crawford & Sanders, 1952; Gajdusek, 1953; Womack & Kass, 1953). A latent period of 3–5 hr. followed by a stepwise increase of infectivity was reported by Schäfer & Munk (1952) in the case of the multiplications of fowl-plague virus on the isolated chorio-allantoic membrane of chick embryos. The heterogeneous nature of the cell suspension used in the present study prevented the

estimation of the multiplicity of infection and, as no attempts were made to prevent re-infection of cells by newly formed virus, no evidence for a step-wise growth cycle could be obtained. Dulbecco & Vogt (1953) described one-step growth curves of poliomyelitis and Western equine encephalomyelitis viruses in cell suspensions.

The relationship between number of cells and virus production in tissue cultures has been studied by several authors (Hallauer, 1932; Plotz, 1933; Rivers & Ward, 1933; Traub, 1933; Fox, 1947; Yanamura & Meyer, 1941; Simms & Sanders, 1942; Ledinko, Riordan & Melnick, 1952; Womack & Kass, 1953). In most of these studies the number of cells was estimated by indirect methods such as the weight, the volume or the nitrogen content of the tissue added to the cultures. The results varied for different systems, but in general it has been found that there is an optimum amount of tissue for maximal virus production. The use of suspensions of isolated cells provides particularly favourable conditions for the study of this relationship, as by this method the number of cells may be estimated by direct count and replicate cultures may be prepared. In the region covered by our experiments a linear relationship was observed between the number of cells added to the cultures and the amount of virus haemagglutinin produced. The fact that with constant cell concentrations the regression line of this relationship shows a slope of approximately 1 (0.998) indicates that under these experimental conditions the virus concentrations obtained are constant for each cell concentration and independent of the total volume of the culture. Under these conditions it may be assumed that throughout the range studied the same average amount of virus haemagglutinin was produced by individual cells. On the other hand, when the number of cells was varied, together with the cell concentration, the slope of the cell-virus regression line gave a value higher than 1 (1.709) indicating that the average amount of virus produced per cell increases with increasing cell concentrations. This fact may represent another manifestation of cellular interactions described for several tissue culture systems (Buchsbbaum, 1932; Ephrussi, 1933; Bloom, 1937; Fischer & Jensen, 1946; Earle *et al.* 1951). It is possible that cellular products diffused into the medium during the period of incubation may favourably influence virus production. The low sensitivity of the haemagglutination test used for virus titrations established the lower limit of cell concentrations studied, as the virus yields in suspensions containing less than 10^6 cells/ml. do not reach haemagglutination level. Dulbecco & Vogt (1953), who used a plaque technique of titration, demonstrated the multiplication of poliomyelitis and Western equine encephalomyelitis viruses in isolated cells. These authors' techniques differed from ours in that the cells were suspended in a nutrient medium.

It should be noted that in the series of experiments in which the total volume of the cultures was varied, the depth of the fluid column was kept low by sloping the tubes during incubation. This precaution was taken in view of the observed decrease of virus yield in cultures in which the fluid column is deep. The influence of the depth of the fluid column on virus production in tissue cultures was studied by Maitland, Laing & Lyth (1932), Yanamura & Meyer (1941), Thompson & Coates (1939), and the results have varied for the different

systems under study. The use of shallow fluid layers is a procedure generally adopted in tissue culture work.

The influence of the size of inoculum on virus production in tissue cultures has been studied by several authors (Fox, 1947; Yanamura & Meyer, 1941; Womack & Kass, 1953; Sanders & Jungeblut, 1942; Dulbecco & Vogt, 1953), and in general it has been observed that the final titres obtained are not greatly affected by the size of the inoculum but the time required for maximal virus production is increased when small inocula are used. Robbins, Weller & Enders (1952), when studying the propagation of poliomyelitis viruses in roller tube cultures of human tissues, obtained relatively smaller titres in cultures inoculated with large amounts of virus. A similar result was clearly demonstrated in the present investigation. Kinetic studies of virus multiplication in cultures started with varying sizes of inoculum are necessary for a better understanding of this phenomenon.

The study of the effect of storage on the capacity of cell suspensions to support virus growth revealed that this property is preserved for longer periods at 37° than at room temperature or at 4°. This result might be explained in part by the occurrence of cellular multiplication at 37°. Cultures incubated at this temperature before addition of the virus revealed after 2 days an irregular sheet of fibroblast-like cells adhering to the glass. In virus-infected cultures the number of these cells was greatly reduced. The cellular sheet of cultures incubated for 2 days in the absence of virus revealed no mitotic figures on microscopical examination after fixation and staining by haematoxylin *in situ*.

The composition of the medium was shown to have a marked effect on the appearance of the cellular sheet formed after incubation. Increasing numbers of fibroblast-like elements were observed in cultures containing increasing concentrations of horse serum. This action of horse serum was accompanied by inhibition of virus haemagglutinin production. The mechanism of this inhibition cannot be explained as due to the depressive action of horse serum on cellular physiological activities as proposed by Fox (1947) for the inhibition of the multiplication of yellow fever virus in tissue cultures.

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