PROPAGATION OF MOUSE ENCEPHALOMYOCARDITIS VIRUS IN ASCITES TUMOUR CELLS MAINTAINED *IN VITRO*

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Received for publication January 16, 1957

MOUSE ascites tumours should provide an excellent source of cells for work with isolated animal virus/cell systems, since they consist of a large population ($1–2 \times 10^8$ cells per ml. of exudate) of separate cells floating free in the peritoneal cavity, and can easily be recovered and suspended in suitable media without the use of enzymes and other deleterious reagents. Although a number of recent papers have described the growth of viruses in such tumours *in vivo* (Koprowska and Koprowski, 1953; also see Moore, 1954, for review of literature), little use has been made of them so far for *in vitro* studies. For example, GD7 virus, NDV, and Bunyamwera virus have been shown to multiply in such cells *in vitro* by Sanders (1953), Moore and Diamond (1955) and Koprowski (1956), but in none of these cases was virus growth correlated with any observable cytological change.

In this paper we describe the growth, and maintenance by serial passage, of the virus of encephalomyocarditis (EMC virus) in ascites tumour cells *in vitro*. High yields of virus were obtained, and virus multiplication was accompanied by cytopathic destruction of the affected cells.

MATERIALS AND METHODS

Tumour

The ascitic form of the Krebs-2 mouse ascites carcinoma was used throughout. It was obtained from the Chester Beatty Research Institute in March 1953, and since that date has been maintained by serial passage in genetically heterogeneous Swiss mice supplied by an accredited dealer. Tumour passages were made at 10 to 12-day intervals by injecting 0·1 ml. of undiluted peritoneal exudate (containing $1–2 \times 10^7$ tumour cells) intraperitoneally into mice 6–8 weeks old.

Preparation of tissue cultures

Mice with well-developed ascites were killed by decapitation, and the ascitic fluid removed. Cells were first separated from the fluid by centrifuging for 10 min. at $210 \times g$, and the ascitic fluid decanted and saved. The cell layer, besides tumour cells, usually contained a variable proportion of red blood cells which could easily be removed by up to three low-speed washings in phosphate-buffered saline (P.B.S.; Dulbecco and Vogt, 1954). After the last washing the cells were suspended in culture medium, and adjusted to a concentration of about $1·0 \times 10^6$ cells/ml. The cell suspension was then dispensed in 1·5 ml. amounts in small, thin-walled, neutral glass culture bottles, 55 mm. long by 20 mm. in diameter. These had a volume of 12 ml. and were closed by a screw cap with a silicone rubber liner. The medium consisted of 65 per cent Earle's saline (Earle, Schilling, Stark, Straus, Brown and Shelton, 1943), 35 per cent mouse ascitic fluid, penicillin (Pen. G, crystalline sodium) 100 i.u./ml., and streptomycin (calcium chloride complex) 0·2 mg./ml. The ascitic fluid was stored at 4° for 3–4 days before use to allow clotting to take place, and the clot was then removed by centrifuging for 15 min. at $1880 \times g$. Cultures were infected directly after setting, using an inoculum of 0·15 ml., and were then incubated at 37° in a horizontal position. They were examined daily for cytopathic effect.

EMC virus

The virus was received originally from Dr. J. Warren, of the Department of Virus and Rickettsial Diseases, The Army Medical Center, Washington, as a lyophilized suspension of infected mouse brain in its 54th intracerebral mouse passage. Since then the virus has had 11 more intracerebral passages in mice at high dilution (10^{-5}, 10^{-6}). The stock virus with which culture passage was started consisted of a 10 per cent suspension of infected mouse brains in 10 per cent horse serum (N.H.S.) (in P.B.S.), stored at −70°. Infected cultures were harvested at the first sign of cytopathic change of the tumour cells, and frozen and thawed (−70°; 37°), and centrifuged before using the supernatant fluid for passage. In this way 10 passages were made in culture at high concentration, followed by 4 passages at limiting dilution. The tumour-propagated virus used in haemagglutination experiments was passed twice more in a culture system in which the cells were maintained in suspension (Sanders, unpublished).

Titration procedure

Titrations were performed in culture, and in mice, serial 10-fold dilutions in 10 per cent inactivated horse serum in P.B.S. always being used. Intracerebral titrations were performed, using 0·03 ml. volumes, in 3–4-week-old mice from the colony used to maintain the tumour. For intramuscular titrations only 21-day-old mice were used, 0·03 ml. volumes being injected into the right gastrocnemius muscle. Five mice (or cultures) were inoculated per virus dilution, and the 50 per cent end-points calculated by the method of Reed and Muench (1938). Mice and cultures were both scored daily, the former for the onset of specific symptoms, and the latter for the appearance of cytopathic change.

Haemagglutination technique

The method used was that of Gard and Heller (1951). All tests were carried out in M.R.C. pattern perspex haemagglutination trays.

RESULTS

Cytopathic changes

Under the conditions of culture described a sheet of tumour cells remained adherent to the wall of the culture vessel for about 6 days. The cells spread out on the wall of the vessel were large disc-shaped structures, with clear nuclei and cytoplasm, the latter showing a small number of prominent refringent granules (Fig. 1). After 6 days uninfected cultures began to undergo spontaneous changes, some cells detaching themselves from the glass, while others assumed bizarre forms; these included the development of spindle-, star-, and crescent-shaped cells, and the appearance of large intracellular vacuoles. The cells at this stage still possessed clear nuclei, while metabolism, indicated by a lowering of pH, still went on. That cell transformation to spindle forms is not a sign of degeneration is supported by Laznitski's finding (1952), that ascites cells of the S37 mouse sarcoma underwent similar changes *in vitro*, while at the same time showing a high proportion of mitotic figures. Acid production, especially in freshly-set cultures, was rapid, and whenever the pH reached about 6·8 as judged by the colour of the phenol red indicator, the medium was routinely replaced with fresh fluid.

Cells maintained in this way were able to support EMC virus multiplication, and at the same time underwent a characteristic cytopathic alteration. The first sign was a loss of their round outline; they then developed crinkled edges and began to shrink, the nuclei becoming pycnotic (Fig. 2). Finally, the cells shrivelled completely, became opaque, and detached themselves from the glass (Fig. 3). The pH of such degenerate cultures shifted rapidly to about 7·8. When

either mouse brain- or tumour-propagated virus was inoculated at high concentration, cytopathic alterations were evident after two days of incubation. For the highest dilutions the onset of the change was delayed up to 6 days. Although this is the time at which spontaneous changes begin to take place in uninoculated cultures (see above), the change in cell morphology in the latter is strikingly different from that produced by the virus, and could easily be distinguished from it.

Passage of virus in vitro

Passage of EMC virus *in vitro* was initiated by inoculating 0·15 ml. of a 10 per cent infected mouse brain suspension into 5 cultures. Two days later all cultures showed cytopathic alteration, and fluid from them was inoculated into a fresh series of cultures. In this way, 10 passages of the virus were made. The fluid harvested from the 10th passage was infective for mice, all 5 animals inoculated intracerebrally with undiluted culture material dying in less than 48 hr. The same material titrated *in vitro* contained $3{\cdot}4 \times 10^7$ T.C.D.$_{50}$/ml. A culture showing cytopathic change at the limiting dilution of 10^{-7} contained virus at a concentration of $6{\cdot}7 \times 10^6$ T.C.D.$_{50}$/ml. Two further passages of the latter material were made *in vitro* at limiting dilution before a pool was made from a large number of cultures. This pool contained $2{\cdot}1 \times 10^7$ T.C.D.$_{50}$ of virus/ml. When the same pool was titrated intracerebrally in mice it contained $1{\cdot}1 \times 10^8$ L.D.$_{50}$/ml., while for intramuscular inoculation the figure was $5{\cdot}3 \times 10^7$ L.D.$_{50}$/ml.

TABLE I.—*Titrations of Mouse Brain Virus and Krebs-2 Tumour Virus*

Virus	Titration system	Titre	I.D.$_{50}$/ml.*
Tumour virus	Intracerebral (i)	$10^{-6.5}$	$1{\cdot}1 \times 10^8$
	,, (ii)	$10^{-6.0}$	$3{\cdot}3 \times 10^7$
	Intramuscular (i)	$10^{-6.2}$	$5{\cdot}3 \times 10^7$
	,, (ii)	$10^{-6.6}$	$1{\cdot}3 \times 10^8$
	Tissue culture	$10^{-6.5}$	$2{\cdot}1 \times 10^7$
Mouse brain virus	Intracerebral (i)	$10^{-7.6}$	$1{\cdot}3 \times 10^8$
	,, (ii)	$10^{-8.2}$	$5{\cdot}3 \times 10^8$
	Intramuscular (i)	$10^{-8.8}$	$2{\cdot}1 \times 10^9$
	,, (ii)	$10^{-7.9}$	$2{\cdot}6 \times 10^8$
	Tissue culture	$10^{-7.5}$	$2{\cdot}1 \times 10^7$

* Expressed in terms of: undiluted culture fluid (tumour virus); 10 per cent infected brain (mouse brain virus).

Titration of mouse brain- and tumour-propagated virus

A series of titrations of mouse brain virus was made *in vivo* and *in vitro*. Using a single pool of virus grown in mouse brain tissue the following titrations were made: (*a*) Intracerebrally in mice; (*b*) intramuscularly in mice; and (*c*) in tumour cells in culture. A parallel series of titrations was made with tumour-propagated virus. The results of the titrations, summarized in Table I, show that tissue culture fluid contains about the same amount of virus as a 10 per cent suspension of infected mouse brain. Further, as a detector of virus the culture system possesses about the same degree of sensitivity as mice inoculated by the intracerebral route.

Haemagglutination and haemagglutination-inhibition experiments

Virus grown either in mouse brain or in Krebs-2 tumour cells in culture strongly agglutinated sheep erythrocytes under the conditions of the test. A 10 per cent suspension of a pool of infected mouse brains contained 190 haemagglutination (HA) units/ml. (geometric mean of 8 observations), while the pooled supernatant from a number of infected ascites tumour cell cultures had a virus content of 95 HA units/ml. (geometric mean of 4 observations).

After 16 passages in cultures of tumour cells, haemagglutination-inhibition tests were performed with tumour-propagated virus, using antisera prepared in rats against three other passage lines of EMC virus :— (*a*) Mouse brain virus ; (*b*) a strain grown in the Crocker mouse sarcoma 180 *in vitro* ; and (*c*) a virus of suspected human origin (" Fantz ")* which is serologically identical with mouse brain EMC. The results are summarized in Table II. The tests show no appreciable serological difference between any of the viruses tested.

TABLE II.—*Haemagglutination-inhibition of Mouse Brain and Krebs-2 EMC Virus*

Antiserum	Virus	H.-I. end-point
EMC (mouse brain virus) . .	Mouse brain .	1/200
	Krebs-2 .	1/100
Fantz (mouse brain virus) . .	Mouse brain .	1/1600
	Krebs-2 .	1/3200
EMC (Crocker virvs) . . .	Mouse brain .	1/1600
	Krebs-2 .	1/1600

DISCUSSION

These results show that EMC virus is capable of growing in a system of non-multiplying mammalian cells maintained *in vitro*. Ascites tumour cells removed from their hosts into a suitable medium do not show any significant number of mitoses, and it has been stated that under such conditions protein synthesis also ceases (Christensen, Riggs, Aspen and Mothon, 1956). Such cells, however, must retain sufficient synthetic ability to be able to support the growth of EMC virus.

Growth of EMC virus in Krebs-2 cells maintained *in vitro* does not appear to be restricted to a single cycle of virus multiplication, as has been suggested for Bunyamwera virus in these cells by Koprowski (1956). It rather resembles the propagation of the related Mengo virus in cells from the Ehrlich carcinoma, also described by this author. However, we did not observe the diminished titre of virus in cultures inoculated with high dilutions of virus, described by Koprowski for this system. For example, fluid from a culture inoculated with undiluted tissue culture virus had a virus content of $3{\cdot}4 \times 10^7$ T.C.D.$_{50}$/ml. ; the fluid from a culture inoculated with a 10^{-7} dilution of this same virus contained $6{\cdot}7 \times 10^6$ T.C.D.$_{50}$/ml., while two passages later fluid from a culture inoculated with a 10^{-5} dilution of infected culture fluid contained virus at a concentration of $2{\cdot}6 \times 10^7$ T.C.D.$_{50}$/ml.

EMC virus propagated in mouse brain has a high virulence for mice injected either intracerebrally or intraperitoneally. We did not find any alteration in infectivity for mice by either route following ten rapid passages of the

* This strain was obtained from the late Dr. G. W. Findlay who himself received it from Professor Hans Bielling who had isolated it in Germany.

virus in tumour cells at high concentration, followed by four passages at limiting dilution; nor was there any change in infectivity for the tumour cells during the same series of passages. Mouse brain virus was also unaltered in infectivity for mice following a comparable number of serial passages in various mouse organs *in vivo* and *in vitro* (Sanders and Taverne, unpublished). Koprowski (1956) has shown that Mengo virus can radically change its infectivity for mice following serial passage in cultures of Ehrlich ascites tumour cells. The first change recorded took place after 38 passages and it is possible that a similar change would have been detected if our passage series had been continued.

SUMMARY

EMC virus multiplies in cells of the Krebs-2 mouse ascites tumour maintained *in vitro*, and can be propagated serially in such a system.

Growth of the virus is associated with cytopathic destruction of the cells.

Tumour-propagated virus agglutinates sheep erythrocytes.

Haemagglutination-inhibition tests performed with EMC virus propagated in either mouse brain or tumour cells *in vitro*, show the two viruses to be serologically indistinguishable.

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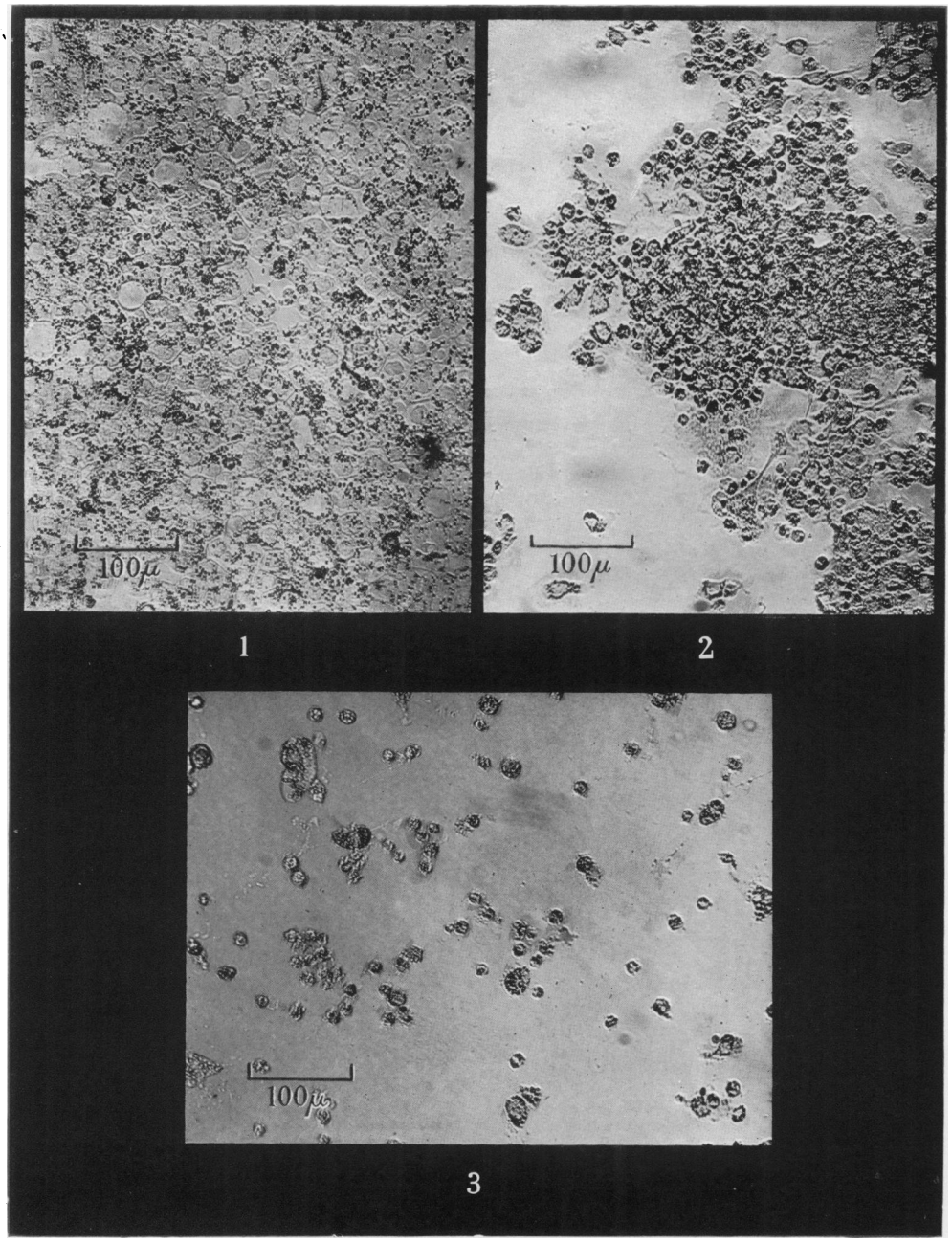
DESCRIPTION OF PLATE

FIG. 1.—Normal uninfected Krebs-2 ascites tumour cells. 48-hr. culture.

FIG. 2.—Krebs-2 ascites tumour cells infected 48 hr. previously with approximately 10^7 $T.C.D._{50}$ of EMC virus.

FIG. 3.—Krebs-2 ascites tumour cells infected 72 hr. previously with approximately 10^7 $T.C.D._{50}$ of EMC virus.



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