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ISOLATION OF SMALLPOX VIRUS IN MONKEY KIDNEY TISSUE CULTURE*

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Rokuro OHASHI, Iwao TANABE and Susumu HOTTA. *Isolation of Smallpox Virus in Monkey Kidney Tissue Culture.* Kobe J. Med. Sci. 13, 235-241, September 1967——A cytopathic agent was isolated from crusts of patients diagnosed as smallpox in Indonesia in 1965, using Japanese monkey kidney tissue cultures. Serial transmissions of the agent through tissue cultures were successful and its growth curves in tissue cultures were determined. The isolated agent was tentatively identified as smallpox virus, based on its biologic and virologic properties.

INTRODUCTION

An epidemic of smallpox occurred in Indonesia in 1965. Isolation of smallpox virus was attempted using monkey kidney tissue cultures. The results obtained will be briefly described in this paper.

MATERIALS AND METHODS

Tissue culture

Kidney tissues from Japanese monkeys (*Macaca fuscata*)** were cellular substrates. Both primary and continuous cell line cultures were employed. The latter was established by one of the authors (I. T.). YLE medium (consisting of 0.1% yeast extract, 0.5% lactalbumin hydrolysate in Earle's balanced salt solution, supplemented with 100 u/ml penicillin and 100 γ/ml streptomycin) was used throughout the study.

Original inoculum

Crusts taken from patients diagnosed as typical smallpox were received from Prof. Soemiatno and Dr. Lo, of Bio Farma (Pasteur Institute at Bandung) August 14, 1965. The specimens were put in a tightly stoppered glass bottle and were immediately transported to Japan. After arrival at Kobe, they were stored in a refrig-

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** Abbreviation: JMK.

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erator at 4°C. December 14, 1965, approximately 20 pieces of the crusts were emulsified by being added with 10 ml of YLE. After a low speed centrifugation, the supernatant was taken and kept frozen in an electric refrigerator at −20°C for 19 weeks. April 28, 1966, the material was thawed and was diluted 10-fold with YLE. Two-tenths ml of each dilution was introduced into each of primary JMK cell culture tubes. One and eight-tenths ml of YLE medium was added and the cultures were incubated at stationary state at 37°C.

RESULTS

Cytopathic effect

In some of the inoculated tubes, cellular degeneration was manifest under low magnification microscope. The results thereof are shown in Table 1.

Table 1 Occurrence of cellular degeneration in tubes inoculated with emulsion of smallpox crusts.

Exp. series	Dilution of original emulsion			
	10^{0}	10^{-1}	10^{-2}	10^{-3}
1	3/3*	0/3	0/3	0/3
2	3/3	0/3	0/3	0/3
3	3/3	1/3	0/3	0/3

* Numerator indicates number of tubes in which cellular degeneration was seen, and denominator indicates number of tubes inoculated. Primary *fuscatus* monkey kidney cultures were cellular substrates.

Degenerative changes produced either in the primary or continuous cell line cultures were essentially the same. Typical pictures of infected and control non-infected cultures are shown in Plate-Figs. 1 and 2.

Serial transmission through tissue culture

From Exp. Series 1 in Table 1, serial transmissions of the agent were carried out. At each passage, the culture fluid was diluted 10-fold with YLE and 0.2 ml thereof was introduced into other tubes. The results up until the 6th generation are summarized in Table 2.

Table 2 Serial subcultures of cytopathic agent derived from smallpox crusts.

Passage	Dilution of culture fluid						$TCID_{50}$(log)
	10^{0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	
2	3/3*	3/3	1/3	0/3			1.75
3	3/3	3/3	0/3	0/3			1.50
4		3/3	3/3	3/3	2/3	0/3	4.25
5			3/3	2/3	0/3	0/3	3.25
6			3/3	3/3	0/3		3.50

* Legends are the same as those in Table 1.
Celluler substrates were: Primary JMK cultures for the 2nd to 5th passages, and continuous line JMK cultures for the 6th passage.

Growth curve experiment

Primary or continuous line JMK cultures received 0.2 ml of fluid from infected tubes, and 1.8 ml medium was added. At intervals after the inoculation, portions

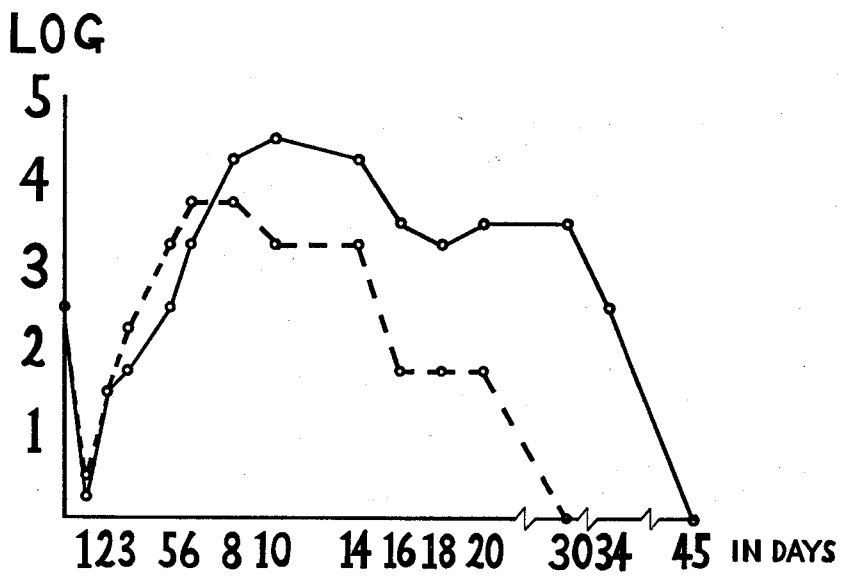

Fig. 1 Growth curve of agent derived from smallpox crusts.
Original inoculum : Culture fluid from the 5th passage of primary JMK cultures.
Ordinate indicates $TCID_{50}$ per 0.2 ml for primary JMK cultures in logarithmic number, and abscissa indicates period of incubation in days.
o——o : Growth curve in primary JMK cultures.
o·····o : Growth curve in continuous line JMK cultures.

of the culture fluid were taken and pooled. The pooled materials were diluted 10-fold with YLE, and each dilution was introduced into JMK cultures to determine ID_{50} values. Examples of the results obtained are shown in Fig. 1.

The growth of the agent in cultures is unequivocal in both instances. A tendency is shown that primary JMK cultures have an apparently greater affinity to the agent than that of the continuous line JMK cultures. It was noted that the cellular populations disappeared almost completely by 2 weeks following the inoculation, but sizable amounts of the active agent were still detectable. This suggests that the agent is fairly stable in the culture fluid incubated at 37°C.

Other biologic and virologic tests

When the infected fluid was introduced onto chorioallantoic membrane (CAM) of fertilized chick embryos, pocks, approximately 1 mm in diameter, were produced after 48–72 hours. The same material was non-pathogenic for 3-week-old white mice intracerebrally and for rabbits intradermally. The agent is ether-resistant and agglutinates chicken erythrocytes. The hemagglutination was inhibited by anti-vaccinia antiserum. Specific adsorption of chicken erythrocytes onto the infected culture cells (hemadsorption reaction) can also be seen. The infected culture cells exhibited positive reaction against anti-vaccinia immunofluorescent globulin. The specific fluorescence was observed in the cytoplasm.

DISCUSSION

It has been reported that variola virus can be grown in a variety of mammalian cell cultures.[1) 2) 3)] However, direct isolation of the virus from smallpox patients has mostly been performed using fertilized chick embryos. In the present studies, an agent was isolated from smallpox cases using JMK tissue cultures. The agent has been successfully cultivated in both primary and continuous line JMK cultures, in which clear cytopathic effect is produced. Although further studies are necessary for the identification of the isolated agent, it is probably the virus of smallpox, based on the origin of inoculum, its affinity to CAM, and some other biologic and virologic properties. Immunologic tests including HI and fluorescent antibody techniques also supported this tentative identification. The affinity of the continuous cell line cultures to the agent is a little inferior to that of the primary cultures, however, the both show essentially the same cytopathic phenomenon. The cell line described here can be used as a cellular substrate for variola virus tissue culture studies.

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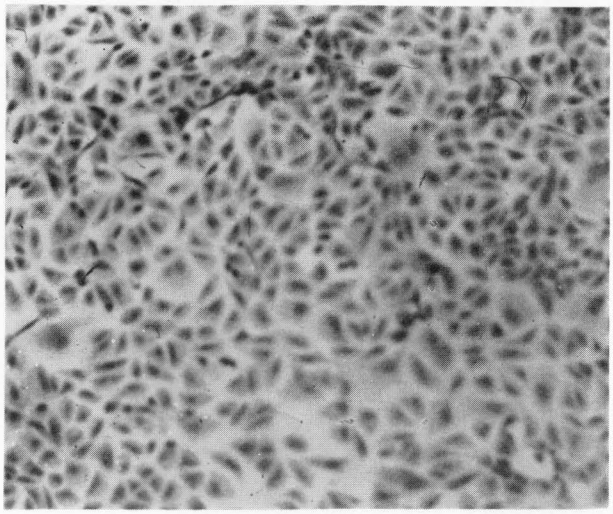
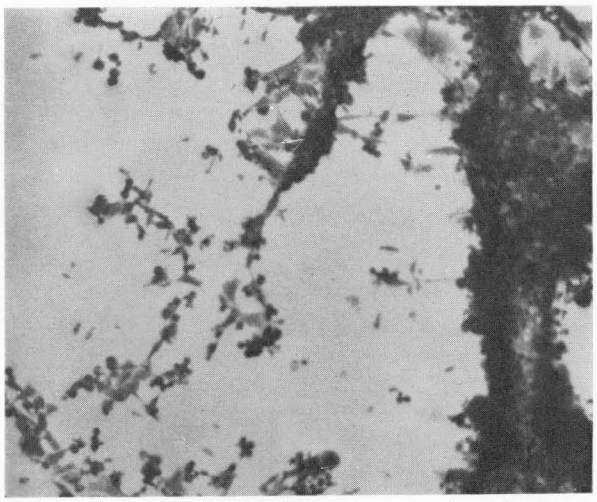


Plate–Fig. 1. Continuous line JMK culture; uninoculated control.
Fixed with methyl alcohol, and stained by May–Grünwald–Giemsa stain. 10 × 10.
A monolayer of cells is seen.



Plate–Fig. 2. Continuous line JMK culture ; inoculated with the agent derived from smallpox crusts.
Fixed with methyl alcohol, and stained by May–Grünwald–Giemsa stain. 10 × 10
Forty eight hours after inoculation. About half of the cellular population has disappeared, and the remaining cells exhibit degenerative changes.