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CHARACTERIZATION OF MULTIPLICATION OF EMBRYO-ADAPTED AVIAN ENCEPHALOMYELITIS VIRUS IN CHICK EMBRYO BRAIN CELL CULTURES*1

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In the cell cultures of chick-embryo brain inoculated with Van Roekel strain of embryo-adapted avian encephalomyelitis virus (AEV), days to reach maximum virus titers were in inverse proportion to inoculum size. In all cases, after having reached maximum virus titers, titers of $10^{2.5}$~$10^{3.0}$ EID_{50}/ml (cell-culture fluids) persisted. AEV antigen, stained directly by fluorescent antibody (FA), was not detected in the infected cells.

The virus infective titers in the brain cell cultures from chick embryos which had been infected with the virus several days after incubation were almost the same as those in the cell cultures inoculated with the same virus. However, in the cell cultures from heavily infected embryos, the viral antigen was detected by the FA test for the first few days of the cultivation.

AEV derived from the cell cultures was more sensitive to an incubation temperature (38.5°C) than that in the infected brain suspension.

INTRODUCTION

There have been reports of the successful propagation of avian encephalomyelitis virus (AEV) in vitro. In 1967 and 1968, MANCINI & YATES reported the successful propagation of a chick embryo-adapted AEV in chick embryo neuroglial[9], chick embryo fibroblastic[10], and chick embryo kidney cell cultures[11]. ABE (1968), and WATANABE & MIURA (1968), of this laboratory, indicated that the cell-culture material of chick embryo brain (CEB) was infective for chick embryos after 5 or 8 serial passages of the Van Roekel strain of embryo-adapted AEV. MATUKA et al. (1970) also reported the successful cultivation in CEB cells of an embryo-adapted AEV established by them. On the other hand, IKEDA et al. (1970) described successful alternate passages of more than 10 times Van Roekel's embryo-adapted AEV in whole chick embryo cell cultures and in chick embryos.

*1 Condensed from a part of a Master's thesis by the senior author, Hokkaido University (an English summary: *Jap. J. vet. Res.*, **19**, 37, 1971) and presented at the 71st Meeting of the Jap. Soc. of Vet. Sci., Apr. 1~3, 1971, Tokyo

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Moreover, recently we[14] described the propagation of the non-embryo-adapted AEV in CEB cell culture. However, there has been no detailed description of the characteristics of the propagation of AEV in the cells.

The present study was carried out to confirm the multiplication of Van Roekel's embryo-adapted AEV in CEB cell cultures, and to characterize the multiplication in the CEB cell cultures from chick embryos infected with the AEV as well as that in the cell cultures incoculated.

Materials and methods

AE virus The seed virus was the Van Roekel strain of AEV (embryo-adapted virus), obtained from National Institute of Animal Health, Tokyo. It had been passed 3 times in 6-day-old chick embryos inoculated into the yolk sacs in this laboratory. The brains of these embryos on the 13th day of incubation were macerated and a 10% suspension was made in Eagle's minimum essential medium containing penicillin (1,000 units/ml) and streptomycin (1 mg/ml). The suspension was then centrifuged for 30 min at 10,000×g. The supernate, possessing a titer of $10^{5.5}$ EID_{50}/ml, was held at −20°C and employed as the stock virus.

Experimental eggs White Leghorn eggs free of AE antibody were obtained from a flock.

Cell culture CEB cells were prepared according to the procedure of Mancini & Yates, with some modifications. The brains of 13-day-old embryos were removed into a Petri dish, washed 4~5 times with phosphate-buffered saline (PBS containing 0.5% lactalbumin) and cut into pieces with scissors. The tissue suspension was rinsed in a Petri dish with 0.25% trypsin solution and trypsinized 3 times in a Erlenmyer flask for 10 min at 30~34°C with a magnetic stirrer. The cell suspension was, then shaken, cooled in an ice-water bath, filtered through 12 layers of gauze and centrifuged for 5 min at 170×g. Before the centrifugation, the growth medium described below or calf serum was added to the cell suspension at about 1 : 10 in order to reduce the loss of cell viability. Packed cells were resuspended in Eagle's minimum essential medium (Eagle's MEM containing Kanamycin 60 μg/ml, Nissan) with inactivated calf serum in the ratio of 20%. The pH of the medium was about 7.2. The suspended cells were diluted in the medium in concentrations of about 1×10^6 cells/ml and dispensed into small bottles (3×5×3 cm), 5 ml to each bottle. If necessary, each bottle contained 4 coverslips for Giemsa or hematoxylin-eosin staining and for the direct fluorescent antibody (FA) technique. The bottles containing cell suspension were incubated at 38.5°C. The media were changed 4 days after the onset of incubation using Eagle's MEM plus 10 or 8% inactivated calf serum

depending on the growth status of the cells.

Virus inoculation The bottles of CEB cells were inoculated with AEV when the cell sheet was almost full, 6～7 days after the preparation. The cells were washed once with PBS before the inoculation. The stock virus diluted in various concentrations as shown in table 1 and figure 1, was inoculated with 0.3 ml per bottle. The virus was allowed to absorb for 1.5 hr at 37°C, and then the inoculum was removed. The cell sheet which had been inoculated was washed 3 times with 5 ml of PBS. Eagle's MEM containing 8% inactivated calf serum was added to each bottle. An appropriate number of bottles were employed as controls. The cell sheet of these bottles was treated similarly with 0.3 ml of PBS without AEV. Fluids and cell sheets from 2 AEV-inoculated bottles were collected and pooled at various intervals (0～5 days) between inoculation and harvesting as shown in table 1. Moreover, the fluids were harvested from the same 2 bottles, daily after inoculation. The fluid harvested were stored at −20°C by the time of titration. Cell sheets from other bottles treated similarly were harvested at intervals of about 5 days for titration of the intracellular AEV. The cell sheet from which the fluid was removed was washed once with 5 ml of PBS, and then 5 ml of the maintenance medium was added for further incubation. Otherwise, the cell sheet harvested was washed and 2.5 ml of PBS was added, and then kept at −20°C. CEB cell-culture materials were obtained in a similar manner from the control bottles.

AEV-infected chick embryo brain (AICEB) cell culture Six- to eleven-day-old chick embryos were inoculated into the yolk sacs with the AEV of $10^{2.5}EID_{50}$. About 15 embryos were used for each experiment. They were cultivated by the same procedure as that of the normal embryos at the age of 13 days. At the time of cultivation and 18 days of incubation, some of the embryos injected were examined for evidence of AEV infection by the FA technique (CEB cells) or gross lesions of the embryos. Cell-culture fluid and cell sheet were harvested and stored by the manner described above. As AICEB cells attached insufficiently to the bottom of cell culture bottle 0 to 2 days after cultivation, the bottles were shaken vigorously to disperse the cells. The resultant cell suspensions were centrifuged at low speed and cell pellets were obtained.

Agar overlay The cell sheet, which had been inoculated and washed, was overlaid with agar by the method of HSIUNG & MELNICK and incubated at 38.5°C. The observation period was for 14 days after agar overlay. The cell sheet in AICEB cell culture was also treated in a similar manner 5 days after cultivation, when the cell sheet was almost full.

Virus titration The fluid harvests from the cell cultures inoculated with AEV or AICEB cell culture were centrifuged at 1,500×g for 30 min. Cell sheets

were frozen and thawed 5 times, and centrifuged similarly, and the supernates were titrated for the intracellular virus. The supernate and ten-fold serial dilutions of the supernate were inoculated into the yolk sacs of five 6-day-old embryonating eggs per dilution. Eagle's MEM with the addition of penicillin (1,000 units/ml) and streptomycin (1 mg/ml) was used as the diluent. The inoculum per embryo was 0.1 ml. All the embryos were examined for evidence of AE infection 12 days after the inoculation. The infection endpoint was determined by the method of Kärber.

The effect of incubation temperature on the infective titer of AEV The stock virus was diluted approximately 1 : 100 with Eagle's MEM, and the cell-culture harvests (infected culture fluids) were approximately 1 : 5. Two ml of the virus or the cell-culture harvests were dispensed into small tubes. Three tubes of each specimen were immediately held at −20°C and used for control. The remaining tubes were incubated at 38.5°C. Three tubes per day were taken out for 7 days of incubation period, and held at −20°C. The virus titration was made on the virus materials in the tubes by the same procedure as that described previously.

AE antiserum AE antiserum was obtained from chickens by administering orally with the non-embryo-adapted AEV isolated in this laboratory. The serum was heated at 56°C for 30 min. It had a log-neutralization index of >2.0 against the Van Roekel strain of AEV and was used for the virus neutralization test for the culture fluids of CEB cells infected.

Virus-neutralization test The undiluted AE antiserum was mixed with an equal amount of the cell culture fluids undiluted or in serial log dilutions. The same dilutions of the harvests were mixed with the diluent without the antiserum. The mixtures were incubated at 37°C for 1 hr. A dose of 0.1 ml of each dilution per embryo was inoculated into the yolk sacs of 6-day-old embryos. Five embryos were used for each dilution.

Direct FA test Young chickens free of AE antibody were administered orally with 1 dose of 2,000 EID_{50} of the Van Roekel strain of AEV. Their sera (log-neutralization index >2.0) were used for the preparation of FA according to the description of KAWAMURA. The conjugated antibody was absorbed twice with acetone-extracted chicken brain powder (0.1 mg/ml) and stored at −20°C until use. CEB cells on coverslips from the CEB cell culture inoculated with AEV or AICEB were washed once with PBS (pH 7.2~7.4), air-dried, fixed with ethanol or acetone at room temperature for 10 min and then stained with the FA at 37°C for 1 hr or at 4°C overnight.

Giemsa staining CEB cells grown on coverslips were stained with Giemsa solution after methanol fixation. If necessary, the preparations fixed with 10%

buffered formalin solution were stained with hematoxylin and eosin.

RESULTS

The infectivity of harvests from CEB cell cultures inoculated Table 1 shows the results obtained from the titration of each of the fluids which were harvested from CEB cell cultures inoculated with large doses of AEV. Initial multiplication of the virus was observed later than 12 hrs after inoculation. Titers of the intracellular virus reached the peak ($10^{2.4\sim3.2}EID_{50}$/ml) 1 day earlier than the fluids, though the maximum titer of the intracellular virus, $10^{3.3}EID_{50}$/ml, was obtained 3 days after inoculation in the cell culture inoculated with $10^{4.0}EID_{50}$/0.3 ml of AEV. Maximum virus titers of the cell-culture fluids ($10^{3.3\sim4.5}EID_{50}$/ml) were obtained 2~3 days after inoculation. In repeated trials almost the same results were obtained. On the other hand, smaller inoculum of AEV ($10^{3.0}$, $10^{2,0}$ and $10^{1.0}EID_{50}$/0.3 ml) resulted in prolongation of time to reach the peak of multiplication as shown in figure 1. In all cases, after having reached the maximum

TABLE 1 *Multiplication of embryo-adapted AEV in CEB cell culture inoculated with large doses of the virus*

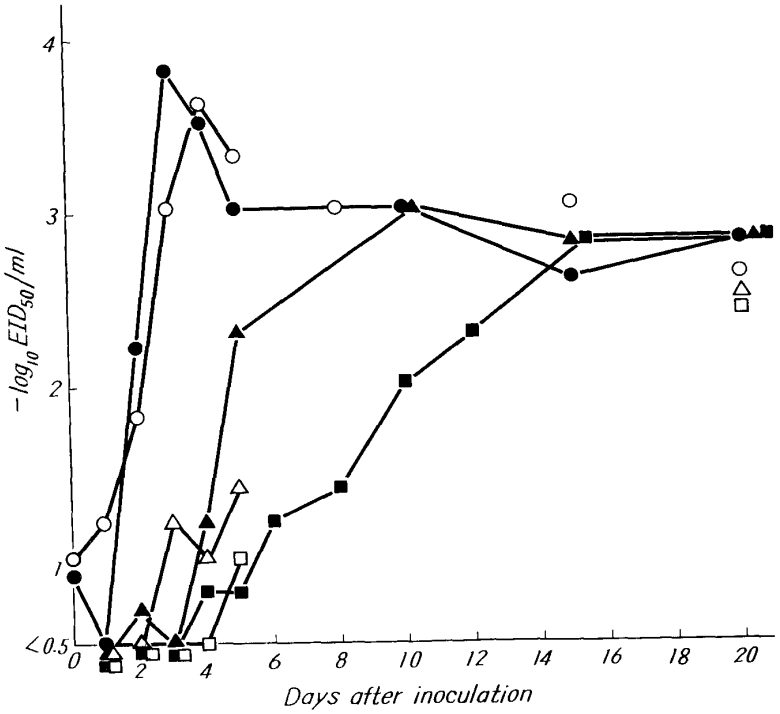
INFECTIVE*1 TITER OF INOCULUM	SPECIMEN EXAMINED	DAYS BETWEEN INOCULATION AND HARVESTING								
		0	1/6	1/3	1/2	1	2	3	4	5
5.0	Cell-culture fluid	2.0*2	1.9	2.7	2.3	2.8	4.5	3.7	4.0	3.3
	Cell phase	2.2	2.0	2.2	2.0	3.2	3.2	3.3	2.5	2.5
4.0	Cell-culture fluid	0.8	2.2	2.7	2.5	2.8	3.2	2.8	2.7	2.8
	Cell phase	2.6	2.0	1.7	1.6	2.4	2.2	1.8	2.4	1.8
4.0	Cell-culture fluid	0.8	.*3	2.3	.	2.2	3.1	3.3	3.2	2.7

*1: Expressed as ($-\log_{10}EID_{50}$/0.3 ml)
*2: Expressed as ($-\log_{10}EID_{50}$/ml)
*3: Not done

virus titers, the infective titers of $10^{2.5\sim3.0}EID_{50}$/ml (cell-culture fluids) persisted till at least 20 days after inoculation and the cell phase also had the titers of approximately $10^{2.5}EID_{50}$/ml on the day. In another trial, the cell culture fluid had infective titer of approximately $10^{3.0}EID_{50}$/ml 28 days at least after inoculation. Infected CEB cells on coverslip were examined at appropriate times by the FA technique. Detection of the viral antigen was negative through the experiments regardless of inoculum size.

The treatment of infected cell-culture fluids with AE antiserum The harvests (cell-culture fluids) at 20 days from cell cultures inoculated with $10^{3.0}$

FIGURE 1 *The virus titers*[*1] *in CEB cell cultures inoculated with smaller doses of embryo-adapted AEV*



● cell-culture fluid
○ cell phase
($10^{3.0}$ EID_{50}/0.3 ml)[*2]

▲ cell-culture fluid
△ cell phase
($10^{2.0}$ EID_{50}/0.3 ml)

■ cell-culture fluid
□ cell phase
($10^{1.0}$ EID_{50}/0.3 ml)

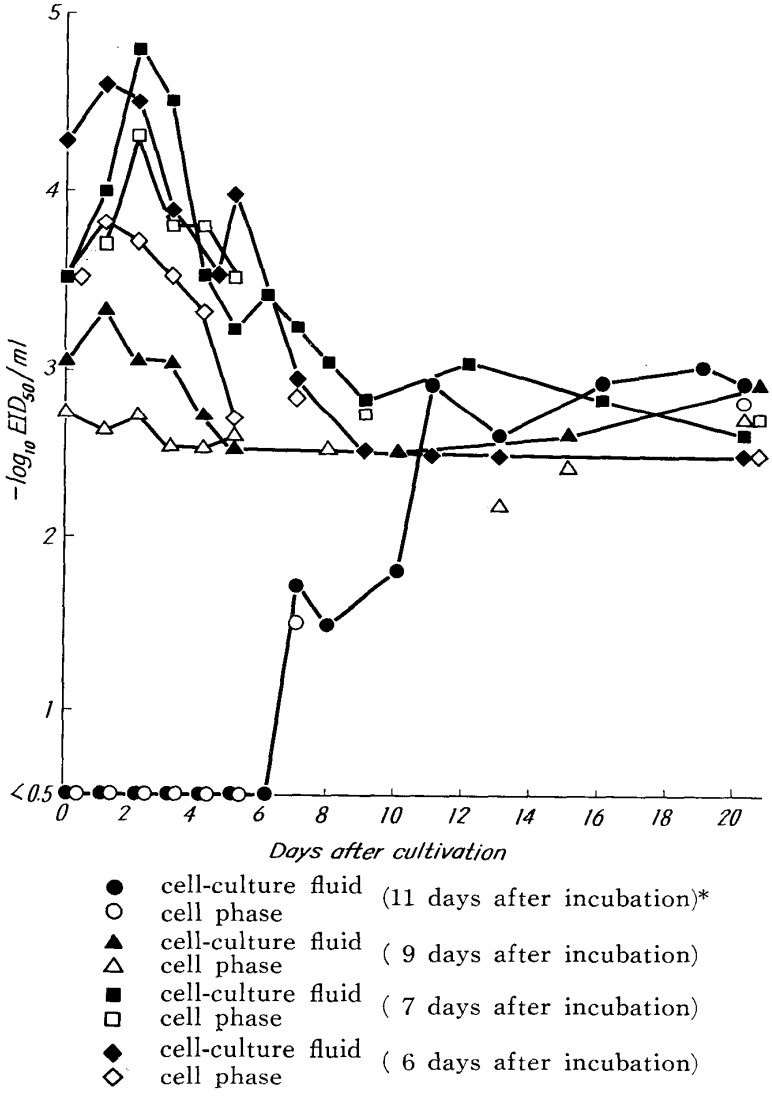
*1: The virus titers later than 5 days after inoculation indicate those of cell-culture fluids harvested daily after inoculation

*2: Inoculum size

and $10^{1.0}EID_{50}$/0.3 ml were perfectly neutralized with the AE antiserum. From the results described above, the multiplication of AEV in CEB cell culture was confirmed, though the virus titer was not so high.

The infectivity of harvests from AICEB cell cultures As shown in figure 2, in the cell cultures from heavily infected embryos which had been inoculated at the age of 6 or 7 days, the harvests had relatively high virus titers ($10^{4.6\sim4.8}EID_{50}$/ml) at the initial stage of the cultivation. Thereafter, the titers decreased gradually. However, after having decreased to $10^{2.5\sim3.0}EID_{50}$/ml, the infective titers persisted till at least 20 days after cultivation. In the case of cultured cells from chick embryos which had been inoculated with the virus at the age of 11 days, the virus was not detected at the initial stage (6 days after cultivation). However,

FIGURE 2 *The virus titers in the CEB cell cultures from chick embryos infected with embryo-adapted AEV at various days of incubation*



*: Age of days at which embryos for cell culture were inoculated with AEV (Cell cultures were obtained from the embryos of 13 day old)

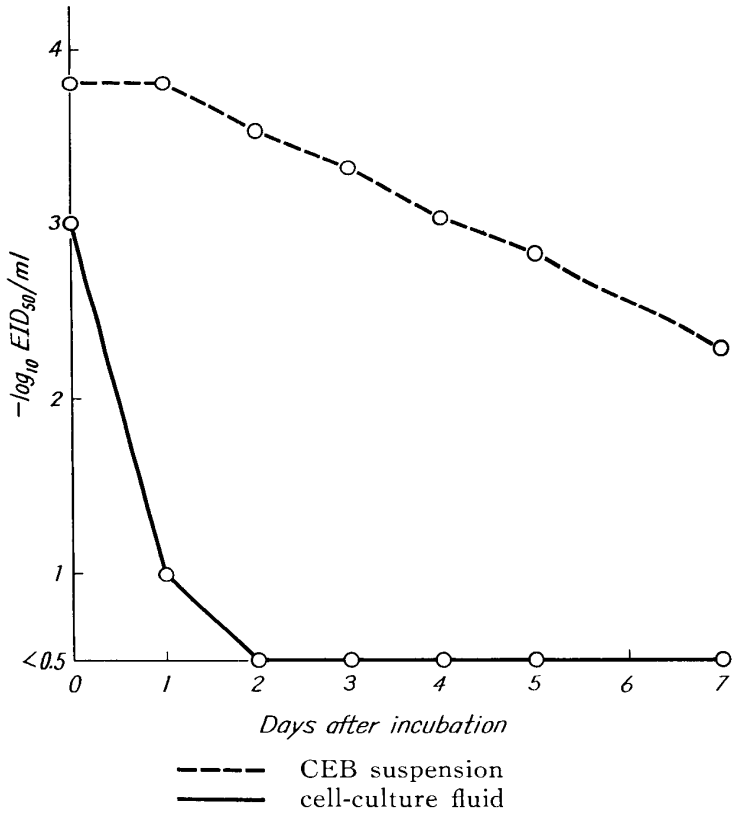
the titer increased gradually day by day, reached $10^{2.5\sim3.0}EID_{50}$/ml, and persisted for long time. On the other hand, in the AICEB cell culture from the embryos inoculated at the age of 9 days, virus titers were less variable from the initial stage to the end of the cultivation and indicated about $10^{3.0}EID_{50}$/ml. Table 2 shows a decline in the specific fluorescence of the cultured cells from chick embryos which had been inoculated with embryo-adapted AEV at various days of incubation. The embryos inoculated at the age of 6 or 7 days manifested

TABLE 2 *AEV antigen by FA technique in the cultured cells from chick embryos inoculated with the virus at various days of age*

AGE OF EMBRYOS AT INOCULATION	DAYS AFTER CULTIVATION										
	0	1	2	3	4	5	6	8	10	11	14–20
6	++*1	++	++	++	+*2	.*4	−*3	.	.	.	−
7	++	++	++	++	++	++	+	+	+	−	−
8	++	.	++	+	+	−	−	.	.	.	−
9	+	+	+	+	+	.	−	.	.	.	−
10*5	−	−	−	−	−	.	−	.	.	.	−
11	−	−	−	−	−	.	−	.	.	.	−

*1: Fluorescence was detected in 50 or more the cultured cells/coverslip
*2: Fluorescence was detected in less than 50 of the cultured cells/coverslip
*3: Fluorescence was not detected in the cultured cells
*4: Not done
*5: Not titrated

FIGURE 3 *Comparative curve of AEV derived from infected CEB suspension and cell-culture fluid at 38.5 C*



AE characterisitic gross lesions. As shown in the table, the specific fluorescence, in the cultured cells from chick embryos inoculated with the virus 6, 7, 8, or 9 days of incubation, was detected by the FA technique from the initial stage of the cultivation. Then, the fluorescence decreased gradually both in number and in intensity and disappeared 5 days or later after the cultivation, excepting a case. The exceptional cell culture from the embryos inoculated at 7 days of incubation gave the distinct fluorescence for 10 days post-cultivation. On the other hand, in the case of CEB cells from the embryos inoculated with the virus at the age of 10 or 11 days, the fluorescence was not observed at all. The fluorescent granules were observed in the cytoplasms of the cells and the size of the granules was variable (figures 4 & 5).

The response of the cultured CEB cells and of the cultured AICEB cells to the infection with AEV Neither the cytopathic effect nor plaque formation was observed in all cases. No inclusion body due to AEV infection was detected in the cells.

The effect of an incubation temperature to the infective titer of AEV As shown in figure 3, the infective titer of the diluted stock virus fell at the rate of about $10^{-0.21}$/day at 38.5°C. On the other hand, AEV derived from the cell cultures (cell-culture fluids) was more sensitive to the temperature.

Discussion

The cultured CEB cells employed for the present study are nearly the same as those described as neuroglial cells by MANCINI & YATES[9]. Most of the cultured cells were of neuroglial cell (mainly astroglia, partly oligodendroglia) at the early stage of cultivation (figures 6 & 7). However, at the later stage, fibroblastic cells were also observed.

The multiplication of embryo-adapted AEV in the CEB cell cultures was confirmed also in this study. The virus titers in the cell cultures were relatively low, but persisted up to the end of cultivation in all cases without any cytopathic effect as described by MANCINI & YATES[9], and WATANABE & MIURA. Whereas, MATUKA et al. established an embryo-adapted AEV strain of high virus titer ($10^{9.0}$EID$_{50}$/ml) and obtained high titers of the cell-cultured AEV ($1.5\times10^{6.0}$EID$_{50}$/ml). It is reasonable to suppose that the high titer of seed virus brought on the higher virus titer of cell culture harvests. However, they did not explain why only their strain had so high infective titer.

In a previous paper[14], we indicated that the initial multiplication of non-egg-adapted AEV in the CEB cell cultures was slow and variable among the strains used, and the infection continued persistently. It took about a week or more for the infected cell cultures to give appreciable virus titers. These

results resemble rather those obtained in the CEB cell cultures inoculated with smaller doses ($10^{2.0}EID_{50}$/0.3 ml or less) of embryo-apapted AEV in the present study.

There have been unsuccessful attempts to pass serially AEV of embryo-adapted or non-embryo-adapted in various organs or cell cultures. Our present data indicated that, as the initial infection of AEV in CEB cell cultures was weak, the incubation period necessary to sufficient virus production was prolonged. Therefore, the failure in serial passage of AEV in the previous attempts appears to have been due partially to inadequate incubation period applied for each passage. Repeated passages of AEV at short intervals might result in the decrease in number or the disappearance of the virus in inoculated cell cultures according to advance of passages.

It remains unsolved why any specific fluorescence was not detected in the cultured CEB cells infected with AEV, and it decreased gradually and disappeared in the AICEB cells; moreover, the virus titers were low and persisted for a long time in these cultured cells. Our previous data[14] on the CEB cells infected with non-egg-adapted AEV showed non detection of fluorescence in the infected cultured cells. Our speculation on the explanation of these facts is as follows: CHEVILLE & MONLUX (1966) stated that, in the chick brains infected with AEV, the earliest detectable fluorescence was found in Purkinje's cells, and was in satellite Bergmann's cells.

We could scarcely find Purkinje's cells and other nerve cells in the cell cultures, and the in-vitro cell culture conditions used, under which cell viability or metabolic activity were lowered, could not maintain the cells, though some nerve cells were detectable in the cultures at the earlier stage. Moreover, the present cultural conditions might reduce the susceptibility of the glial cells, which predominated in the culture, to AEV or the ability of virus replication of the cells.

There is a report[15] as to the heat resistance of AEV. In the present experiment, the infective titer of AEV of a brain suspension kept at 38.5°C, fell at the rate of about $10^{-0.21}$/day, remarkably similar to that reported by von BÜLOW (1964). However, the AEV of cell culture fluids was more sensitive to the incubation temperature than the purified-virus described by him. MANCINI & YATES[9] stated that AEV-infected cell-culture fluid, relatively free of extraneous proteins, will be especially valuable for study of the physical and biological properties of the virus. The present data appear to support their statement.

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EXPLANATION OF PLATE

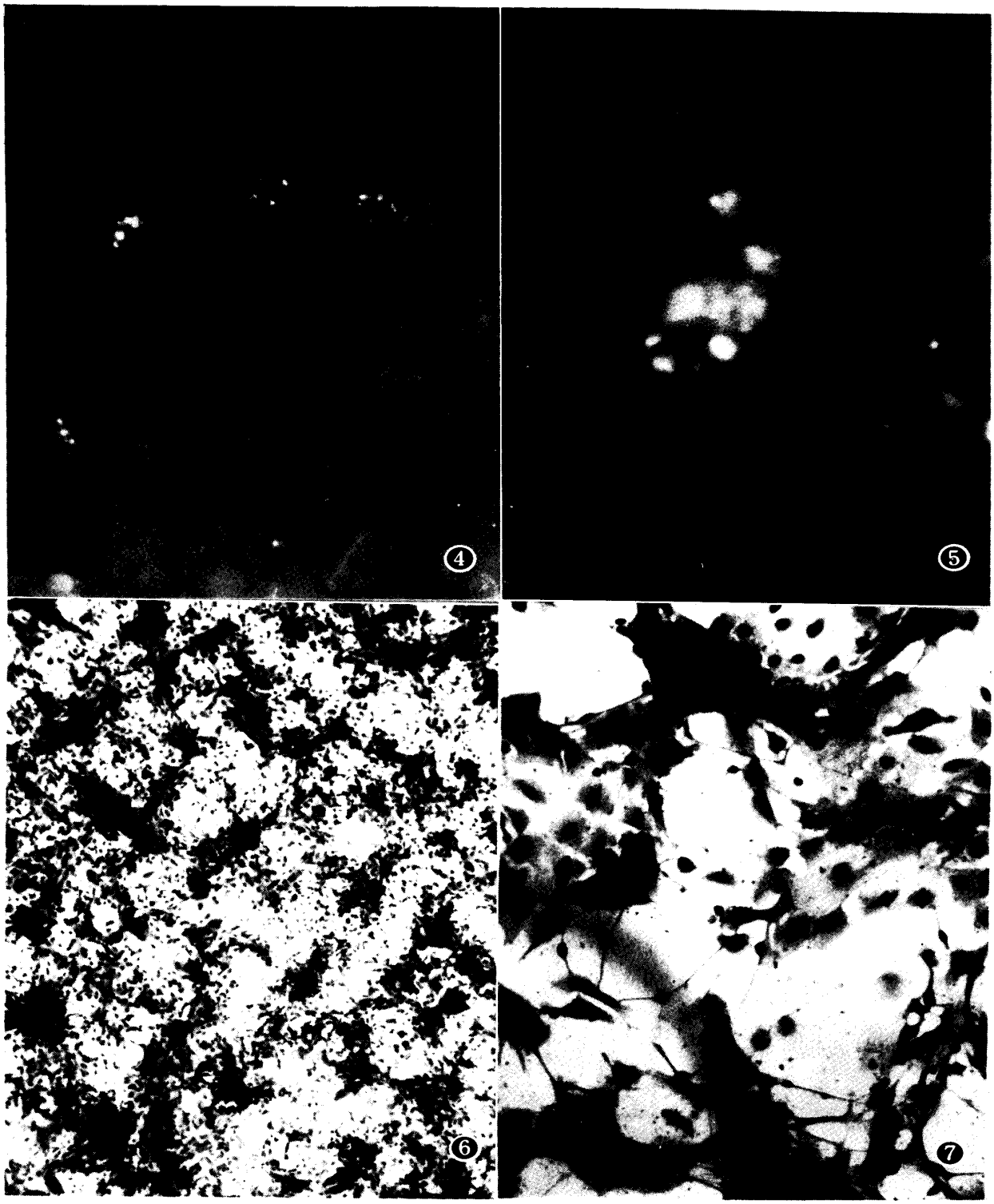
All specimens were made at 4 days of cultivation.

Fig. 4 Fluorescence in the AICEB cells × 200

Fig. 5 Fluorescent granules in the cytoplasms of the AICEB cells × 600

Fig. 6 Colonies in the CEB cell cultures × 50

Fig. 7 Cells in the colonies in the CEB cell cultures × 200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