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Infection of human embryonic skin-muscle tissue culture cells with adenovirus type 12*

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Abstract

,The effect of infection of human embryonic skin-muscle cell cultures with adenovirus type 12 has been studied. When maintained in YLE containing 20 per cent bovine serum, human embryonic skin-muscle tiue culture cells developed little or no cytopathogenic effect for about 50 days after inoculation of adenovirus type 12, though a small amount of virus was always detected in the overlying medium. From day 50∼60, CPE started appearing and spread over 90 per cent of cells accompanied with the increase of virus in the overlying medium. The addition of human serum to the maintenance medium inhibited the virus release. After removal of human serum about 16∼37 days after its addition, virus-and, later, CPE also-again started appearing. The second virus release-and CPE also-was inhibited by addition of human serum to the medium. When maintained in the medium with human serum for about 200 days, the removal of human serum did not result in the appearance of virus or CPE. The virus isolated from the overlying medium of these cells during the whole proce of the experiment was always highly oncogenic to newborn hamsters. Diluted adenovirus-12-immune rabbit serum also showed the effect similar to that of human serum. But, regardle of its much higher antibody titer, the effect of this diluted adenovirus-12-immune rabbit serum was weaker than that of human serum. In one of cell cultures, rapidly growing cells appeared 212 days after virus inoculation. But the available data suggest that these are the cells transformed rather spontaneously in tiue culture than by adenovirus type 12.

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INFECTION OF HUMAN EMBRYONIC SKIN-MUSCLE TISSUE CULTURE CELLS WITH ADENOVIRUS TYPE 12*

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Adenovirus type 12 induces tumors in laboratory animals[1]. Several other types of human adenovirus than type 12 have been also shown to induce tumors in hamsters[2-5]. However, presently nothing is known concerning the oncogenicity of these adenoviruses in man. In our preliminary experiments, the cells of human embryonic kidney and lung tissues cultured *in vitro* responded with marked cytopathogenic effect (CPE) to inoculation of adenovirus type 12 producing a large amount of virus, while the cells of the skin-muscle showed little cytopathogenic effect. These results suggested that human embryonic skin-muscle tissue culture cells might be one of the tissues of choice for the *in vitro* test of the oncogenic activity of these adenoviruses to human cells. Present study was undertaken in this consideration.

MATERIALS AND METHODS

Virus and virus titration : Adenovirus type 12, strain "Huie" was cultured, prepared and titrated as described previously[6].

Tissue culture media : Earle solution containing 0.1 per cent yeast extract and 0.5 per cent lactalbumin hydrolysate (YLE) was used as the basal medium. Before use, this basal medium was supplemented with various concentrations of bovine, human and/or adenovirus-12-immune rabbit sera. Except for adenovirus-12-immune rabbit serum these were all sterilized by filtering Seitz filter pad. Inactivation by heating at 56°C for 30 minutes was done with the bovine serum, but not with the human or adenovirus-12-immune rabbit serum.

Titration of adenovirus-12-neutralizing activity of serum : This was done in the same way as described previously[7], except for that the serum was not heat-inactivated before test. The human sera used were of titers 1 : 4 to 1 : 8, and the adenovirus-12-immune rabbit serum was of 1 : 1024.

Tissue culture of human embryonic skin-muscle cells : The skin-muscle

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tissue of human embryos of 3～5 months of gestation was minced with scissors, treated with 0.25 per cent trypsin at 37°C for 1～2 hours and planted into tissue culture bottles of which culture surface was 100×50 mm. The growth medium was YLE containing 20 per cent bovine serum. The subculture passages of cells were done by trypsinization.

Virus inoculation and cell maintenance : Upon completion of monolayer, cells were washed twice with phosphate-buffered saline (PBS) and inoculated with the virus at about $2\sim10\times10^3$ $TCID_{50}$ per 10^6 cells using YLE with 2 per cent bovine serum as the maintenance medium. On day 2 of virus inoculation, the medium was changed with YLE with 20 per cent bovine serum. Thereafter the medium was changed every 3～5 days with the media described in the section of results. At every or every other medium change, old medium was preserved in freezer (−20°C).

Virus isolation : The isolation of virus from the preserved media was done as follows: 1) 1 ml of preserved medium was inoculated into each of 2 HeLa cell tubes and maintained for 35 days doing medium change with 2 ml of YLE with 2 per cent bovine serum at every 3～4 days; 2) on day 35, the tubes without CPE were frozen-thawed once, its 0.5 ml was inoculated into each of 2 HeLa cell tubes and similarly maintained for 14 days; 3) all these tubes were observed for CPE at every 2～3 days. By this method, about 10^{-3} $TCID_{50}$/0.1 ml of adenovirus type 12 by our usual titration method can be detected[8]. Fluids of HeLa cell tubes showing CPE was titrated and inoculated intraperitoneally into newborn hamsters (0.05 ml per animal) for the test of oncogenic activity.

Complement-fixation test : Adenovirus-12-tumor antigen(s), adenovirus-12-viral antigen(s) and tissue culture cell antigen(s) were prepared in the same way as reported by HUEBNER *et at.* except for that the virus was cultured in HeLa cells[9]. For titration of antigen(s) a positive hamster serum was used at a 1 : 16 dilution representing 4 units of complement-fixing antibody. Two full units of guinea pig complement were used for the test. KOLMER's procedure using 0.05 ml of each ingredient was employed[10], and the complement fixation was done overnight at 4°C.

RESULTS

Infection of human embryonic skin-muscle tissue culture cells with adenovirus type 12 : Representative results of experiments are given in chart 1. When maintained in the culture medium containing bovine serum, rounding of cells was observed only sporadically in 3～10 days after virus inoculation. Then such cells disappeared gradually. About day 51, however, rounding of

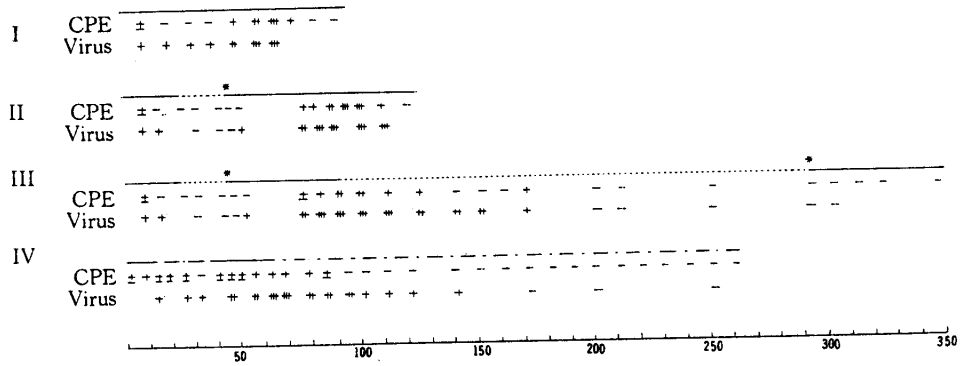


Chart 1 Persistent Infection of Human Embryonic Skin-Muscle Tissue Culture Cells with Adenovirus Type 12

CPE: Cytopathogenic effect. Virus: Virus isolation from overlying medium by inoculating into HeLa cell tubes (+; CPE-development in inoculated HeLa cells on days 31-49. ++; on days 11-30. +++; on days ≦ 10).

———: Maintained in YLE with 20% bovine serum. ---------: Maintained in YLE with 10% bovine serum and 10 or 20% human serum. — · —: Maintained in YLE with 20% bovine serum and 10% adenovirus-12-immune rabbit serum diluted to 1:20 with YLE.

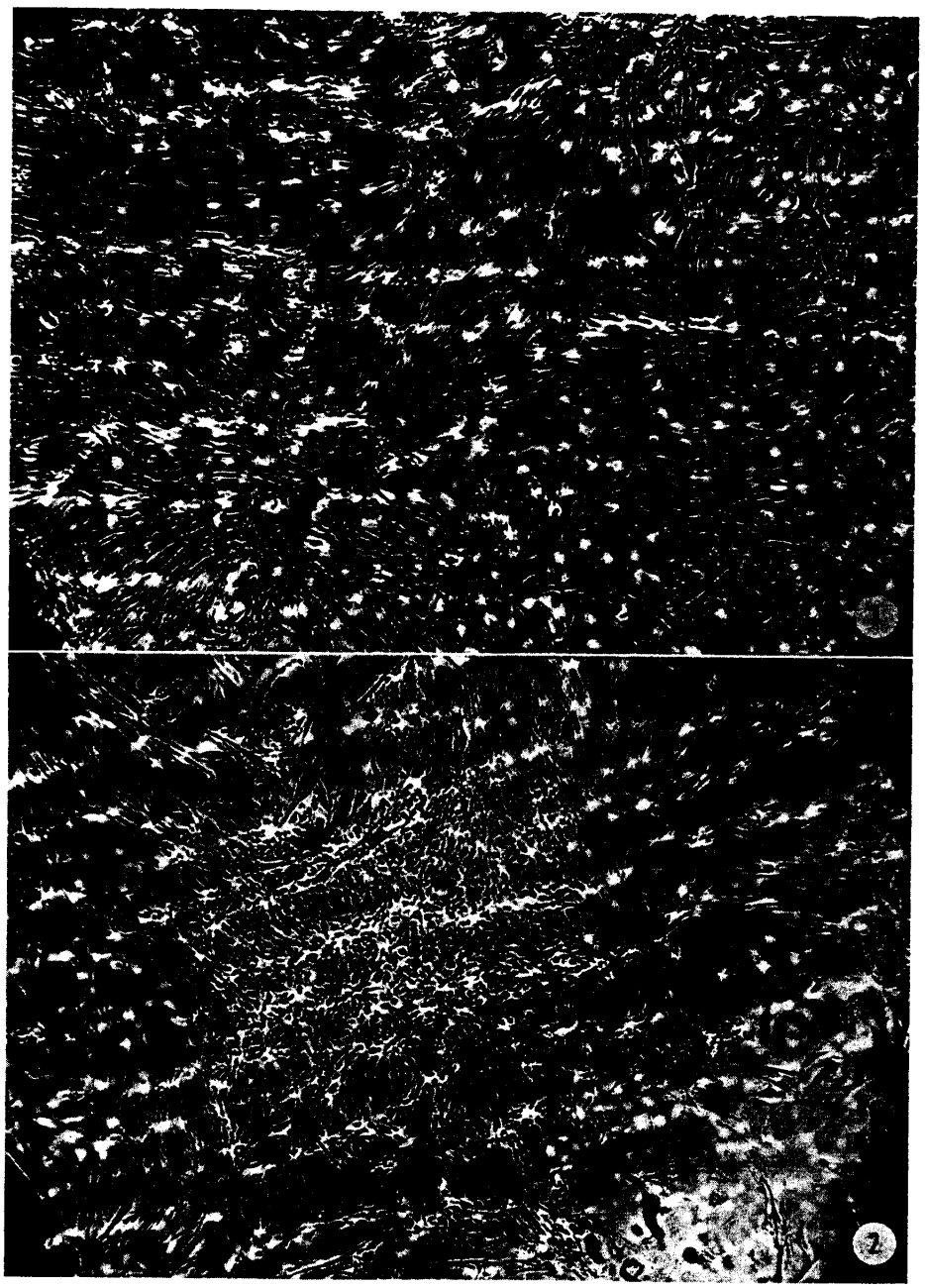
* Maintained in YLE with 5% bovine serum for 4 days, then changed to YLE with 20% bovine serum.

Table 1 Complement-Fixing Antigen(s) in Adenovirus 12 Tumor and New Type of Cells

Antigen	Antigen titer (reciprocal) versus serum of adenovirus-12-tumor bearing hamster[1]
Adenovirus 12	< 8
Adenovirus 12 hamster tumor	≧64
New type of cells[2]	16
Control cells[3]	16

1: Serum of hamster bearing tumor which was used as antigen in this table. 2: New type of cells in Exp. III of Chart 1; 423 days after virus inoculation. 3: Control cells to the above.

cells again started appearing and increased over 90 per cent of cells in 16～23 days. Throughout the whole process, regardless of the presence or absence of recognizable cytopathogenic effect (CPE), at least a slight amount of virus was detected in the overlying culture medium. The amount of virus in the medium reached the maximum when CPE reached the maximum (Exp. 1). When human serum was added to the maintenance medium to 10 per cent, the virus became not demonstrable in the overlying medium and the CPE did not appear. But upon removal of human serum from the medium 16～36 days after its addi-



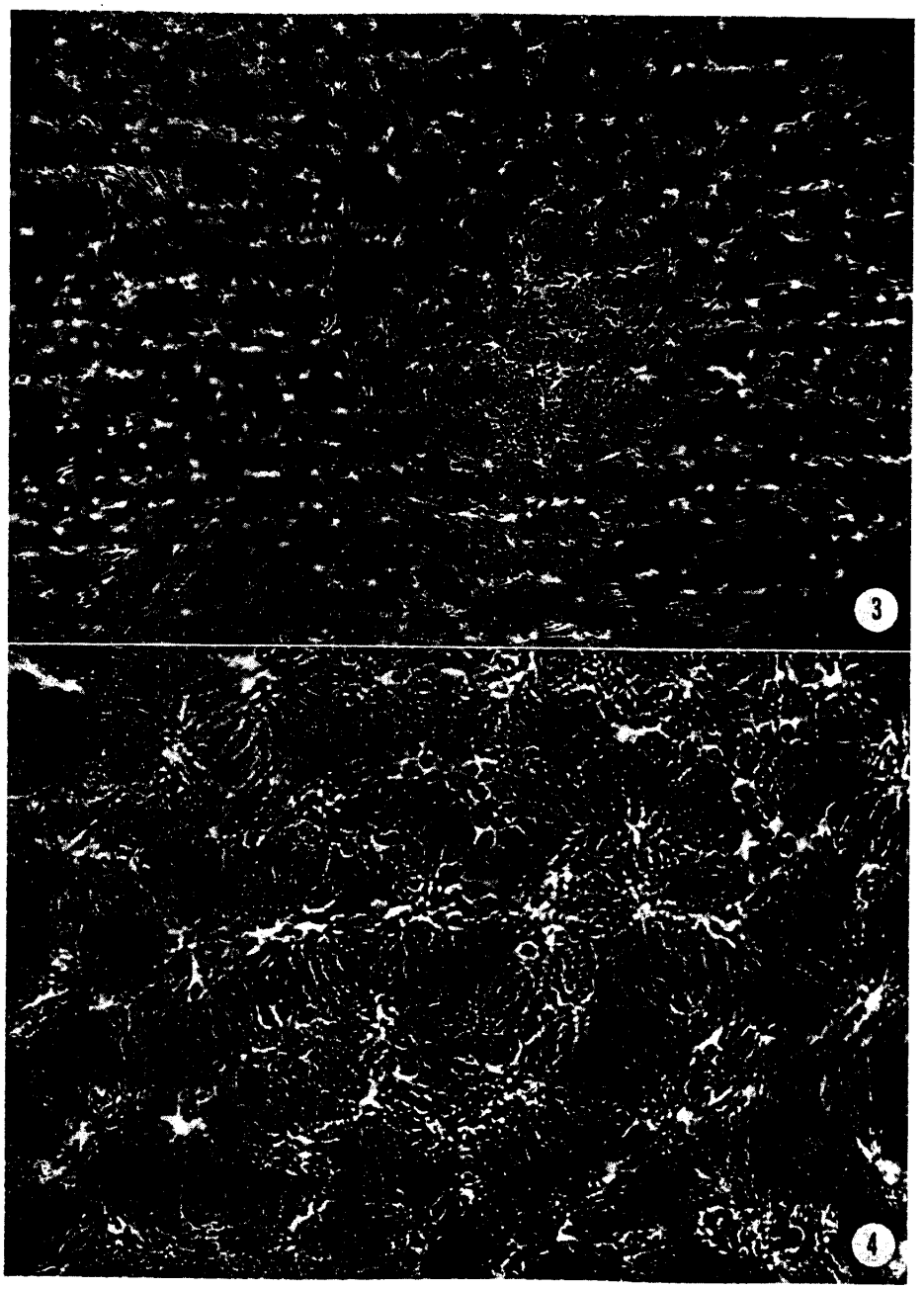
tion, the virus again became demonstrable in the medium in 8 days. The CPE also started appearing again in 36～40 days after removal of human serum and developed to the almost complete CPE-affection as in Exp. I (Exp. II). After the second addition of 10 per cent human serum, the recurring CPE subsided gradually. The virus in the overlying medium also diminished gradually, but was detected for over 90 days. On day 296, 200 days after the second addition of human serum, human serum was removed from the medium, but neither the virus nor CPE was observed (Exp. III). To analyze the effect of human serum on the infection of this virus, adenovirus-12-immune rabbit serum was diluted to 1 : 20 with YLE and added to the medium in place of human serum. Its effect was similar to, but weaker than that of human serum (Exp. IV).

Oncogenic activity of virus recovered from the medium of adenovirus-12-inoculated cell cultures : To test the change of properties of adenovirus type 12 carried in the skin-muscle tissue culture cells for long periods, the virus isolated from the overlying medium of virus-inoculated bottles was inoculated into newborn hamsters. The virus recovered from the overlying medium of Exp. III in Chart 1 on day 52, 129, 143, and 175, of which titers were $10^{1.5}$～$10^{2.5}$ $TCID_{50}$/0.1 ml, produced tumors in 5 of 7, 7 of 8, 5 of 5 and 3 of 4 hamsters respectively.

Morphological appearance of cells : The human embryonic skin-muscle cells cultured *in vitro* were mostly fibroblastic. In Exp. III, a few small areas consisting of big oval cells with rather small nucleus appeared sporadically on day 115. Such cells increased slowly but the growth was grossly not over that of control cells, and did not overgrow the remaining fibroblastic cells. No such cells were observed in the control bottles. On day 212, there were observed several foci consisting of small polygonal cells in virus-inoculated bottles (Figs. 2～4). The growth of these polygonal cells appeared not very fast, but clearly exceeded that of control cells. The center of most foci gradually heaped up (Figs. 5, 6). In a few foci where the surrounding area was free, cells multiplied rather extensively, but even in such foci, the center heaped up later. Upon subculture passage by trypsinization on day 277, the new type of cells propagated well. But normal-like fibroblastic cells were also well cultured, and these two types of cells were mixed. The new type of cells were, however, more sensitive than normal-like fibroblastic cells to the unfavorable conditions such as infrequent medium change or low temperature. This new type of cells were fairly purely collected by the following procedures: 1) subculture this

Fig. 1 Human embryonic skin-muscle tissue culture cells in Exp. III. 213 days after inoculation of control fluid. Live, ×71

Fig. 2 Human embryonic skin-muscle tissue culture cells in Exp. III. 213 days after inoculation of adenovirus type 12. A focus of new type of cells is observed. Live, ×71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mixed culture of both polygonal cells and normal-like fibroblastic cells by trypsinization; 2) maintain the cells without medium change for 5 days when many polygonal cells rounded; 3) wash the cell sheet lightly with trypsin solution twice and collect these trypsin solutions in centrifuge tubes; 4) collect cells by centrifugation and plant in other bottles; and 5) with these new bottles, repeat the whole process again. New type of cells thus collected retained their morphological characteristics as described above for 2 subsequent tissue culture passages, but thence they gradually showed a tendency to take rather fibroblastic form. Their growth was grossly not faster than HeLa cells and, when subculture was done from 1 tube to 2 tubes, a compact sheet was formed in 7 to 10 days in the medium of YLE with 10 per cent bovine serum and 20 per cent human serum.

On day 417, cells of control bottles of Exp. III were transplanted into new bottles. Although cells still retained normal-like fibroblastic appearance, their growth was as rapid as the new type of cells of virus-inoculated bottles.

Complement-fixation test with antigen(s) of new type of cells and serum from adenovirus-12-tumor bearing hamster: As shown in Table 1, the serum from adenovirus-12-tumor bearing hamster reacted in the complement-fixation test with the adenovirus-12-tumor antigen(s), but did not specifically with the antigen(s) of new type of cells.

Sensitivity of new type of cells to adenovirus type 12: On day 354, the tubes of new type of cells of Exp. III were washed twice with PBS and inoculated with 0.25 ml of adenovirus type 12 of $10^{2.5}$ $TCID_{50}$/0.1 ml and maintained in YLE containing 2 per cent bovine serum. Eight days after virus inoculation, weak CPE appeared as observed at the beginning of virus inoculation into human embryonic skin-muscle tissue culture cells.

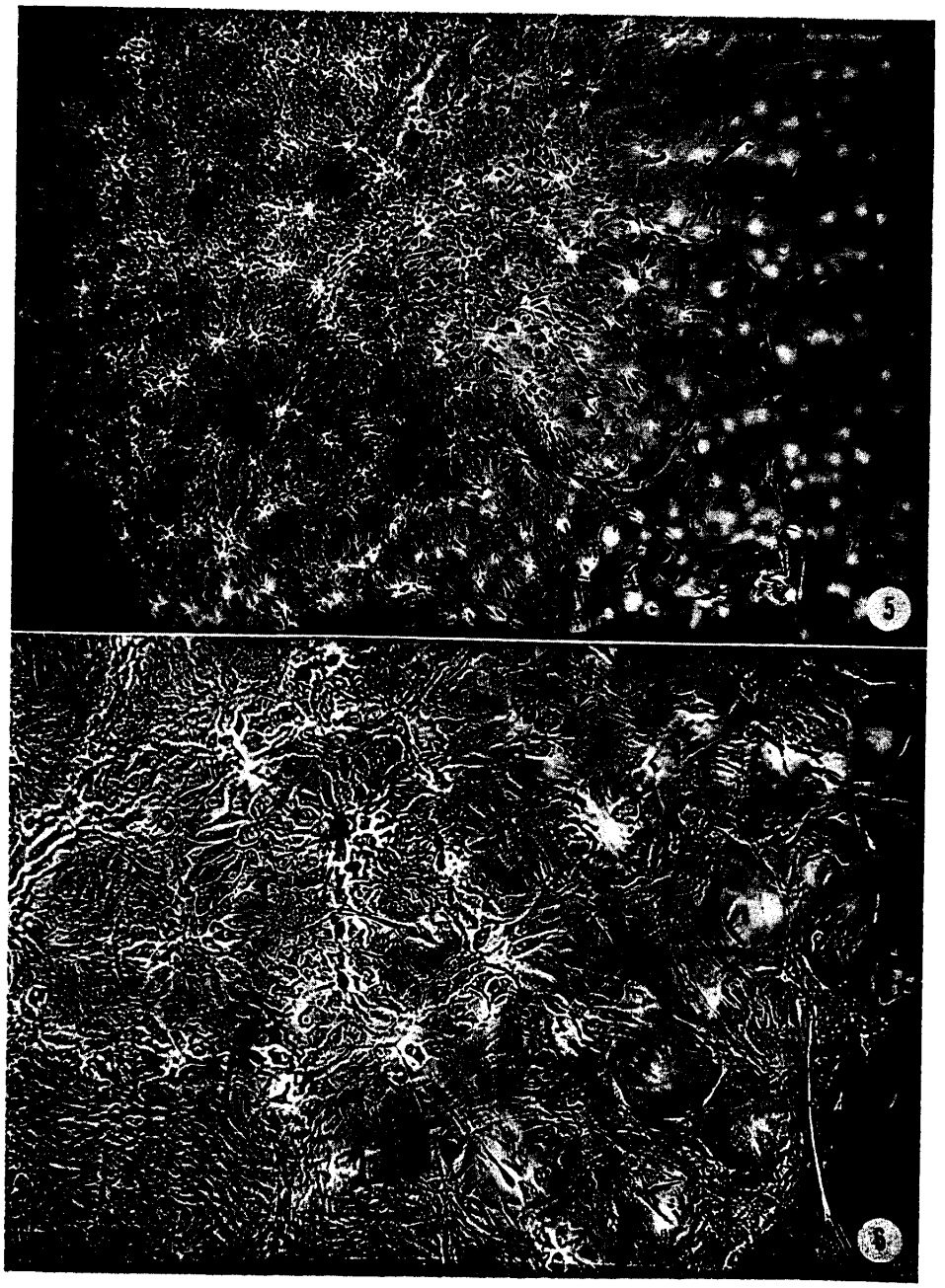
Reproducibility of experiments: Exps. I and II were repeated with another embryonic material, and all showed the results similar to those described in Chart 1. Exp. III was repeated with 3 other embryonic materials and, as for the virus release and CPE development, similar results were obtained. But no such cell foci as observed in Exp. III of Chart 1 appeared in all the 3 repeated experiments.

DISCUSSION

The results of the present experiments suggest that a state of persistent or latent infection without recognizable CPE but with virus release is established between adenovirus type 12 and human embryonic skin-muscle cells cultured *in vitro* in the medium of YLE with 20 per cent bovine serum. Furthermore a

Fig. 3 The same group of cells as in Fig. 2. 213 days after inoculation of adenovirus type 12. Another focus of new type of cells is observed. Live, ×71

Fig. 4 Higher magnification of the focus in Fig. 3. Live, ×179



state of persistent or latent infection without both CPE and virus release is established in the medium of YLE with 10 per cent bovine serum and 10～20 per cent human serum. However, the state of persistent or latent infection in the medium of YLE with 20 per cent bovine serum developed into the manifest infection of almost all cells 2～3 months after infection. In the medium containing human serum, the state of persistent infection developed later into the state of complete cure. These suggest that a state of carrier culture is established not between adenovirus type 12 and a human embryonic skin-muscle cell itself but only between the virus and a population of these cells cultured *in vitro*. The effect of human serum on the establishment of carrier state without CPE and virus release, is partly due to its neutralizing antibody because diluted adenovirus-12-immune rabbit serum showed a similar effect. But the effect of diluted immune rabbit serum was, regardless of its much higher neutralizing antibody titer, weaker than that of human serum: this suggests that human serum contains other factor(s) than neutralizing antibody which is important for the establishment of virus-carrier state. Though the data of the present experiments are not enough to analyze it precisely, the effect of this factor(s) will be due to either one or both of the following two: 1) suppression of virus multiplication in the infected cells, and 2) suppression of the infection to other non-infected cells. Similar results had been previously reported by GINSBERG with adenovirus types 3 and 4 and HeLa cells[11] and VAN HOOSIER *et al.* with adenovirus type 12 and HeLa cells[12]. In their report, however, the observation period is rather short and the state of complete cure in the presence of human serum is not reported.

In one of the experiments reported here, morphologically different and rapidly growing cells appeared. At present, it is very difficult to determine if these are the cells transformed by adenovirus type 12 or transformed spontaneously in tissue culture as reported by other investigators with many kinds of cells cultured *in vitro*[13~17]. But the latter possibility is suggested by the following results: 1) such a phenomenon was not reproducible in the subsequent similar experiments with three other human embryonic skin-muscle materials; 2) development of these cells are observed about 7 months after inoculation of adenovirus type 12 when spontaneous transformation of *in vitro*-cultured cells mostly occurs; 3) upon subculture passages, normal-like cells also propagated well; 4) cells of control bottles also started growing rapidly from about the 13th month of the experiment; 5) these cells were sensitive to reinoculation of adenovirus type 12; and 6) with the serum of adenovirus-12-tumor-bearing hamster,

Fig. 5 A part of focus of new type of cells. Heaping-up to the left. 273 days after inoculation of adenovirus type 12. Live, ×71

Fig. 6 Higher magnification of a part of focus in Fig. 5. Live, ×179

specific complement-fixing antigen(s) was not detected in these cells.

In vitro transformation technique has provided cancer researchers with very useful system for the study of the mechanism of viral carcinogenesis. But prolonged *in vitro* maintenance causes the spontaneous transformation which makes the experimental results very obscure as reported by other investigators and also as observed in the present experiment, whereas some tumor viruses usually produce tumors *in vivo* as late as 6～12 months or more after their infection[8,18].

SUMMARY

The effect of infection of human embryonic skin-muscle cell cultures with adenovirus type 12 has been studied. When maintained in YLE containing 20 per cent bovine serum, human embryonic skin-muscle tissue culture cells developed little or no cytopathogenic effect for about 50 days after inoculation of adenovirus type 12, though a small amount of virus was always detected in the overlying medium. From day 50～60, CPE started appearing and spread over 90 per cent of cells accompanied with the increase of virus in the overlying medium. The addition of human serum to the maintenance medium inhibited the virus release. After removal of human serum about 16～37 days after its addition, virus—and, later, CPE also—again started appearing. The second virus release—and CPE also—was inhibited by addition of human serum to the medium. When maintained in the medium with human serum for about 200 days, the removal of human serum did not result in the appearance of virus or CPE. The virus isolated from the overlying medium of these cells during the whole process of the experiment was always highly oncogenic to newborn hamsters. Diluted adenovirus-12-immune rabbit serum also showed the effect similar to that of human serum. But, regardless of its much higher antibody titer, the effect of this diluted adenovirus-12-immune rabbit serum was weaker than that of human serum.

In one of cell cultures, rapidly growing cells appeared 212 days after virus inoculation. But the available data suggest that these are the cells transformed rather spontaneously in tissue culture than by adenovirus type 12.

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