sional paper chromatograms of extracts of brains of the spastic mice showed no significant alteration of distribution of ninhydrin-reactive constituents by comparison with normal controls.

1. Roberts, E., Eidelberg, E., in *International Rev. of Neurobiol.*, 1960, v2, 279.
2. Baxter, C. F., Roberts, E., PROC. SOC. EXP. BIOL. AND MED., 1959, v101, 811.
3. Wallach, D. B., *Biochem. Pharmacol.*, 1961, v5, 323.
4. Baxter, C. F., Roberts, E., *J. Biol. Chem.*, 1961, v236, 3287.
5. Chai, C. K., *J. Heredity*, in press.
6. Roberts, E., Lowe, I. P., Guth, L., Jelinek, B., *J. Exp. Zool.*, 1958, v138, 313.

Received December 27, 1961. P.S.E.B.M., 1962, v109.

Multiplication and Cytopathogenicity of Simian Vacuolating Virus 40 in Cultures of Human Tissues.* (27246)

HARVEY M. SHEIN† AND JOHN F. ENDERS
Research Division of Infectious Diseases, Children's Hospital Medical Center, Department of Bacteriology and Immunology, Harvard Medical School, Boston, Mass.

The vacuolating simian virus, SV_{40}, was originally found in uninoculated cultures of kidney cells from rhesus and cynomolgus monkeys, in which it multiplies with little or no cytopathic effect(1). Its presence was demonstrated by subinoculation from such systems into cultures of green monkey cells (grivet) in which characteristic cytopathic changes were induced. The agent has subsequently been demonstrated in certain lots of formolized and live attenuated poliovaccines (1,2) as well as in adenovirus vaccine. Eddy and associates showed that injection of fluids from uninoculated rhesus monkey cultures into suckling hamsters was followed on occasion by the development of sarcomas(3). These workers more recently presented evidence that SV_{40} virus in such oncogenic fluids was the responsible factor(4).

Results of serological tests suggest that the virus multiplies in human beings(1,2). Moreover, its intranasal administration to volunteers is reported by certain workers to be followed by respiratory disturbance(5,6). This manifestation was not noted by others(7), although antibody developed in the recipients. Several observations suggest that when given orally to man infection does not occur. Thus SV_{40} was not recovered from stools of patients after oral administration of Sabin poliovaccine containing high concentrations of virus and antibody did not appear in these subjects(1,2). So far attempts to extend the evidence for the capacity of the virus to infect human beings by propagating the agent in primary or stable human tissue cultures have failed. Sweet and Hilleman observed no cytopathic effect in cultures of primary human amnion, stable amnion, Chang liver, Girardi human heart, or HeLa cells(1). Hsiung reported that SV_{40} did not multiply or induce cytopathic changes in cultures of adult human kidney tissue(8) although infectivity was preserved for as long as 58 days (9). In this paper multiplication of SV_{40} associated with characteristic cytopathic effects in primary cultures of human tissues is described.

Materials and methods. SV_{40} Virus. SV_{40} strain VA 45-54 GMK 4, 6/9/61, was obtained from Dr. M. R. Hilleman. *SV_{40} Antiserum.* Heat inactivated rabbit antiserum to SV_{40} strain VA 45-54 GMK 1 with a titer of 1:625 *vs* 1000 TCD_{50} of that strain in grivet monkey kidney cultures was supplied by Dr. Hilleman(1). In neutralization tests equal volumes of 10-fold dilutions of antiserum and 1000 TCD_{50} of virus were maintained at 4°C

* Supported in part by Research Grant from U. S. Public Health Service, Department of HEW.

† Postdoctoral Research Fellow of The National Foundation.

for 2 hours and then added in a volume of 0.2 ml to grivet monkey kidney cultures along with appropriate controls.

Grivet Monkey Kidney (GMK) Cultures. Primary monolayer cultures of kidney tissue of the Green monkey, *Cercopithecus aethiops,* in 12 × 150 mm tubes were prepared from trypsinized cell suspensions and grown in Melnick's lactalbumin-yeast extract medium (10) containing 2% heat-inactivated calf serum. Cultures were maintained with medium 199 containing 2% chick serum and incubated at 37°C in a stationary position. Just prior to inoculation with SV_{40}, fresh maintenance medium was added. Two or 3 days after inoculation, this medium was replaced by one consisting of 45% Hanks' balanced salt solution, 45% bovine amniotic fluid, 5% inactivated horse serum and 5% beef embryonic extract. Medium was changed every 5 to 7 days thereafter. All media contained 50 units of penicillin, 50 μg of streptomycin and 1 μg of amphotericin B (Fungizone) per ml.

Human Fetal (HF) Cultures. Human fetuses of 2 or 3 months' gestation provided tissues for preparation of roller tube cultures. Brain, lungs, skin and muscle, heart, liver, spleen, adrenal, intestine, kidneys and testes were minced separately. Fragments of each tissue were embedded in chick plasma which was then clotted with chick embryo extract. Cultures were incubated in a roller wheel and used as soon as confluent cell sheets had formed, *i.e.,* 7-13 days after explantation.

Primary monolayer cultures of HF kidney tissue in 15 × 150 mm tubes were prepared from trypsinized cell suspensions. For the first 2-4 days after preparation, cultures were incubated in a stationary position. Thereafter they were incubated in a roller wheel. Cultures were used for experiments 1 to 4 weeks after preparation.

Growth medium for all HF cultures consisted of 90% Eagle's basal medium, modified as described by Berg *et al.*(11), and 10% fetal bovine serum (Colorado Serum Co.). Medium was replaced every 2-4 days. Roller tube cultures contained 2 ml of medium; monolayer cultures, 1 ml. Just prior to inoculation with SV_{40} virus, this medium was replaced by medium 199 containing 2% chick serum. One to 3 days later, this medium was in turn replaced by the original growth medium which was changed every 2-4 days. All cultures were incubated at 37°C.

Newborn and 3-Month Post-partum Human Kidney Cultures. Kidney tissues removed at operation from newborn and 3-month-old infants were employed for preparation of trypsinized monolayer cultures. Procedures were the same as described for HF kidney monolayer cultures. *Infectivity determinations* were performed in GMK cultures. Three cultures were used for each 10-fold dilution of viral suspension. Titers were calculated by the formula of Reed and Muench. Cultures were examined for typical CPE daily for 6 weeks after inoculation.

Methods of SV_{40} Passage in HF Cultures. Plasma clot cultures of each tissue were employed in the first passage. Two cultures were each inoculated with 0.2 ml of undiluted infected fluids from GMK cultures. The titer of the inoculum as assayed in GMK cultures was $10^{4.5}TCD_{50}/0.1$ ml. On the first day after inoculation, fluid was removed and cultures washed 3 times with 1.0 ml aliquots of neutralized Hanks' solution. Fresh medium was then added to each culture. Between days 1 and 14, the culture medium was replaced an additional 4 times; between days 14 and 31, it was replaced 5 times. Fluids of the 3rd washing on day 1, as well as fluids removed on days 14 and 31, were titered for infectivity in GMK cultures.

Results. Multiplication in Human Foetal Cells. SV_{40} was found to multiply in each of the human fetal tissues investigated (Table I). In all cases, by 31 days after inoculation, infectivity titers were recorded that exceeded the original titer by 1-3 logs. It was calculated that during this interval the original inoculum was diluted 10^{-13}× through repeated washing and changes of medium.

Because live poliovirus vaccines contaminated with SV_{40} have been administered orally, titers of SV_{40} in HF intestine were investigated in more detail. Similarly, because SV_{40} shows a cytopathic tropism for monkey kidney cells, growth in HF kidney was also studied in more detail. In these sys-

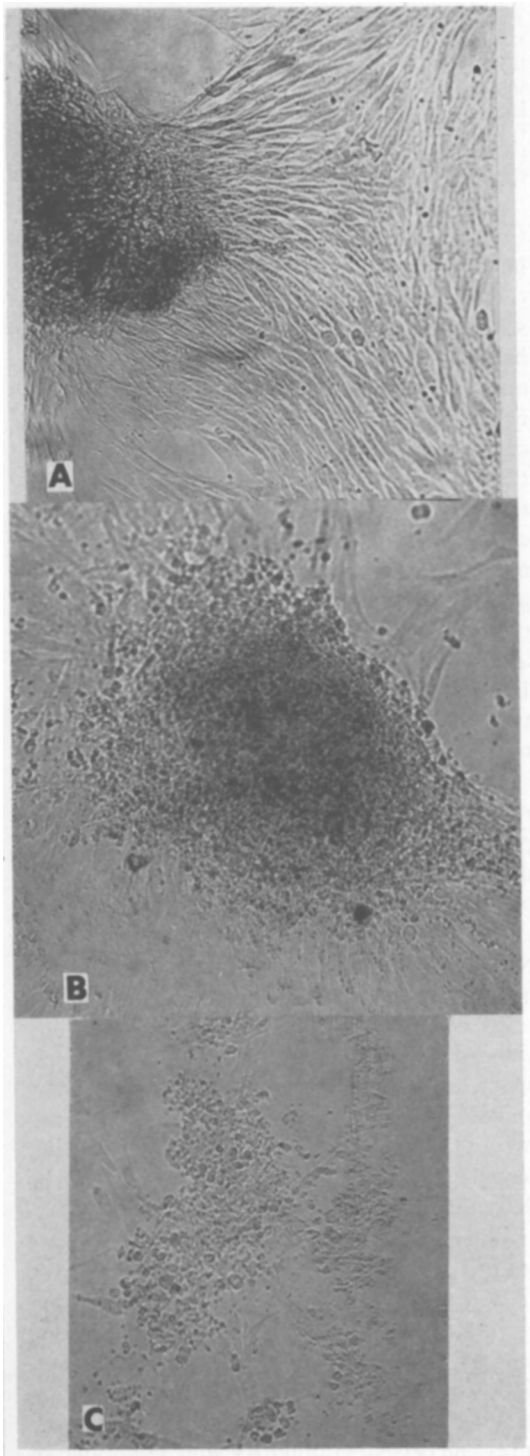

FIG. 1. Unstained HF kidney cultures on 31st day of 2nd SV_{40} passage. Mag. 149 ×. A. Uninoculated control showing largely fibroblastic monolayer. A contracted button of cells is seen in one corner. B. Inoculated culture, showing similar button as in 1A, but with rounded, heaped-up cells, many undergoing necrosis. C. Inoculated culture. Cytopathic effect similar to that in 1B in an area at the edge of the cell sheet.

TABLE I. Multiplication of SV_{40} in Human Fetal Tissues (First Passage).

HF tissue	Titer* of SV_{40} in fluid on day indicated			
	0	1‡	14	31
Kidney	3.5	1.0	≧5.5	≧5.5
Intestine	3.5	1.5	5.8	5.8
Brain	3.5	—	—	≧4.5
Skin-muscle	3.5	—	—	≧4.5
Lung	3.5	—	—	≧4.5
Liver	3.5	—	—	≧4.5
Heart	3.5	—	—	≧4.5
Adrenal	3.5	—	—	≧4.5
Spleen	3.5	—	—	≧4.5
Testis	3.5	—	—	≧4.5
Medium w/o cells†	3.5	—	2.5	1.8

* Expressed as reciprocal log dilution of the end point per .1 ml fluid. All titrations performed in GMK cultures.

† Incubated under the passage conditions.

‡ Cultures washed 3 times on this day. Titer of 3rd wash fluid given.

tems it was found that by 14 days after inoculation viral titer of supernatant fluid increased 100 times. On the 31st day, after several changes of medium, the same titers were obtained.

A second passage in HF kidney was carried out employing as inoculum fluid of the 31st day first passage. Titer of fluid on the 25th day second passage was $\geqq 10^{-6.5}/0.1$ ml after 8 changes of medium. In a third passage in HF kidney, fluid of the 31st day second passage was employed as inoculum. Infectivity titrations were not performed.

Cytopathic Changes in Human Foetal Cells. In none of the inoculated HF cultures were cytopathic changes observed during the first passage, either upon daily microscopic observation or in stained preparations of cells fixed on the 31st day after inoculation. However, changes were observed in areas of fibroblastic growth after 17-23 days in the second HF kidney passage (Fig. 1a-1c), and after 19-28 days in the third HF passage. In affected areas, rounded heaped-up cells were seen. Most of these cells later became necrotic and shrunken, with nuclear pyknosis and cell fragmentation as conspicuous fea-

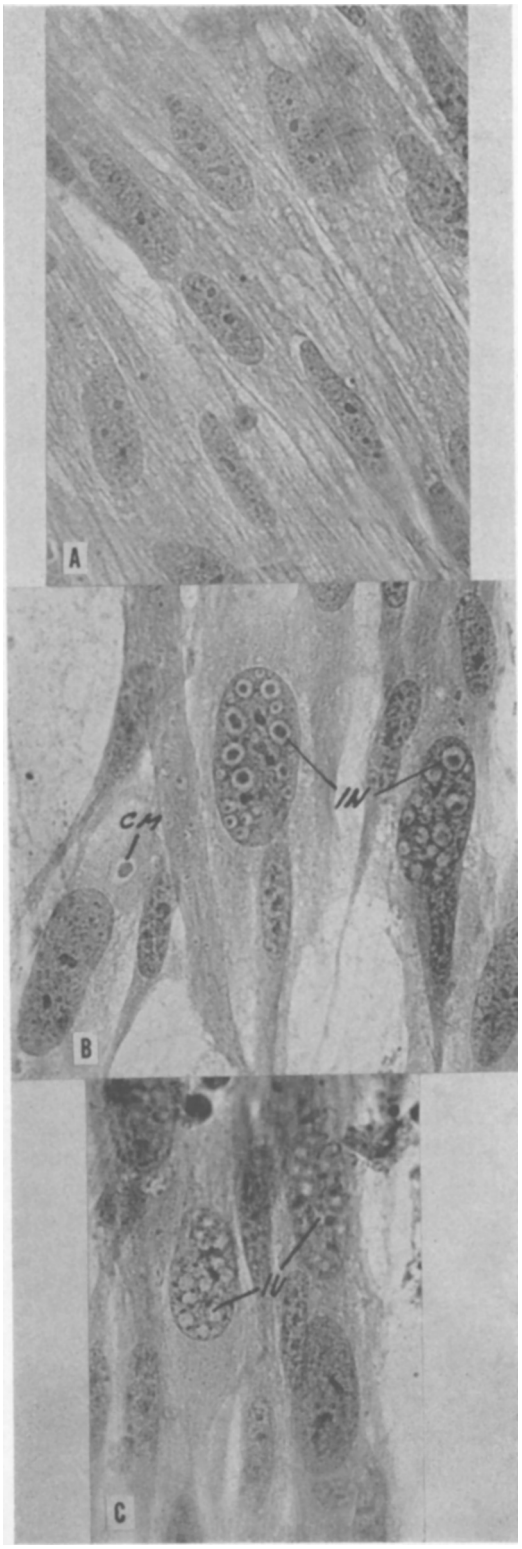

FIG. 2. HF kidney cultures on 39th day of 2nd SV_{40} passage fixed in Bouin's fluid and stained with hematoxylin and eosin. Mag. 749 ×. A. Uninoculated control, showing typical area of fibroblastic growth. B and C. Inoculated cultures, showing cytopathic changes with SV_{40}: Swollen nuclei with eosinophilic inclusions (IN), vacuoles (IV); typical eosinophilic cytoplasmic mass with clear halo (CM).

tures. Involved areas became progressively larger, presenting the appearance of multicellular layers in which the uppermost consisted mainly of degenerating elements. The heaped-up masses blended smoothly into the contiguous monolayer. This appearance suggested that heaping-up was the result of increased proliferation rather than of aggregation of cells secondary to rounding and contraction of the coagulum. Involved areas could be distinguished with the naked eye as more opaque circumscribed masses. Cytoplasmic vacuoles were rarely observed in apparently affected cells. Even 50 to 60 days after inoculation, CPE was not complete. In general the appearance of the cell population suggested that a balance was struck between cell proliferation and destruction.

In one culture at this time, groups of large, rapidly proliferating, epithelial-like cells appeared here and there throughout the predominant fibroblastic-like growth. These cells, which were easily distinguished from elements previously observed in the system, remained closely associated in an expanding mass. Subsequently within this new growth, areas consisting of 2 or more cell layers developed. Whether this phenomenon represents an early stage of cell transformation analogous to that described by Vogt and Dulbecco(12) in hamster embryo cell cultures infected with SE polyoma virus remains to be determined.

When cultures fixed on the 39th day were stained with hematoxylin and eosin, affected cells in scattered areas showed striking nuclear changes (Fig. 2a-2c). These included nuclear enlargement, intranuclear vacuolization, intranuclear eosinophilic inclusions of varying sizes, increased nucleolar-like structures, increased granularity and clumping of the chromatin. There appeared also to be an increased number of mitotic figures. The cytoplasm was free from vacuoles but occa-

sional eosinophilic bodies with clear halos were seen. Nuclear inclusions were seen in only a small proportion of cells in any one area while in others none were observed. Where nuclear changes were most prominent, many heaped-up, rounded cells were present. Large numbers of these cells were clearly necrotic, with homogeneous, deeply eosinophilic cytoplasm and pyknotic, fragmented nuclei. As in unstained material, the general impression was that of a viral infection in which stimulation of cell growth and degenerative changes were associated features. Nuclear changes induced with SV_{40} under these conditions were essentially comparable to those which Gaylord and Hsiung(13) have described in cultures of patas monkey kidney tissue infected with this agent.

In uninoculated control cultures, whether examined in the natural state or after staining, none of these changes were observed.

Multiplication and Cytopathogenicity in Cells of Newborn and Three-Month-Old Infants. Since vaccines containing SV_{40} have been administered routinely to 3-month-old infants, and, infrequently to newborn children, it seemed of interest to determine whether cells from individuals of these ages are also susceptible to the agent. When cultures of kidney tissue from newborns and infants of 3 months were inoculated with 31-day fluid from the 2nd HF kidney passage, cytopathic changes were subsequently seen that were comparable in all respects to those observed in fetal cells. Changes first appeared between 20 and 35 days after inoculation. Cultures of post-natal tissue were thus found to be susceptible to the virus after passage in HF cultures. It was, however, not determined whether passage in the latter system is a prerequisite for growth in infant kidney cells.

Cytopathogenicity for Monkey Cell Systems. When 25- or 31-day fluids from the 2nd HF kidney passage were inoculated into GMK cultures, the CPE was in general similar to that described by Sweet and Hilleman with SV_{40} in this tissue(1). The chief difference consisted in the frequency of cells with cytoplasmic vacuolization. In our experiments with GMK cultures, not more than a small proportion of vacuolated cells was seen, and many cells proceeded to necrosis without exhibiting this effect. In infected cultures, eventually the entire cell population was destroyed, but often, especially with inocula of low infectivity, CPE was not apparent until 4 or 5 weeks after inoculation.

The strain of virus employed failed to induce CPE in primary rhesus monkey kidney cultures. Various procedures were employed that might render the agent cytopathogenic in this system. These consisted in irradiation of the cells prior to inoculation (3,500 r); addition of hydrocortisone (5 μg/ml); increase in bicarbonate concentration and incubation at 32°C. No cytopathic effects were noted.

Identification of the Virus. The comparative infrequency of vacuolization in GMK cultures led to reidentification of the virus, which had been propagated throughout 2 passages in human fetal kidney cells. It was shown that specific SV_{40} antiserum neutralized CPE to titer in cultures of grivet monkey kidney.

Discussion. The demonstration of multiplication of SV_{40} in human cells associated with cytopathic change is of significance from at least two points of view. First, it extends the serologic and clinical evidence suggesting that the agent is infectious for man. For ultimate failure to demonstrate multiplication in a human cell system would leave doubt regarding its infectivity for this host. Secondly, multiplication of the virus under these conditions may provide a useful means of studying its oncogenic potentiality in populations of human cells. Analyses of this sort appear desirable since it has been suggested that the capacity of the agent to induce tumors in newborn hamsters may depend on idiosyncratic factors, while in other hosts, where these are lacking, it may exhibit no oncogenic properties.

Although not conclusive, the observations indicate that SV_{40} stimulates cell proliferation as well as inducing degenerative changes. It is common knowledge that initially a number of viruses stimulate cell division which is later followed by necrosis. Whether in this respect SV_{40} is similar or whether the cell

proliferation observed may be a prelude to subsequent transformation, possibly tending toward the malignant state, remains to be determined. Also for future study is the nature of the target cell. The present results point to the fibroblast. Whether or not this cell will prove to be the only element in which virus multiplies, it may be emphasized here that the cytopathic changes described were evident only in cells of this description.

Summary. Multiplication of SV_{40} virus has been demonstrated in primary cultures of several human tissues. The virus multiplied without cytopathic effect during the first passage in these systems. In subsequent passages in kidney cell cultures, CPE was noted which included nuclear changes, increased cell proliferation and necrosis.

The authors are indebted to Dr. Maurice R. Hilleman, Merck Inst. for Therapeutic Research, for stock SV_{40} virus, SV_{40} antiserum, and GMK cultures. We are also grateful to Mr. John Carabitses for microphotography and to Miss Anne Burket for technical assistance.

1. Sweet, B. H., Hilleman, M. R., PROC. SOC. EXP. BIOL. AND MED., 1960, v105, 420.

2. Magrath, D. I., Russell, K., Tobin, J. O., *Brit. Med. J.*, 1961, No. 5247, 287.

3. Eddy, B., Borman, G., Berkeley, W., Young, R., PROC. SOC. EXP. BIOL. AND MED., 1961, v107, 191.

4. ———, *Virology*, in press.

5. Morris, A. J., Hiatt, C. W., *Conference on Production and Testing Requirements for Live Poliovirus Vaccine*, Nat. Inst. Health, 1960.

6. Sabin, A. B., *Idem.*

7. Morris, J. A., Johnson, K. M., Aulisio, R. M., Chanock, R. M., Knight, V., PROC. SOC. EXP. BIOL. AND MED., 1961, v108, 56.

8. Hsiung, G. D., *Bact. Proc.*, 1961, 151.

9. Hsiung, G. D., Gaylord, W. H., Jr., *J. Exp. Med.*, 1961, v114, 975.

10. Melnick, J. L., *Diagnostic Procedures for Virus and Rickettsial Diseases*, 2nd Ed., Am. Pub. Health Assn, 1956, 97.

11. Berg, R. B., Rosenthal, M. S., PROC. SOC. EXP. BIOL. AND MED., 1961, v106, 581.

12. Vogt, M., Dulbecco, R., *Proc. Nat. Acad. Sci. U. S.*, 1960, v146, 368.

13. Gaylord, W. H., Jr,. Hsiung, G. D., *J. Exp. Med.*, 1961, v114, 987.

Received January 10, 1962. P.S.E.B.M., 1962, v109.

Effect of Anterior Pituitary Preparations on Rat Mammary Gland Growth.* (27247)

HENRY C. DAMM† AND C. W. TURNER
(With technical assistance of Mary E. Powell)
Department of Dairy Husbandry, University of Missouri, Columbia

A number of anterior pituitary preparations have been shown to stimulate varying degrees of lobule-alveolar mammary gland growth when injected into rats alone and with varying amounts of steroids(1,2,3). Most previous studies on experimental effects of pituitary preparations on mammary gland growth in the rat have relied on visual observation as index of glandular growth. Recent technics employing desoxyribosenucleic acid (DNA) as index of growth provide quantitative, objective measurement of growth observed normally and under experimental conditions(4).

Previous studies(5) have demonstrated mammogenic capacity of various anterior pituitary (AP) preparations in the mouse, using DNA as index of growth. Present report deals with progress in determination of mammogenic effectiveness of crude and purified AP preparations in ovariectomized rats.

Materials and methods. Sprague-Dawley-

* Contribution of Mo. Agri. Exp. Sta. Journal Series No. 2333. Approved by the Director. Supported in part by grants from the American Cancer Society and Public Health Service. Lactogen and Growth Hormone kindly supplied by the Endocrinology Study Section, N.I.H.

† Postdoctoral Research Fellow of the National Cancer Institute. Present address: Institute of Pathology, Western Reserve University, Cleveland 6, Ohio.