month observation period, suggest to us the likely possibility of depletion of "stem" cells in mice deprived of thymic tissue. The functional integrity of the thymus may indeed be necessary for permanent reconstitution.

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Received September 2, 1964. P.S.E.B.M., 1965, v118.

Growth and Cytopathic Effect of Rubella Virus in a Line of Green Monkey Kidney Cells.* (29763)

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Parkman, Buescher and Artenstein(1) and Weller and Neva(2) first described the isolation of rubella virus in cell culture systems. Cytopathic changes were, however, not observed in primary cultures of African green monkey kidney cells employed by the former who demonstrated multiplication of the agent by its capacity to interfere with ECHO 11 virus. Although Weller and Neva(2) showed that rubella virus is cytopathic for human amnion cells in primary cultures, the changes are slow to appear and only a few cells are visibly affected at a given time. With a line

* Supported in part by Office of the Surgeon General, Dept. of the Army, and sponsored by Commission on Viral Infections of Armed Forces Epidemiological Board, and in part by Research Grant E-1992, U. S. P. H. S., Department of H.E.W.

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of rabbit kidney cells McCarthy *et al*(3) were largely able to overcome these disadvantages since in this system the virus consistently induces extensive and readily observable cell damage. Nevertheless, for certain purposes it would be desirable to propagate this agent in primate cells susceptible also to a large number of other agents pathogenic for man provided the cytopathic effects of rubella virus were readily apparent. A continuous line of African green monkey kidney cells developed in this laboratory appears to fulfill this requirement as shown by results of experiments to be described in which the growth and cytopathogenicity of 3 freshly isolated strains and a stock strain of rubella virus were studied in this line of cells and, for comparison, in cultures of primary human amnion and African green monkey kidney as well as in another line of African green monkey kidney cells.

Materials and procedures. Cell cultures. The following were employed: (a) primary cultures of African green monkey cells (PGMK) purchased from Microbiological Associates, Bethesda, Md.; (b) primary cultures of human amnion cells (PHA) prepared as previously described(4); (c) line GMK, BSC-1 of African green monkey cells(5) purchased in its 39th passage from Microbiological Associates; (d) a line of African green monkey cells established in this laboratory in 1962 and here designated as line GMK, AH-1. Cells of line GMK, AH-1 have been found capable of supporting growth of several agents pathogenic for primates, *i.e.,* poliovirus, Type I, SV40, measles virus, adenovirus 12. All these agents induced typical cytopathic changes followed by cell destruction and exhibited infectivity titers comparable to those obtained in primary cultures of monkey or human renal and amnion cells. The GMK, AH-1 line was developed by Ann Holloway of the Research Division of Infectious Diseases.

Media. PGMK and PHA cultures were grown in bovine amniotic fluid medium. Eagle's medium(7) containing 10% inactivated fetal calf serum and modified as follows was employed for the growth and maintenance of monkey kidney lines: increase of arginine from 105 to 122.4 mg/l; of inositol from 2 to 4 mg/l; and of $NaHCO_3$ from 2000 to 2200 mg/l; addition of biotin 1 mg/l; substitution of $NaH_2PO_4 \cdot H_2O$ 140 mg/l for $NaH_2PO_4 \cdot 2H_2O$ and of $MgSO_4 \cdot 7H_2O$ 100 mg/l for $MgCl_2 \cdot 6H_2O$. For maintenance after inoculation of virus bovine amniotic fluid medium was used in cultures of PGMK and PHA and modified Eagle's medium containing 2% inactivated fetal calf serum in cultures of green monkey kidney lines. Final concentration of dextrose in Eagle's medium was increased to 0.15-0.20%. With this increase in sugar, cell viability and morphology were well preserved for 2-3 weeks without change of medium. Use of unmodified Eagle's medium or medium 199 with 2% inactivated fetal calf serum has also been found satisfactory. With medium 199 + 2% FCS virus yields of one log higher titer are obtained as compared to those with modified Eagle's medium.

Isolation of rubella virus. Throat washings were collected in Hanks' balanced salt solution from 3 patients seen on the first day of rash. An aliquot of 0.15-0.2 ml of washing was added one hour after collection to each of 4 or 5 cell cultures. Multiplication of virus was demonstrated in PHA, PGMK, BSC-1 cells by the interference technique and in GMK, AH-1 cells by interference and cytopathogenic effect (CPE). In testing for interference, 1000 TCD_{50} ECHO 11 virus was used in cultures of monkey cells and 500 TCD_{50} Sindbis virus (strain AR339) in cultures of PHA. These agents were inoculated one week after addition of rubella virus or of throat washings to be tested for its presence.

Stock virus. For purposes of comparison and control, a representative strain of rubella virus, the Bell strain, in its 60th passage in PHA was employed. This virus was generously supplied by Dr. F. A. Neva of Harvard Public Health School(2).

Sera. Specimens of sera were obtained from patients with typical rubella during the early acute phase and again at intervals ranging from 3 to 18 weeks afterwards. Acute and convalescent phase sera from 2 cases were provided by Dr. F. A. Neva. These sera were

known to have shown a more than 4-fold increase in rubella antibody titers as tested in PHA cultures (Dr. Neva, personal communication).

Neutralization tests. Sera were inactivated at 56°C for 30 minutes and diluted by 2-fold decrements in 0.85% sodium chloride solution. The virus suspension was diluted in maintenance medium to contain 300 TCD_{50}/0.1 ml and mixed with equal volumes of each serum dilution. One-tenth ml of pooled normal rabbit serum (PNRS), employed soon after collection or after storage at −70°C was added to each mixture to enhance the neutralizing effect of anti-rubella virus antibody(2,3). Mixtures were kept at 4°C for 30 minutes, then at 37°C for 1 hour in an incubator. During this time the tubes were frequently shaken. To each of 3 GMK, AH-1 cultures 0.1 ml of each mixture was added. Cultures were examined for cytopathic effect and final readings recorded on the 12th day. Medium was not changed during this period.

Results. Isolation of virus in cultures of GMK, AH-1 cells. Throat washings from 2 patients (Levine and McCombs) were added to cultures of GMK, AH-1 cells. CPE was observed after 15 days and gradually increased thereafter. During 3 subsequent passages cytopathic changes first occurred after about the same interval (13-15 days). In the 5th passage CPE appeared on the 7th day. By the 12-15th day most of the cells were visibly affected or had been destroyed.

The Levine strain and another strain, Shapiro, were also isolated in PHA cultures from the same stocks of throat washings using the interference method. Strain McCombs was independently isolated by this technique in PGMK cultures. When the agents so isolated were added to GMK, AH-1 cultures, CPE was first noted after about 15 days. After 3-4 passages in these cells, however, CPE was seen on about the 7th day in each case. The same decrease in "incubation" period occurred with the stock Bell strain when passaged in this cell line in which it induced the same kind of cytopathic changes.

Cytopathic changes induced in GMK, AH-1 cells. Cellular injury was first manifested by rounding of cells and the appearance of scattered masses of refractile material composed of fragments varying in size and presumably consisting of cellular débris. At about the same time or a little later groups of cells originally epithelioid in appearance became elongated or stellate presenting the aspect of fibroblasts. Subsequently these cells disintegrated or detached from the glass. In cultures infected with virus that had received 5 or more passages in this system, 80% of the cell population was destroyed by the 15th day when nearly all the remaining cells exhibited the spindle-like or stellate form (cf. Fig. 1 a, b, c, d).

At appropriate times, infected cultures were fixed, embedded in collodion and stained with hematoxylin and eosin(8). All the changes just described were apparent and in addition intracytoplasmic eosinophilic inclusion bodies, varying in size and shape and surrounded by a clear halo, were seen in large numbers. Since similar inclusions were infrequently encountered in examination of uninfected control cells, their significance in the infected cultures is unclear. However, the very striking difference in their numbers suggests that if not specifically related to the virus, their formation is stimulated by its presence. Nuclear changes resembling those described by Weller and Neva(2) were not observed (cf. Fig. 2, a, b).

Optimal conditions for demonstration of CPE. It was determined by varying the amount of calf serum in the medium that concentrations exceeding 5% markedly suppressed CPE. A concentration of 2% was found most satisfactory and has been adopted as routine. Frequent renewal of maintenance medium tended to repress CPE. At present, therefore, medium is not changed after virus is added either in infectivity or neutralization tests.

Viral multiplication. The virus multiplied actively in GMK, AH-1 cells as shown by results of infectivity titrations of fluids from cultures at successive passage levels. For this purpose cell cultures were employed to which were added 0.1 ml aliquots of dilutions of infected fluid increasing by a factor of 10. Maintenance medium was used as diluent. Titers based upon readings of CPE taken on

the 12th day were calculated by the method of Reed and Muench(9). With McCombs and Shapiro strains titers of 10^6-$10^{6.7}$/ml were recorded. Repetitions of several of these titrations employing fluid from cultures representing several different passage levels

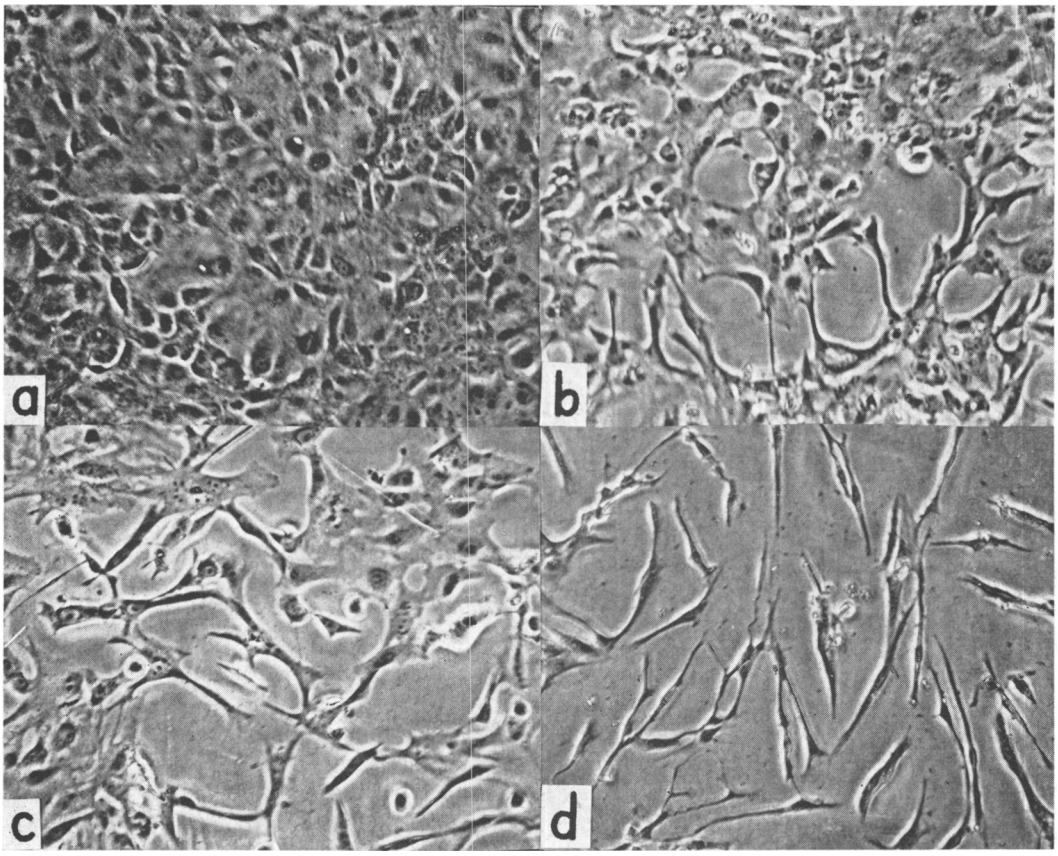

FIG. 1. Cytopathic effects of rubella virus (McCombs strain, 13th passage, inoculum = 100 TCD_{50}) in cultures of GMK, AH-1 cells. Unstained. Mag. × 175. (a) Control: uninoculated; (b) 8 days after inoculation; (c) 12 days after inoculation; (d) 21 days after inoculation.

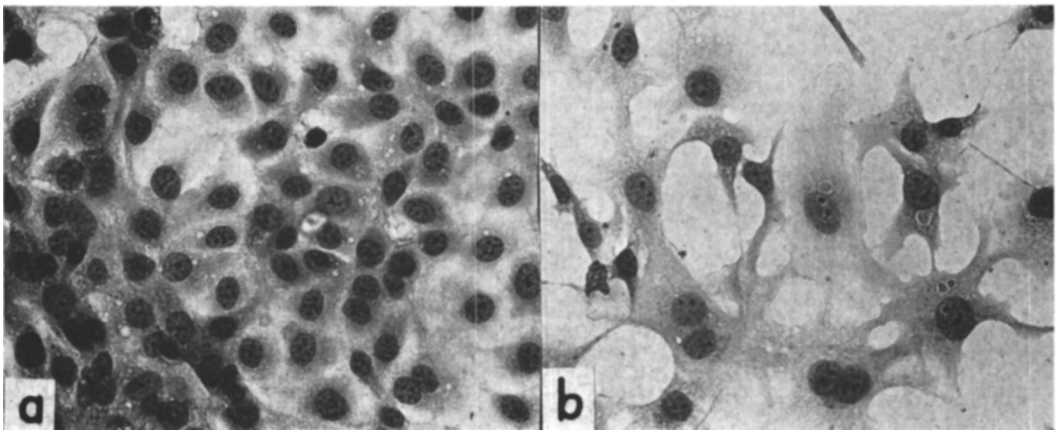

FIG. 2. Cytopathic effects of rubella virus (McCombs strain, 13th passage, inoculum = 100 TCD_{50}) in cultures of GMK, AH-1 cells. Stained with H and E. Mag. × 245. (a) Uninoculated control; (b) 12 days after inoculation.

TABLE I.

Patient	Serum	Antibody titer*
McCombs	Acute	1/8
	Conv	1/128
Blecatsis	Acute	1/2
	Conv	1/1028
Sheldon	Acute	1/2
	Conv	1/64
Chaunyt	Acute	1/2
	Conv	1/128
Liebermant	Acute	1/4
	Conv	1/128

* Expressed as final serum dilution endpoint.
† Sera provided by Dr. F. A. Neva.

yielded almost identical results.

Growth of rubella virus in other cell culture systems. Rubella virus has been isolated and passed several times in PGMK, PHA and BSC-1 cultures without obvious CPE. Use of modified Eagle's medium with 2% fetal calf serum in the primary cell cultures instead of the usual growth medium failed to demonstrate cell injury. The presence of virus was detected either by the interference technique or subinoculation to GMK, AH-1 cultures. Rubella virus grown in GMK, AH-1 cells was shown to multiply in all the other systems but produced CPE in none.

Results of neutralization tests. That this line of monkey cells provided a convenient means of performing serum neutralization tests based on endpoint readings of CPE is shown by results summarized in Table I. In these experiments the McCombs virus was employed and the final readings were made on the 12th day, *i.e.,* the 4th day after the first appearance of CPE.

Some properties of rubella virus propagated in GMK, AH-1 cells. Strain McCombs from the 8th passage in GMK, AH-1 cells was used in experiments to determine its resistance to ether, its capacity to hemagglutinate erythrocytes of several species, to induce hemadsorption and to pass filters of graded pore size.

To a suspension of virus in maintenance medium (infectivity titer $10^{4.5}TCD_{50}$/ml) an equal volume of ethyl ether was added and the mixture allowed to stand for 24 hours at 4°C. After removal of ether by evaporation aliquots of the undiluted and diluted fluid were tested for infectivity in cultures of GMK, AH-1. None was detected.

In the hemagglutination tests 0.5% suspensions in physiological salt solution of fowl, rabbit, guinea pig, sheep, rhesus and African green monkey, rat, mouse and bovine red blood cells were used. Equal volumes of the cell suspension and of fluid from GMK, AH-1 cells infected with the virus (infectivity titer $10^{5.5}TCD_{50}$/ml) were mixed. The mixtures were allowed to stand at 4°C and at 37°C respectively. Readings were made after 1 hour and 24 hours. No hemagglutination was noted in any instance. In attempts to demonstrate hemadsorption erythrocytes of the species mentioned above were added to GMK, AH-1 cultures infected 15 days previously with the McCombs strain. After standing for 1 hour at 4°C the cultures were washed with Hanks' solution and were examined. No hemadsorption was seen.

In filtration experiments Millipore® filters (obtained from Millipore Filter Corp., Bedford, Mass.) were used. Infected tissue culture fluids containing $10^{5.2}TCD_{50}$/ml McCombs virus were passed through filters of 220 mμ, 100 mμ, and 50 mμ pore size. In each case, 5 ml of virus suspension was used. Infective virus was demonstrated only in the 200 mμ filtrate.

The various characteristics of the McCombs strain of virus thus determined are consistent with those described by others for rubella virus(1,2).

Comment. Since the foregoing experiments were performed, we have continued to employ the GMK, AH-1 line in studies of rubella virus, in which it has proven to be a reliable and convenient tool. From the biological viewpoint its susceptibility to the cytopathic effect of the virus, as contrasted with the resistance of primary cultures of African green monkey kidney cells as well as those of the BSC-1 line, presents an interesting subject for further investigation.

Summary. Recently isolated strains of rubella virus as well as a stock strain regularly induce characteristic, easily recognized, cytopathic changes when adapted to a line of African green monkey cells established in

this laboratory. The reaction of this cell line to infection provides a reliable and convenient means for titration of viral infectivity and assay of specific virus-neutralizing antibody.

The author acknowledges the helpful guidance of Dr. John F. Enders in preparation of this manuscript.

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Received September 2, 1964. P.S.E.B.M., 1965, v118.

Osteolathyrism in the Hamster: Pathological and Chemical Changes Produced by β-Aminopropionitrile in Connective Tissues.* (29764)

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The administration of β-aminopropionitrile (BAPN), a potent lathyrogen, has been shown to increase the soluble collagen fractions obtainable from chick embryos(1), from mammalian skin(2,3) and from sponge biopsy connective tissue from rats(4). Since a soluble component of elastin has been reported by Adair *et al*(5), it seemed of interest to determine whether its level also can be affected by BAPN, especially since lathyrogens are known to damage the medial elastic fibers in aortae of growing animals(6-8). Because we have never been able to find any elastin in sponge implant tissue from rats and because the cheek pouch of the hamster has such great extensibility and apparent elasticity, we have studied the effect of BAPN on the growing hamster, especially its effect on collagen and elastin fractions of the pouch.

Materials and methods. Male, Golden Syrian hamsters, 18-28 days of age, were fed finely milled Rockland Rat Stock Diet containing varying amounts of added BAPN fumarate. Unsupplemented Stock Diet was given control groups. All diets were fed *ad libitum*. The animals were sacrificed after varying periods and their mandibles and femurs were examined for gross skeletal lesions. The aorta and one cheek pouch from each animal were dissected out for microscopic examination, fixed in neutral formalin, embedded in paraffin, sectioned and stained with Hornowski's combined elastic and Van Gieson stains. The second cheek pouch was removed and frozen until chemical fractionation could be undertaken (1 to 8 days).†

Soluble and insoluble collagen fractions from cheek pouches were obtained by modifications of methods used by Levene and Gross (1), Jackson(9) and Dasler *et al*(4). The elastin-like fractions were obtained by an adaptation of the procedure of Adair *et al* (5). All fractions were evaporated to dryness at 105°C in an air jet, were hydrolyzed in sealed tubes with 6 *N* HCl at 104°C for 20 hours, and were neutralized with 2.5 *N* NaOH. After filtration and dilution, they were analyzed for hydroxyproline by the method of Neuman and Logan(10). The hy-

* This investigation was supported by USPHS Research Grant AM-01427 from Nat. Inst. of Arthritis and Metab. Dis.

† Pouches frozen longer than 8 days were not used. We have found that rat sponge biopsy tissue yielded consistent soluble collagen values when the sponges were frozen up to 8 days.