allantoic fluid volume of embryonated eggs by dilution of RISA is described. Experimental porphyria in chick embryos results in a marked increase in allantoic fluid volume.

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Studies on Propagation of the Col SK Group of Viruses in Various Tissue Culture Media.* (23413)

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Cultivation of Col SK virus *in vitro* on various cell substrates began with the early work of Jungeblut and Sanders(1,2). Explants of minced embryonic tissues, suspended under controlled oxygen-carbon dioxide tension in ox serum ultrafiltrate, were used for this purpose. The virus multiplied, to varying extent, on brain from mice and guinea pigs or on whole chick material; non-nervous embryonic tissue also supported a basic level of neurotropic virus growth. The virus yield was approximately the same from cells as from culture fluid. While the procedure did not permit demonstration of cytopathogenic effects, Huang(3) discovered that there was less glycolysis and acid production in the infected medium than in non-infected controls. The above results were confirmed by Shean and Schultz(4) who studied the mouse explant requirements of Col SK and MM virus in comparison with those of Theiler's GD VII and FA virus. Growth of MM virus in various extraneural tissues of mice or hamsters was later reported by Chambers, Smith and Evans(5,6), by Evans and Chambers(7) and by Fabiyi(8).

Only sporadic attempts have been made to grow viruses of the Col SK group in tissue cultures of embryonic or adult cells from man or monkey, utilizing modern methods of tissue cultivation. Col SK virus has been reported as growing on cynomolgus monkey testicular cell cultures(9) but the virus apparently does not multiply on cynomolgus monkey kidney (10); growth of MM virus has also been obtained in cultures of testicular cells from rhesus monkeys(11) or from man(6) and, in addition, on human fetal fibroblasts(12). Barski and Lamy(13) reported that Mengo virus multiplies abundantly in tissue cultures of kynocephalus monkey kidney cells, inducing characteristic cytopathogenic effects after 2 days and causing complete destruction of the culture within 4 days; the virus also grew on embryonic human kidney tissue.

Little is known about growth of viruses of the Col SK group on tumor cells. Among malignant mouse cell cultures, L (Earl) cells are said to support non-cytopathogenic growth of EMC virus(14), but Col SK virus failed to multiply on 2 transplantable mouse carcinoma or sarcoma cells studied by Kret (9). On the other hand, mice bearing the Ehrlich ascites tumor are apparently more susceptible to infection with Mengo virus than are normal mice(15); growth of EMC virus in Ehrlich ascites tumor transplants, with marked oncolysis, was reported by Levy and Snellbaker(16). Quite recently, multiplication, with cytopathogenic effects, was described for EMC virus by Eagle *et al.*(17) in KB cell tissue cultures, a human epider-

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moid carcinoma of the floor of the mouth.

The above review suggests that there may be certain differences in growth requirements of the several strains of the Col SK group on fibroblastic or epithelial cell substrates. To provide more information on the subject we have investigated comparatively the cellular responses of L cell, HeLa cell and KB cell tissue cultures to infection with Col SK, MM, F, EMC and Mengo virus.

Materials and methods. The Col SK, MM and F strains were available in this laboratory as mouse brain passage virus. The EMC strain (rat brain passage) and the Mengo strain (mouse brain passage) were kindly supplied by Dr. Joel Warren; we are also indebted to Dr. K. Habel for sending us a sample of the EMC strain grown in L cell tissue culture. L cells were obtained, through the courtesy of Dr. M. Holden, from Dr. Earl. HeLa cells were generously supplied by Dr. J. Syverton. We owe thanks to Dr. H. Eagle and to Dr. B. Mandel for providing us with KB cells. *L cells* were grown, at 37°C, in 200 cc screw-cap square bottles in a medium consisting of 40% horse serum (supplied by the New York City Health Dept.), 40% Hanks balanced salt solution and 20% chick embryo extract, plus antibiotics (200 units penicillin and 100 γ streptomycin per cc). After 7-10 days growth, the cells were trypsinized (0.5% Difco trypsin in Hanks solution for 30 minutes at 37°C) and suspended (200,000 cells per cc) in Hanks solution containing 20% horse serum and 10% chick embryo extract. *HeLa cells* were grown in bottles in a medium consisting of 20% horse serum, 10% human serum and 70% Hanks solution containing 0.1% yeast extract. After 4 days growth the cells were trypsinized and suspended (100,000 cells per cc) in Scherer's maintenance solution containing 10% horse serum. *KB cells* were grown in bottles in Hanks solution containing 10% horse serum, 0.5 cc lactalbumin hydrolysate and 0.1% yeast extract. After 4 days growth the cells were trypsinized and suspended (100,000 cells per cc) in Scherer's maintenance solution with 10% horse serum. All cell suspensions were then distributed in 0.5 ml volumes into small rubber-stoppered stationary tubes and 2-day-old sheets of cells, after replacing the medium, were ready for infection.

The procedures employed gave excellent growth of all cell lines and the sheets could be maintained in tubes for at least 10 days in good condition. The virus inoculum usually consisted of 0.1 cc of various dilutions which was added to a volume of 0.4 cc fluid in each tube. The infected tubes were examined daily for cytopathogenic effects over a period of from 5 to 7 days. Positive tubes were harvested at the peak of cytopathogenicity and the culture fluid was transferred to new tissue culture tubes for a variable number of serial passages; potency of the virus was determined by titration in tissue culture or in mice.

Results. It was found that EMC and Mengo virus grew well on L cells, HeLa cells and KB cells; F virus multiplied only on L cells. None of the 3 substrates supported any growth of Col SK or of MM virus despite repeated attempts to adapt these strains by serial blind passage of either culture fluid or of cells; in fact, Col SK virus was lost, as a mouse-pathogenic agent, between the second and third L or HeLa cell culture transfer. Equally unsuccessful were efforts to obtain multiplication of Col SK virus on HeLa cell cultures which had been pre-treated with hyaluronidase. By contrast, virus titers of considerable height, with full cytopathogenic effects, were realized with EMC, F and Mengo virus on L cells and with EMC and Mengo virus on KB and HeLa cells (Table I).

More detailed experiments were carried out with EMC virus grown on L cells or on HeLa cells. Since transfers were made with relatively high dilutions (10^{-3}) of tissue culture fluid, it may be estimated that the original inoculum had been eliminated after the first 3 serial passages. The virus in initial passages reached a potency of between 10^3 and 10^4 TC ID_{50} per 0.1 cc culture fluid and this titer was maintained over 12 subsequent serial passages; however, comparison of infectivity in tissue culture and in mice indicated that EMC virus grown for 10 serial passages in HeLa tissue culture had suffered a marked loss of its mouse-pathogenicity (Table II).

TABLE I. Growth of Several Strains of the Col SK Virus Group in Various Tissue Culture Substrates.

Virus strain source	L cells					HeLa cells					KB cells				
	Inoculum		No. of passages carried	Cyto-patho-genicity	Virus titer* $TCID_{50}$ (per 0.1 cc)	Inoculum		No. of passages carried	Cyto-patho-genicity	Virus titer* $TCID_{50}$ (per 0.1 cc)	Inoculum		No. of passages carried	Cyto-patho-genicity	Virus titer* $TCID_{50}$ (per 0.1 cc)
	1st passage	Subsequent passages				1st passage	Subsequent passages				1st passage	Subsequent passages			
Col SK 610th mouse passage	10^{-2} 10^{-3}	undil. 10^{-1} 10^{-2} 10^{-3}	4	—	0	10^{-1} 10^{-2} 10^{-3}	undil. 10^{-1} 10^{-2} 10^{-3}	7	—	0	10^{-2}	10^{-1}	2	—	0
MM 260th mouse passage	10^{-2} 10^{-3}	undil. 10^{-1} 10^{-2} 10^{-3}	3	—	0	10^{-1} 10^{-2} 10^{-3}	undil. 10^{-1} 10^{-2} 10^{-3}	2	—	0	10^{-2}	10^{-1}	2	—	0
F 25th mouse passage	10^{-2}	10^{-1} 10^{-2} 10^{-3}	4	+	10^{-2}	10^{-1} 10^{-2} 10^{-3}	undil. 10^{-1} 10^{-2} 10^{-3}	4	—	0	10^{-2}	10^{-1}	2	—	0
Mengo 25th mouse passage	10^{-2} 10^{-3}	10^{-1} 10^{-2} 10^{-3}	4	+	$>10^{-3}$	10^{-2}	10^{-1} 10^{-2} 10^{-3}	3	+	10^{-2}	10^{-2}	10^{-1} 10^{-2}	3	+	10^{-3}
EMC 15th rat passage	10^{-2} 10^{-3}	10^{-1} 10^{-2} 10^{-3}	2	+	10^{-2}	10^{-2}	10^{-1} 10^{-2} 10^{-3}	3	+	10^{-3}					
EMC L cell passage	undil. 10^{-1} 10^{-2}	10^{-1} 10^{-2} 10^{-3}	5	+	$10^{-4.5}$	undil. 10^{-1} 10^{-2}	10^{-1} 10^{-2} 10^{-3}	12	+	$10^{-4.5}$	undil.	10^{-1} 10^{-2}	3	+	10^{-3}

* Harvest from last passage indicated.

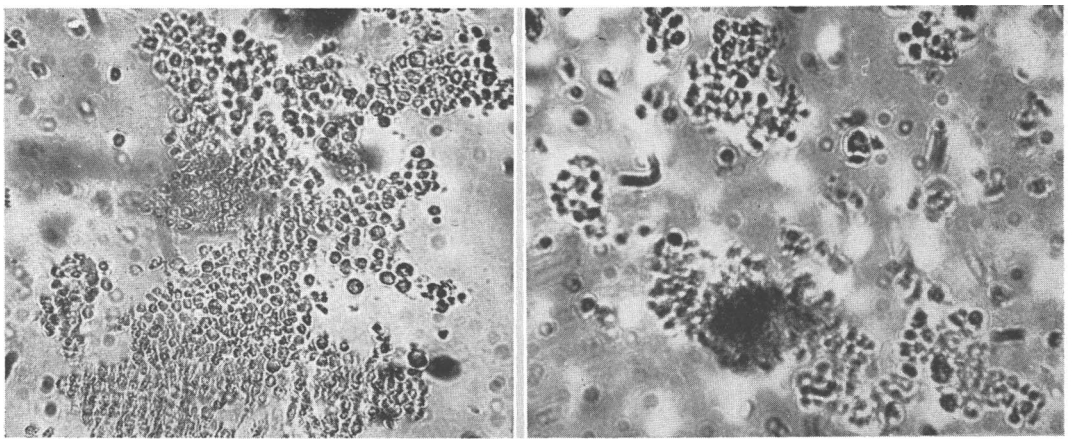

FIG. 1. *Left*—L cell culture infected with L EMC tissue culture virus (V passage). *Right*—HeLa cell culture infected with HeLa EMC tissue culture virus (V passage).

The virus grew to maximum titer in L and in HeLa cell culture tubes within 1-4 days, depending on size of inoculum, and cytopathogenicity became evident within 18-72 hours after inoculation of the sheets. In the early stages, individual cells showed rounding up with increased granularity; later extensive disintegration of the entire cell layer occurred, followed occasionally by detachment of the sheet. While similar in some respects, the over-all picture differed clearly from that produced by the poliomyelitis viruses on HeLa cells. The cytopathogenic effects of EMC virus, in its fifth serial tissue culture passage on L or HeLa cells, are illustrated in Fig. 1.

The process of adsorption and of subsequent multiplication of EMC virus on HeLa cells was studied more closely in the following experiment: A layer of approximately 1 million HeLa cells, on the 7th day of its growth in a bottle, was washed 3 times with Hanks solution and the medium was replaced with 9 cc of maintenance solution containing 10% horse serum. The bottle was then infected with 1 cc undiluted EMC virus tissue culture fluid (IV HeLa bottle passage), resulting in a 10-fold dilution of the virus. The seed virus had a potency of about 10^5 TC ID_{50} per 0.1 cc of fluid providing a ratio of 1:1 between number of susceptible cells and number of infectious particles. Immediately after inoculation a sample of 0.2 cc was withdrawn from the bottle and the lost fluid replaced by 0.2 cc of medium; the bottle was then incubated and another sample of 0.2 cc was withdrawn after 30 minutes, with replacement of the medium. After an interval of 2½ hours from the beginning of the experiment the fluid of the bottle was drained, the cell layer was washed 3 times with Hanks solution and covered with 10 cc of fresh medium, and the bottle was incubated again. Considering this time of the experiment as the zero point of multiplication with subsequent release of active virus particles, samples of 0.2 cc each were removed after 2, 4, 6, 24, 30 and 72 hours, replacing the lost fluid at each interval with 0.2 cc of medium. All samples were then titrated in HeLa tubes and the virus titers expressed in ID_{50} doses per 1 cc of fluid. The data are shown graphically in Fig. 2. It is clear that there occurs in the culture fluid an almost instantaneous loss of nearly 2 logs of virus upon first contact with cells, followed by a further reduction of an-

TABLE II. Comparison of Infectivity of EMC HeLa Tissue Culture Virus for HeLa Cells and for Mice.

HeLa passage	Infectivity in HeLa TC ID_{50} (per .1 cc fluid)	Infectivity in albino mice (10-12 g) ID_{50} intracer. test	ID_{50} intraper. test
I	$10^{-3.5}$	10^{-6}	10^{-8}
XI	$10^{-4.5}$	10^{-6*}	10^{-6}

* Some mice developed paralysis after greatly prolonged incubation periods (10-14 days) following inj. with 10^{-7} dilution.

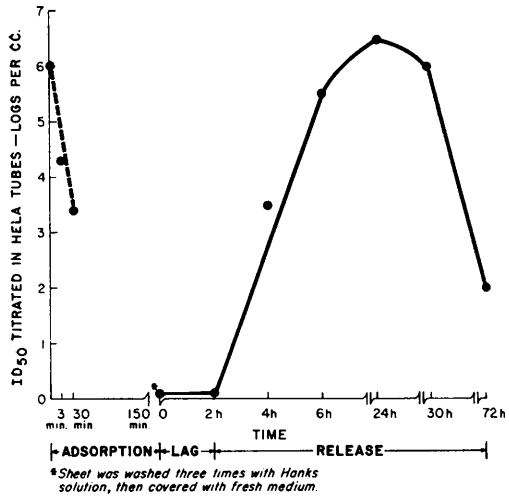

FIG. 2. One step growth curve of EMC tissue culture virus in a bottle of HeLa cells.

other log of virus within the next half hour. Counting from the zero point of virus growth, evidence of multiplication becomes apparent, after a lag period of at least 2 hours, at the 4 hour interval; thereafter the amount of released virus increases rapidly over the next 2 hours, reaching a peak of 6.5 logs at 24 hours incubation. The increments in virus release were in good agreement with synchronous observations on the appearance of cytopathogenic changes over the same period of time. Once the acme of growth had been attained, early deterioration of the virus sets in and the titer drops precipitously to 10^{-2} at the 3-day interval. A similar lability of the virus is noted in making dilutions of infected culture fluids which must be kept on ice to avoid loss of potency. The curve given in Fig. 2 indicates that the entire cycle of virus adsorption, multiplication, and decay in tissue culture is extraordinarily fast, duplicating what is known of the progress of infection in susceptible animals.

To study the formation of hemagglutinin in the tissue culture medium, EMC virus was grown for several consecutive passages on HeLa cell sheets covered with a medium containing 10% horse serum which had previously been absorbed with human O and sheep erythrocytes so as to remove the natural agglutinin for these cells. Hemagglutination tests, using human O red cells as well as sheep red cells, were then carried out with tissue culture fluid harvested at various intervals after infection. The technic employed in these tests was similar to that previously described for hemagglutination tests with mouse brain virus(18), utilizing K veronal buffer as diluent for culture fluid and red cells. As will be seen from Table III, hemagglutinin—at maximum titer of between 1:2 and 1:4—appeared in the culture fluid after 24 hours incubation, with a rapid decline setting in at later intervals. In other words, formation of hemagglutinin paralleled roughly release and decay of infectious virus particles, except for a slight delay during the first 8 hours after infection. The ratio between hemagglutinin titer in tissue culture (1:2) and that of infected mouse brain (1:640) approximately agrees with the proportional difference in infectivity titers of tissue culture virus ($10^{-5.5}$) and of mouse virus (10^{-8}-10^{-9}). Hence there is good evidence that the hemagglutinin of EMC virus is actually identical with the infectious particle. This conclusion is further supported by the fact that HeLa cell tissue culture fluid, in its third serial transfer following inoculation with Col SK virus, failed to hemagglutinate and to infect mice. Therefore, there is no reason to assume that Col SK virus may have a precursor, or incomplete form which is non-infectious but which hemagglutinates.

Identification, by serological methods, of the EMC tissue culture virus grown on HeLa

TABLE III. Hemagglutinin Formation by EMC Virus Grown in HeLa Tissue Cultures.

HeLa passage	Interval since infection of culture	Hemagglutinin titer	
		Human O cells	Sheep cells
I	3 days	1:4	
II	3 "	1:4	1:2
III	3 "	1:4	1:2
IV	8 hr	0	
"	24 "	1:4	
"	72 "	1:2–1:4	
"	96 "	0	
Control: uninoculated medium		0	0
EMC virus mouse brain		1:320–1:640	1:320–1:640

cells was readily accomplished by means of neutralization and hemagglutination-inhibition tests, using a Col SK immune serum and immune sera against the 3 prototypes of poliomyelitis virus; we are indebted to Dr. H. Wenner for making available to us the poliomyelitis typing sera. Specific neutralization as well as hemagglutination-inhibition occurred with infected tissue culture fluids harvested from remote passages, (IV, VIII) and the antiviral potency of the Col SK antiserum used in these tests compared closely with that observed in similar tests with the parent EMC mouse-passage virus. Thus, the neutralization index of Col SK serum was 1:1000 as determined against 100 ID TC_{50} of virus in HeLa tissue culture or against 100 ID_{50} of mouse virus in mice (i.p. test); similarly, 2-4 hemagglutinating units of tissue culture or of mouse virus were completely inhibited by a 1:1000 dilution of the same serum. No neutralization or hemagglutination-inhibition was observed with the poliomyelitis sera.

Discussion. It is clear that different strains of the Col SK group of viruses have different growth requirements when tested in 3 tissue culture media containing mouse or human cells. Thus, whereas L cells, HeLa cells and KB cells supported cytopathogenic growth of EMC and Mengo virus, Col SK and MM virus failed to multiply on the same substrates, and F virus occupied an intermediate position by growing only on L cells; multiplication of EMC virus on KB cells had previously been reported by Eagle *et al.*(17). It is not unusual for individual strains belonging to the same virus group to differ in their ability to propagate in a given tissue culture medium, and the fact itself lends weight to the authenticity of the isolates. It is of interest that HeLa sheets inoculated with non-cytopathogenic Col SK virus were found fully protected against infection with cytopathogenic EMC virus when first and second tissue culture passages were employed which still yielded mouse-pathogenic Col SK virus; no such protective effects, however, could be demonstrated with subsequent serial transfers of Col SK virus which were non-pathogenic for mice. More information on interference phenomena between EMC virus and other viruses in HeLa tissue culture will be reported elsewhere.

The data dealing with the growth curve of EMC virus on HeLa cells are in harmony with similar observations established for other virus-cell systems. The process begins with almost instantaneous adsorption of large amounts of virus on the susceptible cell and is followed, after a short eclipse period, by a rapid increase of infectious virus particles which are released from the injured cell into the ambient fluid. Of special interest is the fact that HeLa cells are also capable of generating viral hemagglutinin, in proportion with the number of infectious units. Heretofore, hemagglutinin production by viruses of the Col SK group in infected animals has been observed only in nervous, and not in extraneural tissues(19). The definite cytopathogenic action and hemagglutinin production of EMC virus in HeLa tissue cultures offer new possibilities for the application of relatively simple serological *in vitro* methods to epidemiological surveys of infections caused by this group of viral agents.

Summary and conclusions. Results of a comparative study of the cell responses induced by several strains of the Col SK group of viruses in various tissue culture substrates are reported. L cells, HeLa cells and KB cells supported cytopathogenic growth of EMC and of Mengo virus, F virus grew only on L cells, and Col SK and MM viruses failed to grow in any of the 3 substrates. HeLa tissue cultures inoculated with EMC virus, in remote serial passage, were infectious for HeLa cells and for mice at an ID_{50} of about 10^5 per 1 cc of culture fluid and had a hemagglutinating titer of between 1:2 and 1:4. Data permitting the establishment of a growth curve of EMC tissue culture virus on HeLa cells are submitted.

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Induced Tolerance and "Homologous Disease" in X-Irradiated Mice Protected with Homologous Bone Marrow.* (23414)

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Several species of mammals can be protected against otherwise lethal doses of whole body X-irradiation by post-irradiation transfusion of bone marrow or spleen. Protection is associated with rapid recovery of the otherwise depleted or destroyed hematopoietic tissues as a result of reseeding and repopulation with cells of the transfused marrow, even if the marrow is of homologous or heterologous origin(1-6).† Mice protected against otherwise lethal doses of irradiation with homologous marrow are thereby rendered tolerant of skin grafts as well as hematopoietic tissue of the marrow donor antigenic type(7,8). Such tolerance of homologous skin and hematopoietic tissue can be abolished by introduction into the tolerant mouse of isologous lymph node or spleen cells from an unirradiated mouse of the same strain(9). The closeness of genetic relationship of the marrow donor and irradiated recipient has a great effect upon both degree of initial irradiation protection and duration of survival(8,10). In otherwise lethally irradiated mice, marrow from guinea pigs or rabbits conferred insignificant protection(8). Rat marrow gave significant but short-term protection. Any type of mouse marrow gave excellent 21-day protection, beyond which isologous marrow gave permanent protection but foreign-strain mouse marrow gave significant late mortality characteristically between the 21st or 30th day and the 60th day post-irradiation. Late mortality was more severe when the foreign strain marrow came from a donor of a different Histocompatibility - 2 genotype (A into C_{57}, Dba into A, A into CBA) than when it came from a donor of the same H-2 genotype (C_3H into CBA) as the recipient. With homologous marrow from an F_1 hybrid into the irradiated parent strain, 2 groups showed excellent pro-

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† Autologous—from same individual; isologous—from another individual of same inbred strain (presumably same genotype) or an identical twin; homologous from an individual of different genotype but of same species; heterologous—from a different species.