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Serial Propagation of 3 Strains of Rabbit Fibroblasts; Their Susceptibility to Infection with Vaccinia Virus.* (22707)

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Tissue culture technics have been employed frequently for the propagation of viruses *in vitro*(1,2). There have been, however relatively few studies that attempt to elucidate in a simplified system which is potentially provided by tissue culture many fundamental problems regarding virus multiplication. To approach problems of this type in a definitive manner, it is necessary to have a continuous supply of uniform experimental material such as is provided by cultures of mammalian cells which can be propagated serially under controlled conditions. Cultures derived from different sources may vary considerably in their viral range and in their response to viral infection in a fashion analogous to laboratory animals. This indicates the need for cultures of more than one cell type as well as cells derived from a variety of animals. These studies were undertaken to investigate the possibility of establishing *in vitro*, strains of fibroblasts derived from rabbit tissues and to compare their relative susceptibilities to infection with vaccinia virus.

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TABLE I. Composition of Cell Culture Media.

Medium	Composition* (V/V) %	%	%
CF	5 CEE	10 NHS	85 V-614
F7		10	90
F15	5 BEE	10	85
F17	10 CEE	20	70
F21	5	20	75
705	5	20	75 703

* The following abbreviations are employed: CEE = chick embryo extract; BEE = beef embryo extract; NHS = normal horse serum; V-614 = solution of Fischer *et al.*(5); 703 = solution 703 of Healy *et al.*(6).

Materials and methods. Media. The composition of the media employed for propagating cells is indicated in Table I. All media were sterilized by filtration through Selas filters of 02 porosity. Chick embryo extract (CEE) and beef embryo extract (BEE) were prepared as described previously(3). *Establishing cell cultures on glass.* Cell cultures were initiated in roller tubes according to the method of Swim and Parker(4). When growth in the roller tubes was well established, the cells were dislodged from the glass by treating with 0.02% trypsin (Nutritional Biochemicals Corp., 2 x crystallized—50% Mg SO_4) in V-614 solution(5) or in solution 703 (6), pH 7.9-8.3, for 10 minutes at 37°C. The

cells were then washed in nutrient fluid, the cell clumps largely dispersed by repeated pipetting. The cells were counted in a hemocytometer and 1.5 x 10^6 were added to T-60 flasks(7). The medium in each flask was replaced 3 times weekly. Subcultures were prepared by the use of trypsin. After treatment with trypsin, the cells were washed in nutrient fluid and added to fresh flasks (8 x 10^5 cells per T-60). *Vaccinia virus.* The CVII strain of vaccinia virus(8) which had been passed 1 to 3 times in cultures of rabbit-muscle fibroblasts was employed. *Titration of vaccinia virus in cell cultures.* One ml aliquots of a suspension containing 5 x 10^4 cells were added to 13 x 100 mm Pyrex tubes. Serial 10 fold dilutions of virus were prepared in solution V-614 and 0.1 ml of each dilution was added to a series of 5 tubes. The tubes were inclined at an angle to 5° and incubated at 37°C in a stationary position. After 24 hours, the tubes were transferred to a roller drum at 37°C. The virus titer ($TC\text{-}ID_{50}$) was determined on the basis of the lowest concentration of virus which destroyed the cells in 9 days by the method of Reed and Muench(9).

Results. Twenty-three strains of fibroblasts derived from tissues of the rabbit, and chick embryo were established *in vitro* and propagated serially in a variety of media. In spite of concentrated efforts to establish the conditions required for the continuous propagation of each strain of cells, only 3 proved to be capable of sustained growth in the media employed. The pedigrees of the successful strains are outlined in Fig. 1. It will be noted that the growth rate of each strain progressively declined after a period of active proliferation. This was accompanied at first by an increase in the number of granules in the cytoplasm of the cells; later, degenerating cells were observed and their numbers increased progressively until the bottoms of the flasks were covered with a dense layer of cellular debris (Fig. 2). Some of the cells continued to metabolize glucose, however, as indicated by the production of acid and fluid replacements were continued at regular intervals. During the 19th week a colony (about 1 mm in diameter) of cells, of normal appearance (Fig. 3), was observed in one of the four flasks in the series derived from adult rabbit testicle. The progeny of the cells from this colony (strain RT6-F17; Fig. 4) have continued to proliferate at a constant rate and are currently in the 57th passage.

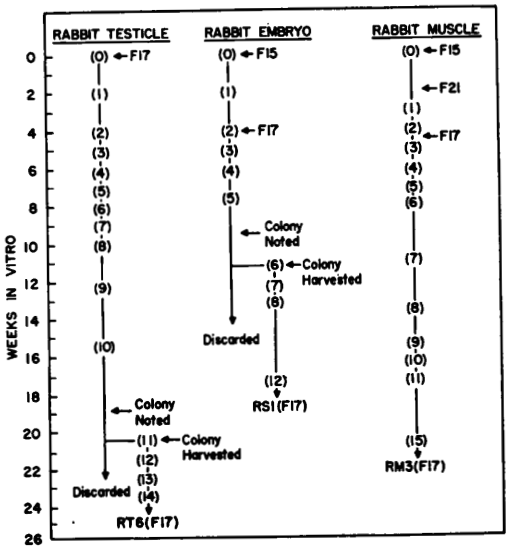

FIG. 1. Pedigrees of 3 strains of rabbit fibroblasts. Figures in parentheses refer to subculture number.

A second strain of fibroblasts was derived from the leg of a rabbit embryo of approximately 15 days' gestation (Fig. 1). During the 10th week three isolated colonies of normal appearing cells were observed in two of the five flasks in this series. The progeny of the cells from one of these colonies (Strain RS1-F17; Fig. 5) are now in the 38th passage.

A third strain of fibroblasts was derived from adult rabbit skeletal muscle. In this instance the degenerative changes which accompanied the decline in growth rate (Fig. 1) were not as extensive as those observed with strain RT6-F17 and RS1-F17. During the 13th week the overall appearance and growth rate of the culture began to improve gradually and after the 16th week it was possible to prepare subcultures at weekly intervals. This strain of fibroblasts, (RM3-F17; Fig. 6), continues to proliferate rapidly after 61 passages.

The other 20 cultures of fibroblasts which

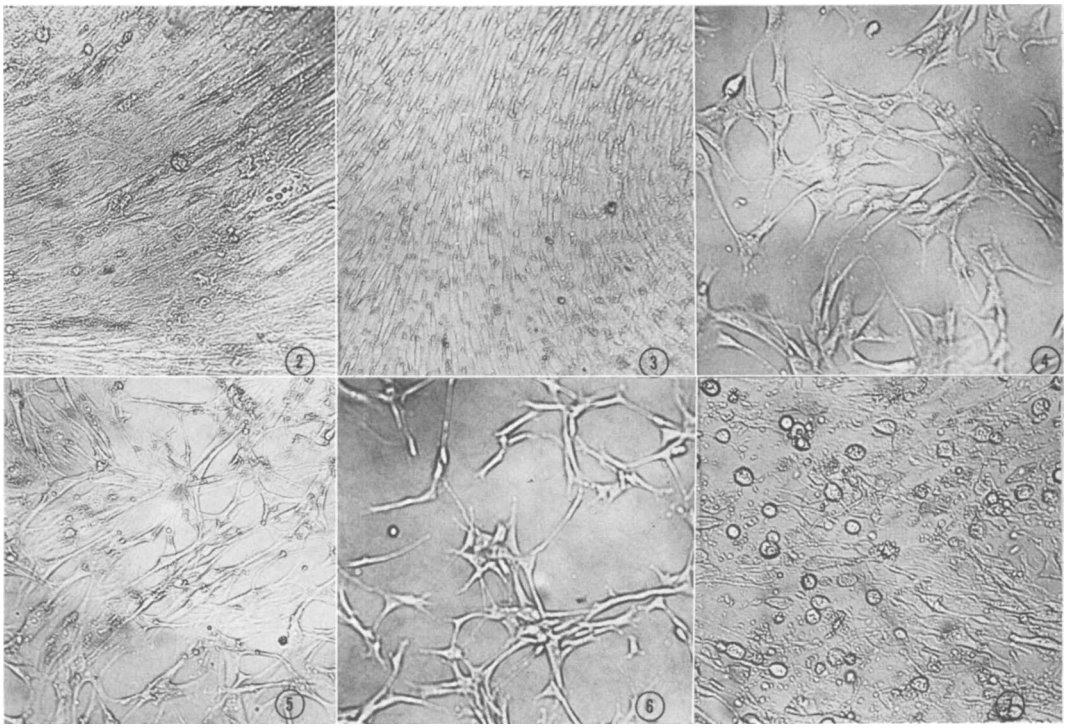

FIG. 2. Culture derived from rabbit testicle after 18 wk *in vitro*.
FIG. 3. Fibroblasts in original colony of RT6-F17 derived from culture illustrated in Fig. 2.
FIG. 4. Rabbit fibroblasts, strain RT6-F17 during 57th subculture.
FIG. 5. Rabbit fibroblasts, strain RS1-F17.
FIG. 6. Rabbit fibroblasts, strain RM3-F17.
FIG. 7. Culture of RM3-F17 three days after infection with 100 TC-ID_{50} of vaccinia virus.

were established (5 rabbit testicle; 2 each rabbit muscle, kidney, liver and lung; 7 chick-embryo heart or skeletal muscle) proliferated rapidly for 8-12 subcultures in a variety of media in addition to those indicated in Table I. After this period, the rate of growth either declined gradually over a period of several weeks or growth ceased abruptly. Numerous attempts to formulate a medium that would prolong the period of active growth of these cells have been unsuccessful. Each strain was maintained by periodic replacement of the medium until no viable or metabolizing cells appeared to be present in the flasks. Several cultures of chick embryo fibroblasts were maintained for 9 months without subculturing but failed to yield cells capable of continuous proliferation.

The following is a summary of the general nutritional requirements of the 3 strains of fibroblasts. Solution V-614 supplemented with CEE extract and NHS (medium F17, Table I) is an excellent medium for all 3 strains of fibroblasts. Media containing less NHS and CEE than F17 are adequate but the growth rate of cells is decreased proportionately whereas, media containing higher concentrations of NHS and CEE than F17 are inhibitory. The medium formulated by Eagle(10) for strain L will replace solution V-614 in F17 medium but the cells fail to proliferate when solution 199(11) or 703(6) is used. CEE is superior to BEE and extracts prepared from various adult rabbit organs are toxic. NHS can be replaced by bovine serum whereas human or rabbit serum is unsatisfactory.

Vaccinia virus multiplies in each of the 3 strains of fibroblasts and this is accompanied by the destruction of cells (Fig. 7). This cytopathogenic action afforded a method for comparing the susceptibility of cell strains to

TABLE II. Relative Susceptibilities of Cell Cultures to Infection with Vaccinia Virus.

Culture	Negative logarithm $TC\text{-}ID_{50}$/ml CF*	F15*	F7*
Lymphosarcoma (MB13)†	6.73	6.50	‡
Chick embryo§	6.08	5.50	‡
RM3 (F17)	5.82	5.12	4.68
RT6 "	5.55	5.55	4.84
RS1 "	5.50	5.38	4.44

* See Table I for composition of media; each value is avg for 4 or more titrations.

† Serially propagated in medium 705 as described previously(3).

‡ Cells degenerated in absence of virus.

§ Fibroblasts derived from skin-muscle of 10-day embryo; serially cultivated in CF; titrations performed at intervals from 3rd to 12th subculture.

vaccinia by employing them for titration of a standard pool of virus.

The relative susceptibilities of the 3 strains of rabbit fibroblasts are presented in Table II, together with results obtained with chick embryo fibroblasts and with lymphosarcoma cells, strain MB13. Strains RM3-F17, RT6-F17, RS1-F17 and chick embryo fibroblasts exhibit the same degree of susceptibility (medium CF) which is less than that observed for MB13 by a factor of approximately ten. The titer observed with MB13 is the same as that obtained in the embryonated egg by the pock method(12). When BEE is substituted for CEE in the medium, the relative susceptibilities of RT6-F17 and RS1-F17 are unchanged whereas, RM3-F17 appears to be less susceptible (compare media CF and F15). On the other hand, when embryo extract is omitted (medium F7) the 3 strains of rabbit cells are uniformly less susceptible. It is of interest that the susceptibility of RT6-F17 (subcultures 13-15; Fig. 1) which was derived from an isolated colony of cells is the same as the parent culture from which it was derived (subcultures 3-6; Fig. 1).

Discussion. The fact that all of the cells derived from normal tissue either ceased to proliferate or multiplied at a sharply reduced rate after an initial period of luxuriant growth is a perplexing problem which is not confined to fibroblasts from rabbit and chick embryo tissues‡(13). This phenomenon has received a minimum of attention primarily because in most instances cultures derived from normal tissues have not been propagated on glass for sufficient periods to observe this phenomenon. For example, certain strains of human fibroblasts cease to proliferate only after 30 subcultures *in vitro*.‡ One interpretation of the results of this study is that the usual tissue culture media are inadequate because they lack essential nutrients or because they are chronically toxic to most fibroblasts.

The results of this survey raise the question of the origin of the fibroblasts capable of continuous proliferation since only 3 of the 23 strains examined yielded such cells and because the latter appeared at a time when most of the cells in the individual cultures were either dead or degenerating. These data are subject to at least 2 interpretations: (1) fibroblasts capable of continuous proliferation were present in the original tissue inoculum and were preferentially selected by serial propagation; (2) in the course of serial propagation, a small proportion of the cells were altered sufficiently by mutation to be capable of sustained growth in F17. The feasibility of the latter is supported by studies of Sanford *et al.*(14) and by the fact that strains of human fibroblasts have been observed to alter their nutritional requirements on prolonged serial cultivation in response to changes in the composition of the medium.‡ Regardless of the mechanism with regard to the origin of the 3 strains of rabbit fibroblasts, it is clear that the proportion of cells derived from tissues of the rabbit and the chick embryo which are capable of sustained growth in the media employed is a very small fraction of the total. Furthermore, it appears that the isolation of permanent lines of fibroblasts from these sources as a routine procedure will require modifications in the composition of the usual tissue culture media.

Morphologically all 3 strains of rabbit fibroblasts are similar and each has maintained its characteristic appearance throughout the period *in vitro*. The most striking difference observed between strains RM3-F17, RT6-F17 and RS1-F17 is that the rate of growth of the former in F17 (12 fold per week) is about twice that of the other 2

‡ Unpublished experiments.

strains (Table II).

Summary. Twenty-three strains of fibroblasts derived from normal rabbit and chick embryo tissue were serially propagated on glass in a variety of media. All except 3 strains failed to proliferate for more than 8 to 12 subcultures. The 3 successful strains were derived from adult testis, embryonic skin-muscle and adult muscle respectively and continue to proliferate at a constant rate after 38 to 61 subcultures. Vaccinia virus multiplies in all 3 strains of fibroblasts resulting in the complete destruction of cells.

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Studies on Utilization of Sulfate Sulfur for Growth of the Chicken.*† (22708)

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It has been reported(1) that addition of sodium sulfate to a purified diet devoid of inorganic sulfur improved growth and feed efficiency of young chickens. Studies with S^{35}-sulfate have demonstrated that small amounts of sulfate sulfur are incorporated into cystine in the growing chicken(1,2) and laying hen (3), and larger portions into taurine by the chick embryo(4). In addition, the feathers of birds fed $Na_2S^{35}O_4$ contain a radioactive organic fraction which is not a sulfur amino acid and which has not been identified(1).

The purpose of these studies was to verify the growth response from sulfate sulfur, to determine whether such a response results from the conversion of sulfate sulfur to sulfur amino acids and to identify the radioactive compound appearing in the feathers after the feeding of $Na_2S^{35}O_4$.

Growth. The basal diet was a modification§ of that used by Gordon and Sizer(1), which contains 15% casein and 10% gelatin as the protein‖ source. Female New Hampshire X Silver Cornish crossbred chicks were fed the basal diet for 1 week. They were then weighed, divided into groups of 13 chicks of equal weight distribution and fed the experimental diets for 3 weeks. The chicks were reared under electrically heated brooders in wire-floored cages, in an air-conditioned room. Addition of 0.5% Na_2SO_4 to the basal

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† An abstract of part of this work was published in *Poultry Sci.*, 1955, v34, 1209.

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§ Wesson oil or lard was used as the fat. $MgCO_3$, $MnCl_2$ and copper acetate were used instead of the corresponding sulfates.

‖ The protein furnished about 0.54% methionine and 0.06% cystine. Since the estimate corresponds closely to the 0.132% total sulfur found by analysis the diet probably contained no inorganic sulfur.