TABLE V. Radioactivity Recovered from Rats Given 1 mg Tritiated D (Expressed in dpm).

	24 hr	7 days	14 days	21 days	28 days
Organs	1.4×10^7	8.4×10^6	6.4×10^6	4.4×10^6	3.5×10^6
Intestinal contents	2.1×10^7	3.9×10^7	6.7×10^6	3.2×10^6	1.7×10^6
Blood	8.3×10^5	7.6×10^5	2.4×10^5	2.5×10^5	2.2×10^5
Urine	2.0×10^7	2.4×10^7	3.7×10^7	3.4×10^7	3.7×10^7
Feces	3.4×10^7	2.9×10^8	3.9×10^8	4.8×10^8	5.0×10^8
Total radioactivity	8.9×10^7	3.6×10^8	4.4×10^8	5.3×10^8	5.5×10^8
% of dose recovered	13	54	66	79	82

high quantity of tritiated material was found in fatty tissue, suggesting that fat may serve as depot for the slow release of D. Radioactivity appeared in the blood one minute after the intramuscular injection of D in oil. The maximum concentration appeared within 15 minutes and was circulating 28 days after the material was given. Approximately 82% of the total radioactivity given was recovered on day 28.

1. Fried, J., Sabo, E. F., Grabowich, P., Lerner, L. J., Kessler, W. B., Brennan, D. M., Borman, A., Chemistry and Industry (London), April 15, 1961, p465.

2. Lerner, L. J., Brennan, D. M., Borman, A., Proc. Soc. Exp. Biol. and Med., 1961, v106, 231.

3. Lerner, L. J., Brennan, D. M., Yiacas, E., DePhillipo, M., Borman, A., Endocrinology, 1962, v70, 283.

4. Lerner, L. J., DePhillipo, M., Yiacas, E., Brennan, D. M., Borman, A., ibid., 1962, v71, 448.

5. Kelly, R. G., Peets, E. A., Gordon, S., Buyske, D. A., Anal. Biochem., 1961, v2, 267.

Received January 7, 1965. P.S.E.B.M., 1965, v118.

Development and Characteristics of the Rabbit Kidney Cell Strain, LLC-RK$_1$* (30044)

ROBERT N. HULL, ARTHUR C. DWYER, WILLIAM R. CHERRY AND O. JEANNETTE TRITCH

Lilly Research Laboratories, Indianapolis, Ind.

Primary rabbit kidney cell cultures have been found useful for cultivation of some viruses, and, in particular, those in the herpes group. One strain of embryonic rabbit kidney cells ERK-1 developed by Westwood *et al*(1) was described as being sensitive to poliovirus following a "transformation" which occurred. The attempt to establish a strain of rabbit kidney cells in our laboratory was initiated in 1959 for the dual purpose of providing the laboratory with such a cell strain and to attempt duplication of the finding of Westwood *et al.*

Material and methods. Tissue cultures. All rabbit kidney tissue cultures were prepared by trypsin dispersion of the kidney cells from 3-week-old New Zealand white rabbits by a procedure essentially the same as that described by Younger(2). Medium 199(3) supplemented with horse serum was used as a nutrient. Culture populations were quantitated by the nuclei enumeration procedure of Sanford *et al*(4).

Viruses. All viruses employed in this study were from stocks carried in these laboratories as tissue culture adapted strains. Virus titrations were performed by inoculation of 0.5 ml quantities of log dilutions of virus into each of 10 cultures/dilution. Titers were determined by the calculation of 50% end-

* Strain LLC-RK$_1$ is now under study by the Cell Collection Committee of the Tissue Culture Association as a candidate strain for replacement in the American Type Culture Collection. Until such placement is made, the culture is available only from our laboratory. The supply is restricted to those investigators who agree, upon receipt, not to further distribute the culture.

points by the method of Reed and Muench (5).

Results. The kidneys from several 3-week-old rabbits were trypsinized, pooled, and planted in tube cultures on October 28, 1959. The cells were nourished with medium 199 containing 5% horse serum and 100 units of penicillin and 100 μg of streptomycin/ml were added. The cell planting medium contained 0.42 g/liter of sodium bicarbonate. A medium change was made after 2 days of incubation, and the sodium bicarbonate content was increased to 1.68 g/liter Nine days after planting the culture medium was again altered to contain 2.1 g/liter of sodium bicarbonate, and the horse serum was replaced by 6.25% rabbit serum. Following an additional week's growth with 3 medium changes, 4 of the original tube cultures were trypsinized, pooled, and subcultured to 4 tubes. The medium was again modified to contain 5% rabbit serum and 1.12 g/liter of sodium bicarbonate. At approximately 2-week intervals, additional subcultures were made until passage no. 6 was obtained. At this point 5% horse serum was added in place of the rabbit serum supplement in the medium. The strain was continued henceforth as a stock culture and is currently in its 201st passage. The use of antibiotics in the medium on stock cultures was discontinued after the 53rd passage.

On a routine basis, cultures of strain LLC-RK$_1$ were split 1 to 4 at weekly intervals with medium changes performed every other day. Following this procedure, a 16-ounce bottle culture with a floor area of 98 sq cm, contained 30-40 million nuclei following 1 week's growth. When growth was allowed to continue for 15 days, a total population of nearly 80 million nuclei was obtained. A typical growth curve in T-15 flask cultures is seen in Fig. 1. In this instance, starting with a population of 101,111 nuclei, a maximum population of 4,722,221 nuclei was reached following 14 days' growth. This represented a 46.7-fold increase with a generation time of 60.7 hours. It appeared that the cultures did not reach log phase growth until the 8th day.

In the preparation of tube cultures for assay work, it was determined that a minimal

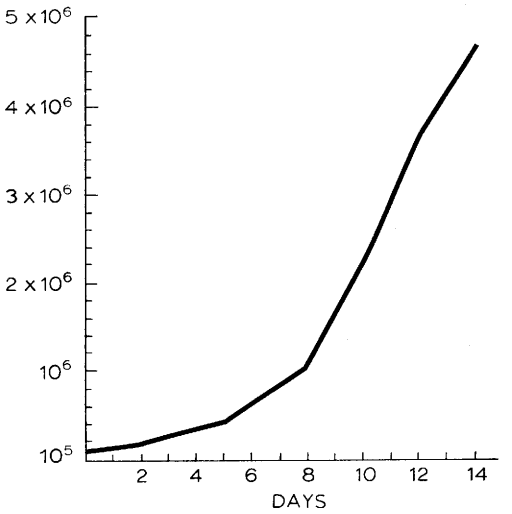

FIG. 1. Growth curve of strain LLC-RK$_1$ in T-15 flask cultures.

inoculum of 100,000 nuclei/tube was necessary in order to have sufficient growth of cells for use 3 days hence. Inocula of 50,000 nuclei/tube required 7 days' incubation to produce adequate cell sheets, while inocula of 25,000 or less failed to proliferate in the medium under the conditions employed. On the basis of the bottle cultures, population figures reported above, one 7-day-old 16-ounce bottle stock culture can provide inocula for 300-400 tube cultures.

Strain LLC-RK$_1$ was tested frequently for PPLO contamination but all such assays were negative. Likewise, there was no evidence of any viral or other microbial contamination. The cells became vacuolated when cooled to room temperature for several days, but all efforts to demonstrate the presence of a viral agent by subinoculation into other cell types and into primary cultures of rabbit kidney cells failed. Fixed and stained cultures showed no abnormalities indicative of a virus infection.

According to Vara and Pesonen(6) the diploid number of chromosomes for rabbit cells is 44. Counts obtained from the cell strain averaged very close to that number. At the 192nd passage level, chromosome counts were in the range of 39-46 with an average of 42. Average counts obtained on earlier passages ranged from 40-46.

The malignant potential of strain LLC-

RK$_1$ was assessed by inoculation of baby rabbits and suckling hamsters. This phase of the study is only briefly summarized as many of these tests are still in progress. A total of 14 rabbits was inoculated either intracerebrally or intramuscularly (IM) with viable cells from passage levels 22, 28, 155 and 191. Some rabbits died while on test, but no evidence of malignancies was detected in any of these animals by gross and histopathological examinations. Some of these rabbits, still surviving, have been observed for over 1 year and have remained free of tumors or obvious malignant disease. Four young rabbits inoculated IM with the 191st passage cells were surviving and without evidence of tumors after 2½ months observation. Ninety-six suckling hamsters were inoculated subcutaneously in the interscapular area with cells at passage levels 163, 165, and 167. Many of these were lost during the first 3 weeks primarily due to cannibalism by the mothers. A total of 17 inoculated hamsters have been held for periods of 8-10 months without incidence of malignant disease. Those which died were all negative for malignancy by gross and histopathological examination.

The morphology, growth rate, chromosome number, and virus susceptibility of strain LLC-RK$_1$ remained reasonably stable throughout its growth *in vitro*. Particular attention was given to the possible development of sensitivity of the cell strain to poliovirus. The strain was observed for its ability to support the growth of type I poliovirus at the following culture passage levels: 7, 10, 11, 12, 16, 19, 21, 24, 32, 36, 40, 55, 56, 60, 65, 90, and 91. In some instances, non-specific cell damage occurred which was probably due to toxic effects, as such cytotoxicity could not be passed. Back titrations in monkey kidney cells of supernatant fluids from cultures showing such cell damage failed to provide evidence for virus proliferation.

Like primary rabbit kidney cells (pRK), strain LLC-RK$_1$ is highly susceptible to viruses of the herpes group. A comparison of the sensitivity of the cell strain to that of pRK for B virus and Herpes simplex is seen in Table I. In this series of 8 titrations, strain LLC-RK$_1$ was equally as sensitive as the primary cultures to infection with these

TABLE I. Comparative Sensitivity of Strain LLC-RK$_1$ and Primary Rabbit Kidney Cultures to B Virus and Herpes Simplex.

	B virus		Herpes simplex	
Test No.*	pRK	LLC-RK$_1$	pRK	LLC-RK$_1$
1	6.0	6.0	8.5	8.5
2	5.5	5.6	6.0	6.0
3	8.0	8.0	6.5	6.5
4	7.5	6.5	6.6	6.8
5	6.3	6.2	6.6	6.3
6	5.8	6.0	6.5	6.5
7	6.2	6.2	7.5	7.0
8	6.1	5.6	6.5	7.0
Avg	6.4	6.2	6.8	6.8

* The same lot of virus was not used in all assays.

2 viruses. The cytopathic effect (CPE), however, in cell strain cultures inoculated with terminal dilutions of the viruses was delayed by 1-2 days in comparison to its time of appearance in pRK. The Herpes simplex virus produced the typical large multinucleated giant cell type of CPE generally associated with this group of viruses, but the strain of B virus employed did not do so. Similar observations were made in pRK cells infected with this virus. In monkey kidney cells, B virus uniformly produced the multinucleated giant cell type of CPE, but the CPE elicited by the virus in either pRK or LLC-RK$_1$ cells consisted mainly of a rounding and clumping of cells very much like the CPE seen in cultures infected with adenoviruses.

A number of viruses other than those belonging to the herpes group were tested for growth in strain LLC-RK$_1$. Of 24 different viruses studied, 10 were found to produce a CPE and to proliferate in this cell strain. Table II identifies the agents studied and

TABLE II. Virus Spectrum of Cell Strain LLC-RK$_1$.

Susceptible to:	Non-susceptible to:
Vaccinia	Poliovirus I
Herpes simplex	Coxsackie B-5, A-9
B virus	ECHO 4, 9, 10
Pseudorabies	Measles
Marmoset agent	Mumps
SA-8	Parainfluenza 1
SA-1	Asian influenza (Jap 305)
Rubella	Influenza B (Maryland)
A-1 hepatitis	Adenovirus 2
RKV	Varicella
	Vesicular stomatitis

indicates the results obtained. Three additional herpes viruses are listed under the susceptible column including: pseudorabies, the marmoset agent recently described by Holmes *et al*(7) and by Melnick *et al*(8), and SA 8, a vervet monkey virus reported by Malherbe and Harwin(9). Although the cell strain was not directly compared to pRK, or other cell types, in reference to sensitivity to these viruses, it was found to be quite adequate for growth and assay purposes. Titers of 10^8 or greater/0.5 ml were frequently obtained. Strain LLC-RK$_1$ was employed in one study to produce an experimental pseudorabies virus vaccine which was demonstrated to be protective in cattle.

SA-1 is a virus of vervet monkeys first isolated by Malherbe(9). It is a "foamy virus" essentially identical to F.V.1 of rhesus monkeys described by Rustigian *et al*(10). SA-1 grew to titers of 10^2-10^3/0.5 ml in strain LLC-RK$_1$ and produced the typical large syncytial masses characteristic of this group of viruses. The cell strain, however, was slightly less susceptible to this virus than were primary cultures of vervet monkey kidney cells.

Strain LLC-RK$_1$ was used routinely in the laboratory for cultivation and assay of the A-1 hepatitis virus reported by O'Malley *et al*(11) and for the RKV agent described by Hartley and Rowe(12). The A-1 hepatitis virus produced a rather non-descriptive type of CPE reaching titers of 10^8 or greater/0.5 ml in some instances. An 8-10 day assay was necessary to determine the final titer of a given pool of virus. The RKV virus grew slowly; 10 days or more of incubation were necessary for development of CPE even from an inoculum of undiluted virus. Virus titrations were held for 14 days. Maximal titers obtained with the RKV agent were in the order of 10^4/0.5 ml. The CPE of this virus was very much like that produced by S.V.$_{40}$ virus in vervet monkey kidney cells. Many small vacuoles appeared in the cytoplasm of infected cells and eventually these areas of the culture underwent necrosis.

The possibility of using strain LLC-RK$_1$ for growth of rubella virus was suggested by the report of McCarthy *et al*(13) who reported the growth and assay of rubella virus in the RK-13 rabbit kidney cell strain developed by Beale at Glaxo Laboratories. Rubella virus was successfully cultured in LLC-RK$_1$ cells and was carried through 14 serial passages. Titers up to $10^{4.5}$/0.5 ml, as determined by the vervet monkey kidney interference test, were obtained. Rubella virus produced some CPE in the rabbit kidney cell strain. Detailed studies will be published later.

Of those viruses which were found not to be infectious for LLC-RK$_1$ cells, some produced cytotoxic effects, but no virus multiplication occurred. This was especially true for adeno-, measles and ECHO 10 viruses.

Discussion. Strain LLC-RK$_1$ was carried in continuous culture for a period of 5 years. During this time, there was no alteration of cell growth or morphology or of other properties which would indicate a "transformation" of the culture. In this respect, our experience in the development of a rabbit kidney cell strain was unlike that described by Westwood *et al*(1). The latter authors, however, initiated their strain from cells of embryonic rabbit kidney while strain LLC-RK$_1$ was derived from the kidney of newborn rabbits. Doubt, however, has been registered in the reports of Brand and Syverton(14) and of Coombs *et al*(15) that ERK-1 is a rabbit cell strain.

Reports such as those mentioned above have suggested that many continuously propagated cell strains, either through contamination with other cell types or by mislabelling, have lost their original species identity. Brand has made a compilation of such findings(16). Thus, it is now necessary to confirm the identity of all cell strains described in the literature. We have described(17) a method for identification of 22 cell strains maintained in our laboratory by taking advantage of their varied sensitivities to a selected group of viruses. In this respect, it was noted that strain LLC-RK$_1$ was similar to the rat tumor strain, LLC-WRC$_{256}$. More recent investigation, however, has revealed that these 2 strains can be distinguished by the sensitivity of LLC-RK$_1$ to rubella virus. The rat strain also is highly malignant for baby rats which is not true for strain LLC-RK$_1$. The chromosome number of these 2 strains is also very

similar as the rat strain has a model number of 41. Cells of the rat strain which was derived from the Walker rat carcinoma, however, contained the large metacentric marker chromosome characteristics of this tumor. The average chromosome number for strain LLC-RK$_1$ is also very close to the diploid number of 42 for monkey cells and fairly close to the 46 of human cells. The virus spectrum of strain LLC-RK$_1$, however, is quite unlike that of either of these 2 species. Thus, although we have not offered immunological proof at this time that strain LLC-RK$_1$ is of rabbit origin, we feel that the other characteristics described serve to differentiate it from any of the other strains carried in our laboratory with which it might have become contaminated or otherwise confused.

The pH, or amount of sodium bicarbonate buffer, in the culture medium was found to be important for optimal cultivation of this cell strain. A pH of 6.9 obtained by inclusion of 1.12 g/liter of sodium bicarbonate was most satisfactory for initiation of cell growth. After the cell sheets were developed and while the cultures were used for virus growth, or assay, the cells were maintained best with a sodium bicarbonate concentration of 1.68 g/liter. Growth of B virus and Herpes simplex was enhanced by further increasing the buffer content to 2.1 g/liter, although this level was not optimal for maintenance of good cell sheets. Frequent medium changes were necessary in the growth phase of the cultures. Tube cultures on virus assays were maintained up to 5 days without intermediate medium renewals, but if assays were to be held for longer periods, medium changes at 5-day intervals were found necessary for continued survival of the cultures.

This rabbit kidney strain proved useful for the study of a number of viruses. It compared quite favorably with pRK for growth and assay of the herpes virus group. Further, the cell strain was successfully employed for recovery of Herpes simplex virus from human infections. It was used in preparation of an experimental pseudorabies virus vaccine which was field tested in cattle. Conceivably, it could be used to advantage in the growth of some viruses for production of vaccine for human use. Studies to date have failed to show any malignant tendencies of the strain and no adventitious agents have been isolated from it.

Summary. A strain of rabbit kidney cells, LLC-RK$_1$, was developed and carried in serial passage for 5 years. It maintained a near-normal chromosome number and was found not to be transplantable to either young rabbits or suckling hamsters. It was highly susceptible to the herpes group of viruses and also supported the growth of vaccinia, SA-1, rubella, A-1 hepatitis, and RKV. It was insusceptible to the human enteroviruses, influenza and parainfluenza, measles, mumps, adenovirus, varicella, and vesicular stomatitis.

The authors wish to express their gratitude to J. C. Nash, L. A. Dye, G. Butorac and J. H. Bodenmiller for technical assistance in performing virus assays. We are further indebted to G. W. Weaver for chromosome preparations and to C. G. Culbertson for histopathological examinations.

1. Westwood, J. C. N., MacPherson, I. A., Titmus, D. H. J., Brit. J. Exp. Path., 1957, v38, 138.

2. Younger, J. S., Proc. Soc. Exp. Biol. and Med., 1954, v85, 202.

3. Morgan, J. F., Morton, H. J., Parker, R. C., ibid., 1950, v73, 1.

4. Sanford, K. K., Earle, W. R., Evans, V. J., Waltz, H. K., Shannon, J. E., J. Nat. Cancer Inst., 1951, v11, 773.

5. Reed, L. J., Muench, H., Am. J. Hyg., 1938, v27, 493.

6. Vara, P., Pesonen, S., Acta Obst. et Gynec. Scand., 1947, v27, 215.

7. Holmes, A. W., Caldwell, R. G., Dedmon, E., Deinhardt, F., J. Immunol., 1964, v92, 602.

8. Melnick, J. L., Midulla, M., Wimberly, I., Barrera-Oro, J. G., Levy, B. M., ibid., 1964, v92, 596.

9. Malherbe, H., Harwin, R., S. A. Med. J., 1963, v37, 407.

10. Rustigian, R., Johnston, P., Reihart, H., Proc. Soc. Exp. Biol. and Med., 1955, v88, 8.

11. O'Malley, J. P., Meyer, H. M., Smadel, J. E., ibid., 1961, v108, 56.

12. Hartley, J. W., Rowe, W. P., Science, 1964, v143, 258.

13. McCarthy, K., Taylor-Robinson, C. H., Pillinger, S. E., Lancet, 1963, v2, 593.

14. Brand, K. G., Syverton, J. T., J. Nat. Cancer Inst., 1960, v24, 1007.

15. Coombs, R. R. A., Daniel, M. R., Gurner, B. W., Kelus, A., Nature, 1961, v189, 503.
16. Brand, K. G., Mammalian Chromosomes, Newsletter, 1961, No. 6, 8.
17. Hull, R. N., Tritch, O. J., Nat. Cancer Inst. Monogr., 1962, No. 7, 161.

Received November 23, 1964. P.S.E.B.M., 1965, v118.

Transaminases in Blood, Urine and Cerebrospinal Fluid of Normal and Unilaterally Nephrectomized Dogs.* (30045)

CLAIR M. HIBBS AND E. H. COLES (Introduced by G. K. L. Underbjerg)
Department of Pathology, Parasitology and Public Health, Kansas State Univ., Manhattan

Transaminases are intracellular enzymes normally found in body fluids as a result of the normal catabolic processes and/or pathologic processes. Quantitative determinations of these enzymes have been used extensively in human medicine to detect cellular destruction. The development of simplified laboratory procedures for transaminase determinations has stimulated increased use of such tests in the study of animal diseases.

Serum transaminase levels have been reported in dogs(2,6,9). However, a few reports concerning the levels of these enzymes in urine and cerebrospinal fluid of the canine have been published.

This study was undertaken in an attempt to establish and compare glutamic oxalacetic and pyruvic transaminase levels in blood, urine and cerebrospinal fluid of control and unilaterally nephrectomized dogs.

Mean serum glutamic oxalacetic transaminase (SGOT) of mongrel dogs was found to be 39 $\pm$ 3.5 units(6) using the method of LaDue, Wroblewski and Karmen(4,5). Other investigators reported a mean level of 22.7 $\pm$ 5.4 Sigma-Frankel units SGOT in dogs 9 months of age and mean SGOT level of 18.6 Sigma-Frankel units in the dog(2,10). Mean serum glutamic pyruvic transaminase (SGPT) levels of 21.8 $\pm$ 6.2 and 22.5 Sigma-Frankel units have been reported(2,10).

Presence of lysed red blood cells in serum resulted in greater glutamic oxalacetic transaminase (GOT) activity. However, significant changes were not found in transaminase levels when serum and plasma from heparinized and oxalated blood were studied. No significant changes were found in serum collected at various times during the day(14).

Urine samples from apparently normal humans were found to contain slight, insignificant amounts or less than 1 unit/ml GOT (1,8,15). Little or no transaminase activity has been found in dog urine. It was postulated that either the enzyme molecule was too large to pass through the glomerular filter or tubular cells were capable of completely reabsorbing the enzyme after filtration(3).

Normal cerebrospinal fluid glutamic oxalacetic transaminase (CFGOT) in infants was reported to be 1 to 10 units/ml with a mean of 4.5 $\pm$ 2.2 units(7). Cerebrospinal fluid glutamic oxalacetic transaminase level in dogs was 0.66 uM/ml/hr $\pm$ 0.08 using the method of Karmen(4,15).

Methods. Dogs of mixed breeds, both sexes, less than 2 years of age, and vaccinated against canine distemper were used. Group I dogs were non-nephrectomized controls. Group II consisted of dogs which had been subjected to surgical unilateral nephrectomy.

Tissue destruction due to surgery is reported to cause a transient elevation of SGOT (12, 13, 17). This elevation may occur within one to four days after surgery. To avoid this, samples were not collected from dogs in Group II for one month following nephrectomy. Tissue destruction associated with surgery was, therefore, not a factor in any alteration of transaminase levels in Group II dogs.

* Contribution No. 200, Dept. of Veterinary Med., Agri. Exp. Station, Kansas State Univ., Manhattan. Supported in part by research grant from Mark L. Morris Foundation.