Biol. Chem., 1949, v180, 755.
5. Philpot, F. J., Pirie, A., Biochem. J., 1943, v37, 250.
6. Matsui, K., J. Biochem., 1965, v57, 201.
7. Bessey, O. A., Lowry, O. H., J. Biol. Chem., 1944, v155, 635.
8. Burch, H. B., Lowry, O. H., Padilla, A. M., Combs, A. M., J. Biol. Chem., 1956, v223, 29.
9. Yagi, K., Nagatsu, T., Nagatsu-Ishibashi, I., Ohashi, A., J. Biochem., 1966, v59, 313.

Received September 5, 1967. P.S.E.B.M., 1967, v126.

Converted-Cell Focus Formation in Culture by Strain MC29 Avian Leukosis Virus.* (32552)

A. J. LANGLOIS AND J. W. BEARD
Department of Surgery, Duke University Medical Center, Durham, N. C.

Lack of a ready means for bioassay of avian tumor virus infectivity is a continuing impediment to quantitative studies of the agents. With procedures for titrating Rous sarcoma virus (RSV)(1) and a few leukosis agents(2,3) by host response *in vivo*, comprehensive experiments became feasible though tedious and expensive. Problems of RSV titration were immeasurably advanced by exploiting the phenomenon of focal morphologic conversion of chick embryo cells (CEC) infected by the agent in culture(4,5). The linear relation of focus number to virus concentration afforded the basis for simple and convenient infectivity assay(5). In marked contrast, CEC response to the leukosis viruses—those responsible for the leukemias and other hematopoietic diseases—was entirely different(6,7). Although these agents infected CEC, conversion was slow and uncertain(8), and none of them studied earlier produced distinctive foci. Assays by the Rous interference factor (RIF) test(6,9,10) or enumeration of infectious centers detectable with fluorescein-conjugated antibodies(6) yielded useful data but were inconvenient and time-consuming.

Recent studies, however, disclosed singular results with strain MC29 leukosis virus(11, 12). This agent which causes myelocytomatosis, renal growths and hepatomas(11-13) infects CEC cultures and, in high virus-cell multiplicity exposure, regularly effects rapid and massive cell conversion(14,15). On the other hand, with appropriate multiplicity and culture conditions, strain MC29 causes foci in CEC monolayers appearing at approximately the same rate and almost as distinct as those induced by RSV. This report illustrates the characteristics of strain MC29 leukosis virus focus formation and applicability of the phenomenon to bioassay of the agent.

Methods and materials. Strain MC29 was isolated(11) in 1960 in Sofia, Bulgaria. As a leukosis agent, the strain is characterized primarily(11,12) by the induction of myelocytomatosis with which there may be associated renal tumors and hepatomas(13). Virus for the present studies was from CEC cultures derived initially(15) by passage from the blood plasma of chickens diseased(12) with the agent. Assays were made on 4 culture-fluid pools—115, 202, 694 and 698, Table I—collected from conventional cultures(15) without agar.

Chick embryos were from eggs of chickens† free of Rous resistance inducing factor (RIF). Primary CEC cultures were prepared by trypsinizing 10- or 11-day old embryos with 0.25% trypsin in phosphate buffered saline (PBS), pH 7.4. Culture vessels, 100 × 20 mm plastic Petri dishes (Falcon Plastics, Los Angeles, Calif.), were seeded with 8 to 10 × 10^6 cells in 10 ml of growth medium like that used by Calnek(8). After 3 to 4 days,

* This work was supported by USPHS Research Grant C-4572 from Nat. Cancer Inst.; by the Annie Mabel Sherris Memorial Grant for Cancer Research from Am. Cancer Soc., Inc.; and by the Dorothy Beard Research Fund.

† The chick embryos used in this work were from White Leghorn eggs obtained from Dr. Roy Luginbuhl, University of Conn., through the Research Resource Program of Nat. Cancer Inst.

TABLE I. Characteristics of Pools of Tissue Culture Virus Titrated as Shown in Fig. 5 and 6.

Virus pool	Virus particles /ml $\times 10^8$	Interval after CEC infection, days	Interval of virus production, hr	Virus particles/infectious unit $\times 10^8$
115	8.3	7	24	2.2
202	21	15	24	7
694	6	7	4	5
698	19	7	8	13

the confluent monolayer was taken up with 0.05% trypsin, and the cells were sedimented, resuspended in growth medium and counted with a hemocytometer. The suspension was diluted with growth medium to a concentration of 1.5×10^6 cells/5-ml aliquots. Fifteen ml of suspension were added to a series of 50-ml conical centrifuge tubes to each of which 0.3 ml of an appropriate virus dilution was added. The virus-cell preparations were mixed with a Vortex Jr. Mixer (Scientific Industries, Inc., Queens Village, N. Y.), and 5 ml of each mixture were seeded on 3 plastic 60 × 15 mm culture dishes.

After about 16 hours, the medium was decanted, and the cells were washed with 5 ml of PBS and covered with 0.9% agar in growth medium. Three ml of medium without agar were added 4 days after infection. Incubation was at 38.5°C in a 5% CO_2 humidified incubator.

Seven days after infection, the agar was removed, and the cell layer was washed with PBS, fixed for 5 minutes with 4 ml of absolute methanol, exposed to May-Grünwald stain for 10 minutes and then counterstained for 20 minutes with Giemsa stain. The preparation was rinsed with distilled water and air-dried. Focus counts were made on images of the whole dish projected on a white background with a modified slide projector (Delinascope Projector, American Optical Co., Instrument Division, Buffalo, N. Y.). Coverslips were placed in some cultures to obtain cell specimens for study with the microscope (14,15). The preparations were fixed with Carnoy's fluid and stained with May-Grünwald-Giemsa.

RSV for comparative studies was obtained from Dr. W. R. Bryan of the National Cancer Institute.

Physical numbers of virus particles in the various pools were determined by sedimentation of the agent on agar and direct counts in the electron microscope(16).

Results. Exposure of CEC to strain MC29 in high multiplicity virus-cell relationship in conventional cultures—50 virus particles/cell —is followed by massive, rapid culture conversion(14,15). For 2 to 3 days after infection, the rate of growth of infected cultures is similar to that of cells not exposed to virus. Thereafter, growth of infected cells continues at essentially exponential rates in contrast to the control cultures in which cell multiplication slows and ceases usually after 2 to 3 weeks. Conversion is seemingly complete within 3 to 5 days after infection to yield cultures of uniform morphology.

With small doses of virus and an agar overlay, conversion occurs in foci detectable within 48 hours. Fig. 1 illustrates a typical group of morphologically altered cells 7 days after exposure to virus. A similar group of cells 4 days after infection shows an epithelium-like morphology, Fig. 3, with characteristically very distinct, round nuclei with 1, 2 or more very large nucleoli. These features of strain MC29 foci and converted cells are similar in principle to those related to RSV infection but differ considerably in some morphologic aspects from the analogous RSV structures, Fig. 2 and 4. Loosening of the focus cells to form holes in the monolayer seen frequently with RSV foci did not occur with strain MC29 virus. The staining reaction of strain MC29 focus cells differed markedly from that of RSV foci. Pyronin Y and azure blue stain(17) useful with RSV foci did not yield high contrast with strain MC29 cells. May-Grünwald-Giemsa stain, however, provided considerable contrast, Fig. 1, although the edges of the foci merged somewhat diffusely with the unconverted cells.

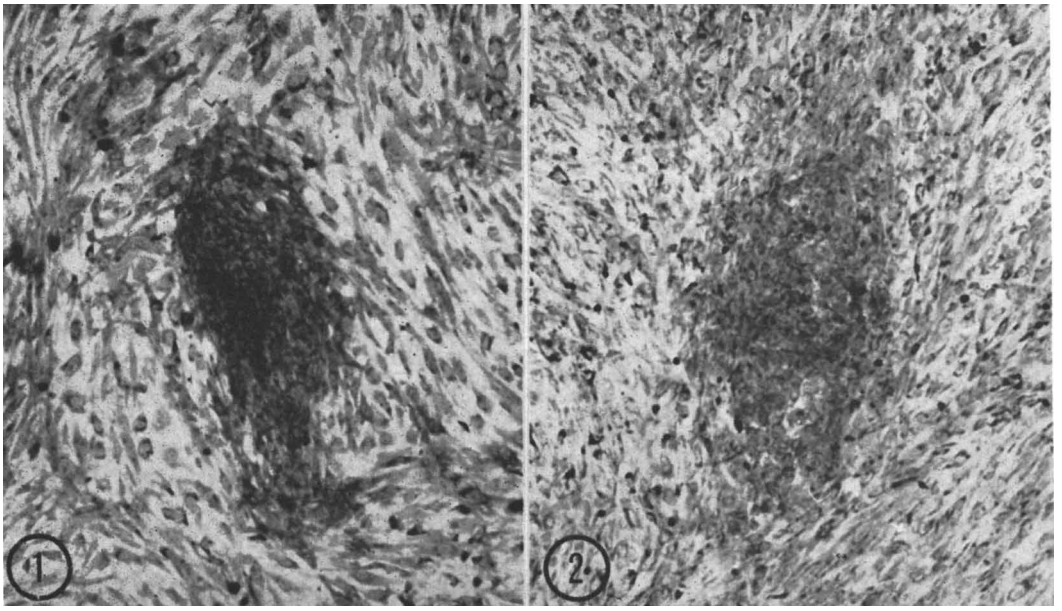

FIG. 1. Focus of converted CEC 7 days after exposure to strain MC29 virus. × 52.5.
FIG. 2. Focus of converted CEC 7 days after exposure to RSV. × 52.5.

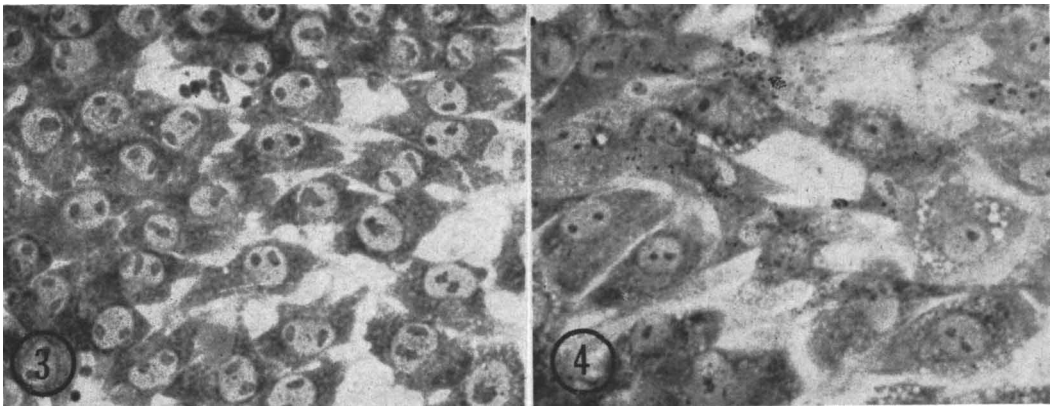

FIG. 3. Morphology of MC29 converted CEC from focus like that of Fig. 1, 4 days after exposure to virus. × 328.
FIG. 4. Morphology of RSV converted CEC from focus like that of Fig. 2, 7 days after exposure to virus. × 328.

The formations were nevertheless sufficiently distinct for counting.

Fig. 5 shows the results of a series of titrations on the same pool, 115, Table I, of strain MC29 virus from previous tissue culture passage. While the individual titration series showed some variation (Fig. 6), there was obviously a direct and close relationship between the dose of virus particles and the number of foci. There appeared to be a slight but definite difference between the levels of responses of the 2 different batches of CEC studied.

Further analogous studies were made on 3 other different pools of tissue culture virus. Fig. 6 shows the results which illustrate the variations between the individual estimates. The close relationship between virus-particle number and the number of foci is again evident.

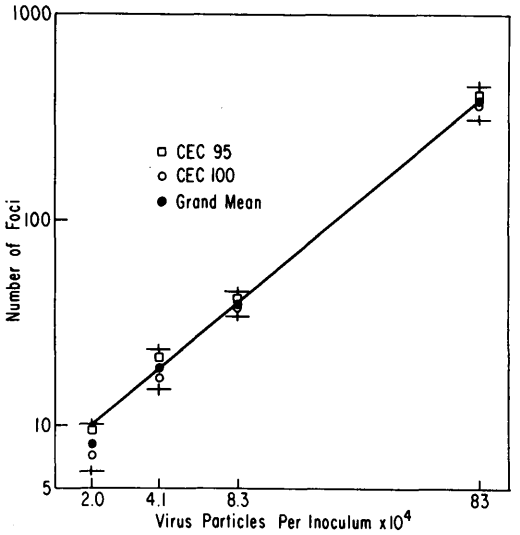

FIG. 5. Precision of estimate of focus-formation by a single pool, 115 (Table I), of strain MC29 virus in 10 separate titrations in 2 different batches of CEC from embryos from the same chicken population. The open squares are the mean values of 4 titrations with 3 plates for each dose in CEC pool 95; the open circles are analogous values for 6 titrations with CEC pool 100. The closed circles are the means of the mean of the squares and open circles. The horizontal bars indicate the 95% confidence limits, and the line was drawn by sight through the grand mean values obtained with the doses from 4.1 × 10^4 to 83 × 10^4 virus particles per inoculum.

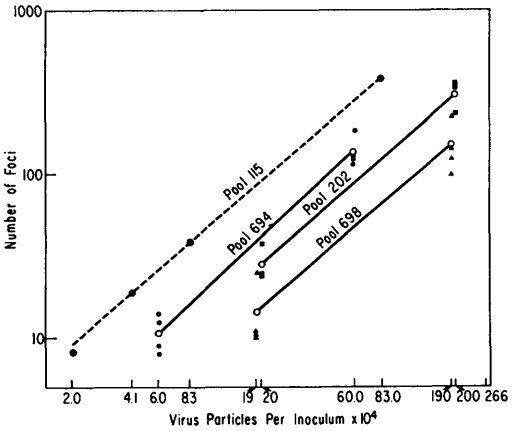

FIG. 6. Relation of dose of previous passage tissue-culture virus particles to number of foci induced in CEC monolayers. The results with pool 115 are the same as those of Fig. 5. Pools 694 and 698, Table I, were from the same series of cultures taken after 4 and 8-hour culture periods, respectively, and titrated in the same batch of chick embryo cells. Pools 115 and 202 were from different culture series and were titrated in different batches of CEC. Each titration was made with a separate set of dilutions carried separately through all maniputations. The lines were drawn through the respective mean values indicated by the open circles.

The ratios of the number of virus particles to foci of converted cells observed under the conditions of the experiments in the different studies are given in Table I. Variation in this series of studies was approximately 6-fold, extending from 2.2 × 10^3 to 13 × 10^3 particles/infectious unit. A comprehensive study of factors influencing variation was not made. Pools 694 and 698 were obtained from the same cultures collected at 4- and 8-hour intervals of incubation and were titrated in the same batch of embryo cells. Pools 115 and 202 were different sets of cultures and were titrated in different batches of CEC.

Discussion. Response of CEC monolayers to strain MC29 avian tumor virus differs markedly from that to other leukosis virus strains. A distinctive feature is the rapid morphologic conversion and exponential growth(14,15) of CEC after exposure to the virus not thus far observed with any other leukosis virus strain(8,14,15). The present studies illustrate a second singular characteristic. Although strains other than MC29 induce CEC conversion(8,14,15), the process is gradual and not associated with the formation of distinctive foci.

The foci of cells converted by strain MC29 were not as sharply distinct as those occurring with RSV. Nevertheless, the cell collections were readily recognizable in projected images of the entire stained culture dish and clearly distinguishable by color difference from clumps of unchanged cells. The reliability of focus detection and enumeration is attested by the intimate relationship of virus-dose to focus-number illustrated in Fig. 5 and 6.

Several avian tumor virus strains are known to contain 2 or more agents capable of inducing quite different neoplasms. A notable example is RSV preparations which include an agent causing sarcomas and a group of Rous-associated viruses (RAV) which induce leukosis(18). It is possible that strain MC29 is likewise a mixture of agents which might explain the unique behavior of a leukosis virus strain in the induction of foci. The

phenomenon would be most unusual, however, since sarcoma association with strain MC29 infection is virtually nonexistent(11, 12), and the non hematopoietic diseases are limited to epithelial growths of the kidney and liver. Studies on potential multiplicity of agents in strain MC29 are in progress.

The infectious or focus forming unit of strain MC29, Table I, observed in many experiments has been consistently in the range of about 10^3 to 10^4 physical virus particles. As reported, 50 such virus particles per cell were sufficient to cause essentially complete replacement of the cells initially present with a morphologically uniform population of altered elements within 3 to 5 days. It appears probable that infection may have been spread by virus newly formed in the conventional cultures. Liberation of virus by converted cells is recognizable by physical particle count within 48 hours after infection at which time the rate of particle liberation is about 100/cell/hour(15).

Accurate assay of strain MC29 infectivity makes available a unique and very valuable model for studies of cell-virus interrelationships in the induction of the neoplastic process. This is the only avian virus-tumor system which affords access to both control uninfected cells (CEC) and, simultaneously, to cells in the continuous process of rapid progressive morphologic conversion in high proportion to unchanging elements. This is in contrast to the RSV system in which cells in the early processes of conversion are in relatively small proportions(5).

Summary. Infection of chick embryo cell monolayers in high multiplicity with strain MC29 leukosis virus in conventional tissue culture induces rapid, massive morphological conversion within 4 to 7 days. Treatment of the cells in appropriately lower inoculation multiplicities results in the occurrence of infectious centers distinguished as foci of converted cells. When the infected cells are overlaid with agar, the number of foci occurring in 7 days is closely proportional to virus dose. This relationship is applicable to bioassay of strain MC29 leukosis virus with reproducibility and precision of results compatible with practical quantitative studies on the agent. The bioassay procedure and the rapid massive culture conversion make available a unique avian virus-tumor system for studies on cell-virus interrelationships in the induction of neoplasia.

1. Bryan, W. R., J. Nat. Cancer Inst., 1956, v16, 843.

2. Eckert, E. A., Beard, D., Beard, J. W., ibid., 1954, v14, 1055.

3. ———, ibid., 1956, v16, 1099.

4. Manaker, R. A., Groupé, V., Virol., 1956, v2, 838.

5. Temin, H. M., Rubin, H., ibid., 1958, v6, 669.

6. Vogt, P. K., In Viruses, Nucleic Acids, and Cancer, Williams & Wilkins Co., Baltimore, 1963, p374.

7. Baluda, M. A., Goetz, I. E., Ohno, S., ibid., 1963, p387.

8. Calnek, B. W., Nat. Cancer Inst. Monogr., 1964, v17, 425.

9. Rubin, H., Vogt, P. K., Virol., 1962, v17, 184.

10. Vogt, P. K., Rubin, H., ibid., 1963, v19, 92.

11. Ivanov, X., Mladenov, Z., Nedyalkov, S., Todorov, T. G., Yakimov, M., Bull. de l'Institut de Pathologie Comparée des Animaux, 1964, v10, 5.

12. Mladenov, Z., Heine, U., Beard, D., Beard, J. W., J. Nat. Cancer Inst., 1967, v38, 251.

13. Heine, U. I., Mladenov, Z., Beard, D., Beard, J. W., Program 24th Meeting Electron Microscopy Society, 1966, pB21.

14. Sankaran, S., Ph.D. Dissertation, Duke University, 1967.

15. Langlois, A. J., Sankaran, S., Hsiung, P. L., Beard, J. W., J. Virol., 1967, v1, 1082.

16. Sharp, D. G., Beard, J. W., Proc. Soc. Exp. Biol. & Med., 1952, v75, 75.

17. Siminoff, P., Reed, F. C., III, Virol., 1963, v21, 284.

18. Vogt, P. K., Adv. Virus Res., 1965, v11, 293.

Received September 5, 1967. P.S.E.B.M., 1967, v126.