Strain MC29 Avian Leukosis Virus Release by Chick Embryo Cells Infected with the Agent* (33823)

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Unlike other avian leukosis viruses, strain MC29 (myelocytomatosis) (1–3) initiates rapid, continuous processes of infection and morphologic alteration (4) of chick embryo cells (CEC) *in vitro*. Morphologic change may be evident within 48 hr after CEC exposure to virus and complete 24 hr thereafter (5, 6). With low virus-cell multiplicities, altered-cell foci are proportional to virus amount (7) as with Rous sarcoma agent (RSV) (8, 9).

Some attributes of CEC growth and morphology and associated MC29 virus release were reported (5–7, 10). Virus was estimated by electron microscope counts of physical particles measurable from about 48 hr after cell infection. However, release of infectious virus was detectable within 12 to 18 hr. In further work described here, virus was measured in terms of infectivity—focus-forming units (FFU)—also, for correlation with physical-particle release. Studies in parallel were made with RSV.

Materials and Methods. Viruses. Strain MC29 was established in tissue culture with filtered plasma from birds with myelocytoma (7). RSV was the Bryan high titer strain[1] from lot TV4 12/67.

Cultures. Primary CEC cultures and secondary virus-assay plates (7) were prepared from embryos[1] free of resistance-inducing factor (RIF) (11). Growth medium (12) was 82% M199, 8% newborn-calf serum (Grand Island Biological Co., Grand Island, N. Y.) and 10% tryptose phosphate broth (Difco Laboratories, Detroit, Mich.) with penicillin, 50 units/ml; streptomycin, 50 μg/ml; and amphotericin B, 0.5 μg/ml. Growth medium with 0.9% agar was used for overlay in virus assay (7).

Stock viruses. Primary cultures (5×10^6 cells/100 × 20-mm plate) (Falcon Plastics, Los Angeles, Calif.) 24 hr old were inoculated with 0.2 ml of previous-passage MC29 tissue culture virus or RSV. Fluids were changed 2 days later, removed again the next day, and the cells were washed twice with 5-ml vol of phosphate buffered saline (PBS). Ten ml of fresh growth medium were added, and 6 hr later the supernatants were pooled separately, centrifuged cold at 3020*g* for 10 min, parceled in 1- and 5-ml ampoules, and stored at —78°. The respective stocks contained 4.68×10^8 particles/ml or 8.55×10^5 FFU/ml—546 particles/FFU— of MC29 virus; and 1.21×10^8 particles/ml or 8.56×10^5 FFU/ml—141 particles/FFU—of RSV.

Assays. Trypsinized cells were counted in a hemocytometer, and virus particles were estimated with the electron microscope (13). Virus titration was as described for strain MC29 (7) and RSV (9).

Virus stability. Aliquots of the respective virus stocks diluted 1:50 with growth medium were distributed in ampoules loosely covered with aluminum foil. Two zero-time ampoules of each agent were sealed and stored at —78°, and the other ampoules were placed in the CO_2 incubator at 38.5°. At 6-hr intervals, 2 ampoules from each group were sealed and stored at —78°.

Cell growth and virus release. In the experiment reported here, 156 primary cultures were plated as above. After 24 hr, 3 cultures were trypsinized, and the average cell density/culture was determined. The medium was

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[1] Rous sarcoma virus was obtained from Dr. E. Bernstein, University Laboratories, Highland Park, New Jersey; and chick embryos were from white leghorn eggs from Dr. Roy Luginbuhl, University of Connecticut. The materials were available through the Research Resource Program of the National Cancer Institute.

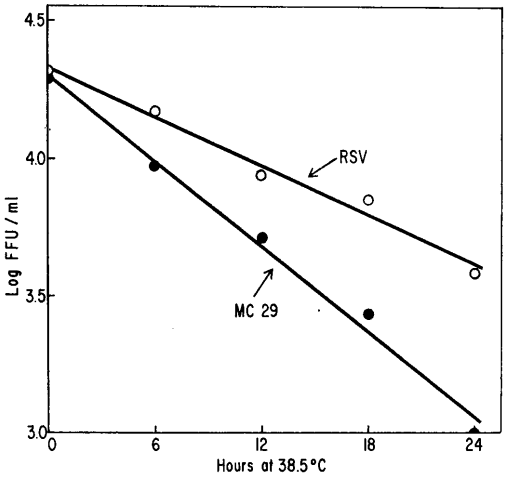

FIG. 1. Stability of MC29 and Rous sarcoma viruses: the agents in cell-growth medium were distributed in ampoules loosely closed with aluminum foil and placed in a humidified CO_2 incubator at 38.5°. The zero-time samples were sealed and kept at −78°. At 6-hr intervals ampoules were removed, sealed and also stored at −78°. Each point in the graph represents the average of measurements on 2 separate samples.

decanted from the remaining cultures, and the nonconfluent cell layers were washed with 5 ml of PBS. Fifty-one cultures were treated with 0.2 ml of stock MC29 agent—0.06 FFU (33 particles)/cell; and a like number with a similar volume of RSV—0.06 FFU (8.5 particles)/cell. After 30 min at 38.5°, the cell layers were washed twice with 5 ml of PBS, and 10 ml of growth medium were added. The remaining 51 cultures were treated in the same way but without exposure to virus.

Three cultures from each group were terminated 6 hr after injection and at 6-hr intervals thereafter. Cultures for next sampling were washed with 5 ml of PBS, and 10 ml of fresh growth medium were added. The various sample fluids were thus in contact with the cells for only 6 hr before harvest. Other cultures were changed daily. Fluids for particle counts were centrifuged at 3020*g* for 10 min and diluted 20-fold in 2% glutaraldehyde. Samples for infectivity assay were stored in sealed ampoules at −78°.

Results. MC29 virus stability under simulated cultured conditions but without cells, Fig. 1, was notably less (half-life, 6 hr) than that of RSV (half-life, 10.5 hr) measured in the same study. These values were applied to calculations of virus particles/FFU, Table I.

Population doubling times (PDT) of both control and RSV-infected cells, Fig. 2, were the same (42 hr) throughout the study. The like PDT of MC29-infected CEC, however, deviated sharply at 60 hr to only 24 hr. As before (6) the rate increase was related to morphologic alteration first noted here at 66 hr. These growth rates which were higher than those previously observed (6) with normal and MC29-infected CEC (120 and 42 hr, respectively) were associated with change in source of newborn-calf serum.

Virus release/6-hr culture-time increments is illustrated as numbers of particles and FFU, Fig. 3, and corresponding rates/cell/hr

TABLE I. Ratio of Physical Particles to Infectious Units (FFU) of MC29 and Rous Sarcoma Viruses.[a]

After virus inoculation (hr)	MC29 (particles/FFU)		RSV (particles/FFU)	
	Not corrected	Corrected[b]	Not corrected	Corrected[b]
30	—	—	465	384
36	—	—	214	176
42	310	225	173	143
48	534	387	365	301
54	755	547	104	86
60	1970	1428	286	236
66	1170	848	360	297
72	1540	1116	423	349
78	1530	1109	201	166
84	1010	732	306	252
90	1280	928	326	269
96	1410	1022	227	187
102	950	689	200	165
Av	1219	878	282	232

[a] These values were calculated from the data of Fig. 3 and corrected for infectivity inactivation during the respective culture periods of 6 hr (Fig. 1). Values obtained less than 30 hr after infection were not included, since virus-particle number could not be accurately determined before that time.

[b] Integration of the net rate of virus inactivation yielded infectivity survival values of 72 and 82% for MC29 and RSV for 6-hr intervals assuming constant rates of virus release during these periods.

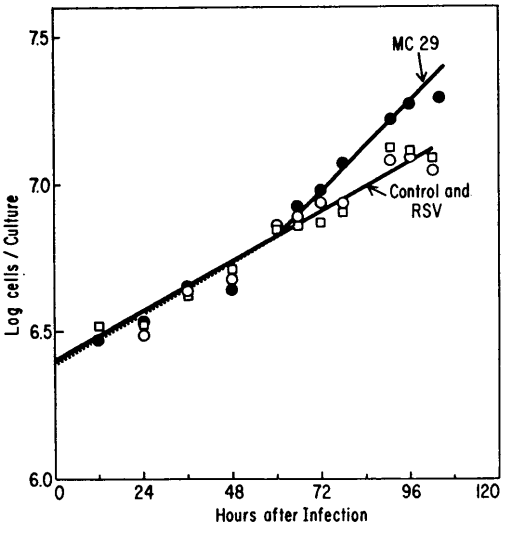

FIG. 2. Growth of noninfected cells and cells infected, respectively, with MC29 and Rous sarcoma viruses: the data were obtained from the cell counts in the same experiment as that of Figs. 3 and 4.

are shown in Fig. 4. Similarity of the curves of Fig. 4 to those of Fig. 3 indicated small influence of cell increase, Fig. 2. In the cultures uniform initially with respect to CEC number and virus inoculums, the rates of MC29 virus-particle release exceeded those of RSV particles. The relationships of FFU release, however, were reversed, and the output of RSV FFU was greater than that of MC29 virus. Release of RSV infectivity was first notable at 18 hr and that of particles at about 30 hr; for MC29 the corresponding times were 18 and 48 hr. Infectious MC29 virus-increase with time was about 150-fold from about 0.001 to a maximum of 0.15 FFU/cell/hr corresponding to about 230 to 300 physical particles/cell/hr at approximately the time of complete morphologic alteration. At peak production, about 2×10^9 particles or 2×10^6 FFU were released/ml of culture fluid in 6 hr with slight later rate decline due, probably, to cell crowding during the 102-hr period. Release rates become constant with regular changes of medium and division of cultures.

In comparison, maximum RSV release was about 10^9 particles or 3.5×10^6 FFU/ml of culture fluid corresponding to a rate of about 1.0 FFU/cell/hr or about 150 to 200 VP/cell/hr. The rate did not decline, possibly because the RSV-infected cells did not grow to crowding (see Fig. 2).

There was no trend of change in the quality of particle infectiousness relative to change of virus release rates of either agent. Variations in the ratios of particles/FFU, Table I, included those inherent in the methods of sampling, titration, and particle counting as well as possible changes in biologic activity. In the entire experiment, the average actual number of particles/FFU of MC29 was 1219, but the number after correction for inactivation (Fig. 1) was 878/FFU. The corresponding values for RSV were 282 and 232. Thus on the basis of particles/FFU, RSV

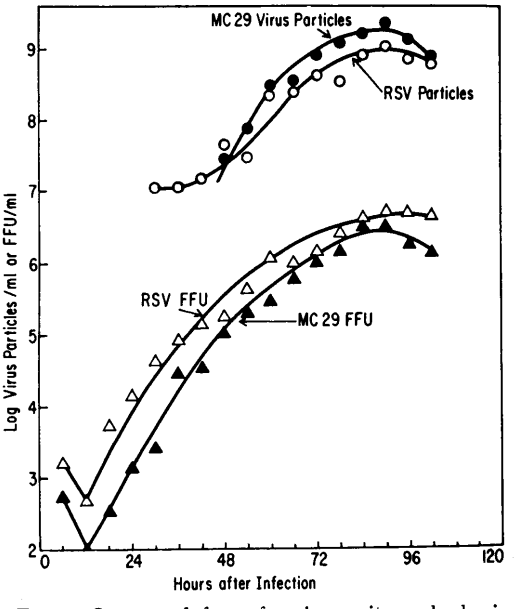

FIG. 3. Output of focus-forming units and physical particles of MC29 and Rous sarcoma viruses in chick embryo cell cultures: medium poured from triplicate MC29 and RSV cultures at 6-hr intervals after expousre to virus were pooled and sampled for virus-particle count and infectivity measurement. Fresh medium was placed on those cultures to be sampled at the next interval so that all specimens were exposed to 38.5° for only 6 hr. Titrations were made of multiple dilutions of the individual samples with cultures prepared from the same population of cells. Each virus-particle value is the average of 2 determinations. All infectivity values charted represent the observed data corrected for temperature inactivation as indicated in Fig. 1 (see Table I).

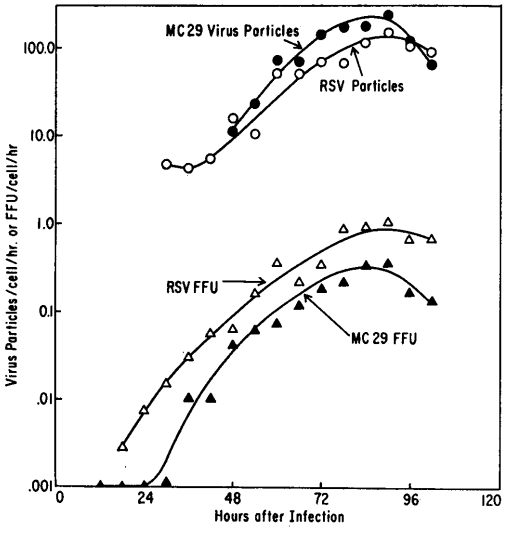

FIG. 4. Rates of liberation/cell/hr of physical particles and infectious units (FFU) of MC29 and Rous sarcoma viruses by chick-embryo cell cultures. After removal of medium for the analyses as described in Fig. 3, the cells from the triplicate cultures were suspended with trypsin, pooled, and counted. With these data, the rates of virus liberation were calculated from the values of the particle and infectious-unit numbers given in Fig. 3.

infectivity was more than 3-fold that of MC29 virus.

Discussion. Because of titration and other difficulties, estimates of avian tumor virus stability have varied somewhat with differing conditions (14). Direct comparison of strain MC29 and RSV, however, could be made under like conditions of culture procedures and infectivity measurement. The results demonstrated again relative lability of another avian tumor virus and showed that the quality of strain MC29 preparations responsible for focus formation was distinctly less stable than the corresponding property of the RSV samples.

Growth rates of RSV-infected CEC have differed in various studies (14–16) probably related largely to culture conditions. Strain-MC29 altered CEC have grown at rates consistently greater than those of noninfected CEC, but variations observed were from 42 hr in other studies (6) to 24 hr PDT seen in the present work. In this case the difference was traced definitely to the quality of newborn-calf serum. The MC29 CEC grew more rapidlly, also, than RSV CEC.

Rates of virus release as determined both by direct particle and FFU estimates were the resultant of infection by introduced virus and of progressive infection by newly liberated virus of CEC not infected by the initial low virus doses (0.06 FFU/cell); by probable rate changes with onset and development of individual cell response; and the attainment of constant rates of virus liberation by morphologically altered elements in protracted studies (5, 6). The maximum rate of RSV liberation was about 1.0 FFU/cell/hr (150–200 particles/cell/hr) in comparison with FFU rates of 0.01 (17), 0.1–0.2 (18), 0.16–1.6 (19) and 0.08 (20) previously described. RSV particle-release studies have not been reported. The rate of MC29 virus release [av 230 particles/cell/hr (0.15 FFU/cell/hr)] was far greater than the rate of about 30–60 BAI strain A virus particles/cell/hr released by myeloblasts (21, 22). The values, however, were comparable with rates of 123 and 107; 63 and 129; and 295 and 570 in 2 separate studies each with BAI A, ES4, and R leukosis virus strains in CEC estimated by particle count (23). Little is known of the basic factors determining rates of virus synthesis, but a relation has been seen between the individual cell-donor hosts and rate of BAI A strain output by myeloblasts (22).

It is evident that the electron microscope estimates were a measure of the total number of morphologically similar virus particles liberated into the supernatant culture fluid. In the studies on the high-titer RSV, the micrographs provided no means for distinguishing between particles with the RSV genome and the non-RSV RAV particles known (14) to be present in varying numbers in tissue culture RSV preparations. Thus, calculated numbers of physical particles/FFU in the RSV preparations, much lower than those previously reported (24), included not only particles presumably containing the RSV genome but nonfocus-forming RAV particles as well. Various studies have suggested RAV concentrations

approximately 10-fold those of actual RSV. In consequence, values of RSV-particle output/cell/hr and of RSV particles/FFU would be less, by some presently unknown factor, than those cited here, Table I. This, however, does not affect in any way the validity of the data on virus-particle output/cell/hr in the RSV preparations or on the total number of virus particles/FFU. Further, these considerations have no bearing on the values of rate of infectious unit (FFU) output/cell/hr which are independent of the number of RAV particles in preparations containing an excess of RAV. All of these relationships might be equally applicable to interpretation of the MC29-strain data, because the strain may be a mixture of subgroups (25) of avian tumor viruses, one or more of which is not measurable by focus formation. Significant estimates related to virus species can be made only on separated isolates not yet available. It is notable that, contrary to previous interpretations (17), attainment of constant average rate of virus release, as shown by physical particle count, was not a function of liberation–inactivation equilibrium, Instead, the limiting rate appeared to be dependent on cell-virus concentration interrelationships and culture conditions (6).

Accuracy of the results was conceivably affected by a variety of factors difficult to assess. For example, it was possible that particles elaborated prior to the beginning of each 6-hour study interval were carried over into the respective supernatant-fluid specimens. This potentiality was greatly lessened by thoroughly washing the culture with PBS at the beginning of the interval as described. Particle aggregation was another hazard to accurate titration. Examination of each culture fluid at a dilution of 1:20 for electron microscope counts, however, revealed but occasional groups of loosely associated virus particles. Such associations would not be expected to persist through the repeated pipettings requisite for preparing the higher dilutions used in the infectivity titrations. In addition, each value cited was the average obtained with 3 separate cultures. It is evident that all of the values were subject to numerous variations inherent in the techniques employed, but the results, nevertheless, provide useful information not thus far available for study on the strain MC29 and RSV systems.

Summary. Release of strain MC29 avian leukosis and Rous sarcoma (RSV) viruses by infected and/or morphologically altered chick embryo cells was measured by counts of physical particles and infectious units (FFU). Particles and FFU were liberated in parallel to a maximum rate at 3–4 days after low multiplicity culture inoculation. The ratios of particles/FFU, corrected for virus inactivation, were 878 and 232 for MC29 and RSV, respectively. Morphologically altered MC29 and RSV cells had a population doubling time of 24 and 42 hr, and the half-lives of the 2 agents were 6 and 10.5 hr, respectively, under simulated culture conditions.

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Tensions of O_2 and CO_2 in Gas Pockets of Germfree Rats* (33824)

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Animals devoid of bacteria provide a means for finding out whether the usual bacterial flora have influence on "normal" physiological functions. The effectiveness of two of the primary physiological systems, the pulmonary and cardiovascular systems, can be evaluated by the end result of their function, that is, the tissue O_2 and CO_2 tensions. Cardiac output (1) and metabolic rate (1, 2) have been reported to be 20–30% below normal in germfree rats. However, these changes are not necessarily associated with changes of tissue gases, for tissue pO_2 and pCO_2 depend on the balance between local blood flow and local metabolism.

A simple direct technique which can be utilized to measure tissue gases in unanesthetized animals is the subcutaneous gas pocket. Gas is introduced into contact with tissue and left until the O_2 and CO_2 in the pocket come near equilibrium with their dissolved counterparts in the tissue (3, 4).

Materials and Methods. Rats were kept in three plastic film isolators at the Lobund germfree laboratories and maintained according to established procedures. In each isolator were 6 males and 6 females, 60–70 days of age. Twelve control rats in one of the isolators had been born as germfree but were removed from the germfree environment at 35 days of age and kept under ordinary laboratory conditions until the first day of the experiment. Thus they were "conventional" rats with normal bacterial flora, but during the experiment were kept in the same kind of environment as the germfree rats in the other two isolators.

A pocket of 30 ml of air was formed on the back of each rat with hypodermic needle and syringe. A sample for analysis on the Scholander 0.5-ml apparatus (5) was taken from the pocket of each rat 2 days later and additional air was injected into each pocket. Then the 12 germfree rats in one of the isolators were monocontaminated with a normal rat intestinal bacterium, *Clostridium difficile,* by giving the culture with a stomach tube and mixing the culture in the drinking water. Gas samples were taken from these monoinocculated rats the following 2 days, and from each rat in the other two isolators 4 days after forming the pockets. Routine bac-

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