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In Vitro Chick Embryo Cell Response to Strain MC29 Avian Leukosis Virus

D. P. BOLOGNESI, A. J. LANGLOIS, L. SVERAK, R. A. BONAR, AND J. W. BEARD
Department of Surgery, Duke University Medical Center, Durham, North Carolina 27706

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Strain MC29 avian leukosis (myelocytomatosis) virus induced infection, elaboration of virus, and morphological alteration in chick embryo cells in vitro. Virus liberation began within 18 hr, morphological change was detectable at about 40 hr, and the cultures could be completely altered within 80 hr after infection. Altered cells were about half the volume and grew at approximately twice the rate of uninfected elements. The output of virus estimated by electron microscopy was about 140 particles per cell per hr. Deoxyribonucleic acid remained constant, but ribonucleic acid increased in both infected and control cells in adjustment to culture environment. The rates of uptake and incorporation of ^{3}H-uridine and the incorporation of ^{3}H-thymidine increased in the infected cells with onset of morphological change but were unaffected by processes of infection and virus elaboration per se. Incorporation of a ^{14}C-amino acid mixture was slightly greater in the infected than in control cells. The speed of continuity of infection and massive morphological alteration constitute a unique response to avian tumor viruses, and the system gives promise of singular value for detailed studies of the processes of infection and morphological change.

Strain MC29 avian leukosis virus (10) induces neoplastic response chiefly of the myeloid hematopoietic tissue in the intact chicken (10, 15). In contrast to the profound myeloblastic leukemia caused by BAI strain A (7), however, the strain MC29 disease is not notably leukemic but is characterized principally by neoplastic proliferation of myeloid cells at the level of myelocytic differentiation. The cells may be distributed as diffuse growths, myelocytomatosis, or form localized, solid tumors, myelocytomas. Frequently associated with such myeloid neoplasms are renal adenocarcinoma and cystadenoma. Unlike the responses to avian leukosis strains previously studied are morphological alterations of hepatocytes and formation of frank primary tumors of the liver (U. Heine, D. Beard, Z. Mladenov, and J. W. Beard, *in preparation*).

Investigation of strain MC29 was recently extended to examination of its influence on chick embryo cells (CEC) in vitro. As observed with other avian tumor viruses (4, 14, 23-26; S. Sankaran, Ph.D Thesis, Duke University, Durham, N.C., 1967), exposure of CEC to strain MC29 results in the induction of infection, the elaboration of virus, and morphological alteration (12) of the culture. With low multiplicities of virus and an agar overlay, the cells form heaps or foci (11) somewhat similar to those observed with Rous sarcoma virus (RSV). Also like the response to RSV, the cells altered by MC29 virus grow in layers which are indicative of loss of contact inhibition. In this respect, the behavior of the MC29 cells differs from that of CEC responding to other leukosis viruses, such as BAI A, R, and ES4 strains which retain contact inhibition and form uniform monolayers (A. J. Langlois, S. Sankaran, and J. W. Beard, *in preparation*).

A singular aspect of the response to MC29 virus, however, is the apparently exceedingly high rate of morphological change which may yield uniformly altered cultures (12) within 74 hr after cell exposure in appropriate virus particle multiplicity. This finding suggests high incidence of cell response, and, more important, direct continuity in a high proportion of the cells of those successive processes concerned with cell-virus interaction in initiation of infection, elaboration of virus, and alteration of cell morphology. Several strains of avian sarcoma viruses cause CEC infection and rapid morphological change, but the time required for alteration of the whole population of cells appears to be greater than with MC29 (21, 22). Leukosis viruses other than strain MC29 (4, 12; S. Sankaran, Ph.D Thesis, Duke University, Durham, N.C., 1967) apparently induce a high incidence of infection with virus elaboration, but altered cells appear only after protracted culture periods and death of most of the elements initially exposed to the virus.

In strain MC29 cultures, on the contrary, the population grows progressively without notable loss of cells (12). The potential value of such a system for investigation of the continuous processes of infection and conversion is apparent.

In the experiments reported here, studies were made on the growth characteristics of CEC exposed to strain MC29 (11, 12). Exploratory investigations were made, also, on the kinetics of virus liberation and on some aspects of the physiological processes concerned particularly with cellular metabolism of ribonucleic acid (RNA), deoxyribonucleic acid (DNA), and protein. Morphological and other biological features of the system will be described in another report (A. J. Langlois, S. Sankaran, and J. W. Beard, *in preparation*).

MATERIALS AND METHODS

Strain MC29, isolated in Bulgaria (10), was brought to this laboratory in 1965 for further study of its etiological activity in the intact chicken (15). Virus for tissue culture was then obtained for passage (11) from the blood plasma of chickens diseased with the MC29 agent. All of the experiments reported here were made with a single pool (115-67) of tissue culture fluids. The pooled culture fluids, passed through a 0.3-μ Millipore filter (Millipore Corp., Bedford, Mass.) and stored at -70 C in sealed ampoules, contained 8.3×10^8 virus particles per ml, as determined by electron microscopic count (19).

Eggs for embryos were from a laying flock free of resistance-inducing factor (16). Primary cell cultures were prepared (4) from decapitated, eviscerated, and trypsinized 10- or 11-day-old embryos seeded at 4×10^6 to 10×10^6 cells per plastic petri dish (100 × 20 mm; Falcon Plastics, Los Angeles, Calif.). The cells which attached to the dish, chiefly fibroblasts, were grown in 10 ml of Calnek's growth medium (4) at 38.5 C in a humidified 5% CO_2 atmosphere. These primary cultures were infected at 24 hr with 33 virus particles per cell.

Radioisotope labels. At the time of infection and at daily intervals thereafter, the culture medium was removed from all dishes, the cells were washed with phosphate-buffered saline (PBS) (6), and fresh medium was added. Each day some cultures were treated with labeled compounds (New England Nuclear Corp., Boston, Mass.): uridine-5-^3H, 26 c/mmole; thymidine-5-methyl-^{3}H, 16.1 c/mmole; or ^{14}C-amino acid mixture, 40 mc/milliatom of carbon. At cell harvest, the medium was sampled for radioactivity and virus particle count (19). The cells were washed with PBS, removed with trypsin, sedimented, and then resuspended in PBS. A sample was taken for cell count, the cells were sedimented again, and the pellet was frozen in a dry ice-alcohol bath and stored at -20 C. In some supplementary studies of labeled-compound uptake from the medium, successive 0.2-ml samples of medium were taken from the same culture dish over the interval under study.

Analysis of cells. Fractionation of labeled cells was effected with procedures adapted from those of Scott et al. (18). The fractions were examined for radioactivity in a liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.) and for ultraviolet absorption. Acid-soluble nucleotides were obtained by three successive extractions of cells with 0.5 ml of 0.3 M $HClO_4$ at 0 to 4 C. For extraction of RNA, the residual cell pellet was incubated for 60 min with 0.5 ml of 1.0 M NaOH at 23 to 24 C. Protein and DNA were precipitated by addition of 0.1 ml of 6.5 M $HClO_4$ to the preparation chilled to 0 to 4 C. The supernatant fluid from centrifugation and two pellet wash fluids of 0.5-ml volumes of 0.3 M $HClO_4$ were combined. DNA in the insoluble material left from RNA extraction was hydrolyzed by heating a suspension of the residual pellet in 0.5 ml of 0.3 M $HClO_4$ at 90 C in a water bath for 30 min (29). The precipitate was removed by centrifugation, and the supernatant fluid and 0.5 ml of 0.3 M $HClO_4$ used to wash the pellet were combined for DNA analysis.

Amounts of RNA in μg were computed by multiplying the difference between the optical density values measured at 260 and 320 mμ, respectively, by the volume of the extract and the factor 32.8. This factor was obtained from the data for the nucleotide ratios of the whole RNA of avian myeloblasts of myeloblastic leukemia (27) and the extinction coefficients of nucleotides at 260 mμ in acid solution (3), assuming no residual hypochromicity in the partially hydrolyzed RNA (9). An approximate estimate of acid-soluble nucleotide was made with the same factor, although the proportions of various nucleotides, nucleosides, and bases in the soluble pool were unknown. The corresponding factor for calculation of DNA quantity was 31.2, determined in like manner from nucleotide ratios of chicken cell DNA (27) and extinction values (3).

Protein (13) and radioactivity were determined on ^{14}C-amino acid-labeled cells, extracted three times with cold 0.3 M $HClO_4$, sedimented, and then dissolved in 1.0 ml of 1 M NaOH.

Cell and virus-particle enumeration. Cell samples in PBS were mixed with equal volumes of 2% glutaraldehyde and stored at 2 to 4 C for later counts in a hemocytometer. Culture fluids for virus particle counts were diluted with appropriate volumes, usually 20-fold, of 1% glutaraldehyde and stored in the cold. Counts were made by electron microscopy of virus particles sedimented on agar (19).

Cell volume. Cells suspended in PBS without Ca^{++} or Mg^{++} were centrifuged to equilibrium packing, $2{,}000 \times g$ for 30 min, at 2 to 4 C in a Van Allen blood volume index tube (28) with the bulb calibrated and filled. The tubes were coated with silicone (Siliclad; Clay Adams, Inc., New York, N.Y.) to prevent sticking of the cells to the lower part of the bulb.

Morphological alteration. Cells growing on cover slips placed in appropriate cultures were fixed in methanol and stained with May-Grünwald-Giemsa. The proportion of altered cells on each cover slip was determined by counts in random areas with a limited-field ocular at a magnification of 450 ×. All counts, 500 cells each, were made on triplicate preparations.

RESULTS

Kinetics of cell growth and virus liberation. Response of CEC to strain MC29 is characterized in part by elaboration of virus and marked change in cell growth rate. The results of a typical experiment (Fig. 1) showed that the control-cell growth was essentially constant with a population doubling time (PDT; 5) of about 136 hr throughout the 5-day study. The growth rate of the virus-treated cultures was approximately the same at first but then, after about 48 hr, increased abruptly to a PDT of about 42 hr. In longer experiments, growth of the altered cells continued at an approximately constant exponential rate for as long as 2 months (12). In contrast, untreated control cells died in 2 or 3 weeks under the conditions of study.

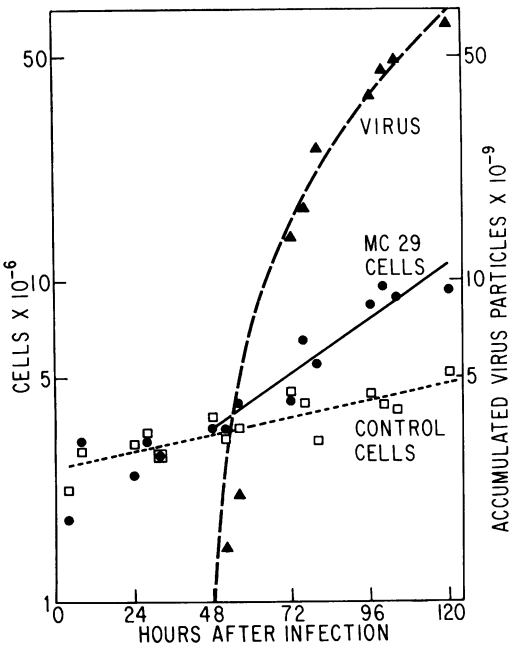

FIG. 1. *Characteristics of cell growth and virus liberation. Cultures in 100 × 20 mm dishes containing 4 × 10⁶ cells were exposed to 33 strain MC29 virus particles per cell. Medium in these and corresponding control cultures was changed at 24-hr intervals, but the cells were not transferred to new dishes during the 5-day study. At 4, 8, and 24 hr after each medium change, a group of cultures was harvested for cell count and virus particle count in the culture fluid. Each control-cell point represents the mean of counts of four dishes, and each MC29 cell and virus point show the mean of two cultures. Straight lines were fitted to the cell data by the method of least squares, with the 4- to 120-hr values for control cells and the 48- to 120-hr values for MC29-treated cells. The line was drawn through the virus points as previously described (2), on the assumption of logarithmic growth of the MC29 cells at the rate shown (42-hr PDT) and a constant rate (kv) of virus liberation (141 virus particles per cell per hr). Apparent virus particles in the supernatant fluids of the control cultures never reached the base line of the graph.*

Virus particles free in the medium of the virus-treated cultures first reached levels sufficient for counting by electron microscopy (about 10^7 particles per ml) at about 48 hr. Values of the accumulated numbers of virus particles liberated thereafter are charted in Fig. 1. The characteristics of the average rates of virus appearance in the culture fluid are indicated by the close fit of the experimental points to the curve drawn (2) on the assumption of a constant rate of particle liberation per cell per hour and logarithmic cell growth. The *kv* (2) calculated for the interval of 48 to 120 hr in this experiment was 141 particles per cell per hr. That this *kv* was not fully representative of all particles liberated has been indicated in other experiments. Electron micrographs of cells sectioned in situ attached to the dishes of similar cultures (U. Heine, *unpublished data*) revealed many particles attached to the surface of the dish and trapped about and between adjacent cells. Such particles can be suspended also by trypsinizing the cultures.

The data from experiments on the time course of virus liberation could also be plotted, as in Fig. 2, to emphasize differences in the rates of virus output related to time after change of culture medium. As in other experiments, virus liberation was demonstrable in the cultures at about 48 hr, and it appeared that the rate of liberation was greater in the first daily 8-hr period than in the last 16 hr. Total numbers of virus particles per culture increased in the three intervals between daily changes of the medium from 48 to 120 hr of culture (Fig. 3). The highest concentration attained was about 2.5×10^9 particles per ml in the interval between 96 and 120 hr. Occasional particles of approximately the size of virus were evident in some fluids from cultures of normal cells.

Kinetics of morphological alteration. As reported earlier (12), morphological alteration of the cells may be detectable within about 48 hr after exposure to virus and essentially complete at about 3 days. For a detailed study of this feature of response, virus-treated cultures prepared with three cover slips in each dish were terminated at successive intervals, and the cover slips were removed and stained. Counts were made of 500 cells in random areas of each cover slip, and the averages of the three values for each interval are given in Fig. 4. Since the percentage of morphologically altered cells as a function of time appeared to follow a sigmoid curve, the data were

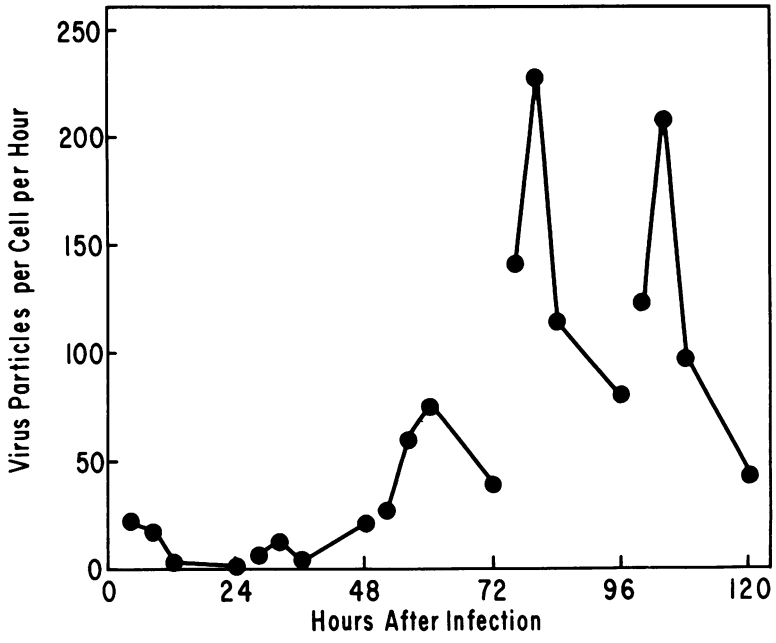

FIG. 2. *Rate of virus particle liberation relative to change of culture fluid at 24-hr intervals in an experiment similar to that of Fig. 1. Cell and virus-particle counts were made on two cultures at 4, 8, 12, and 24 hr.*

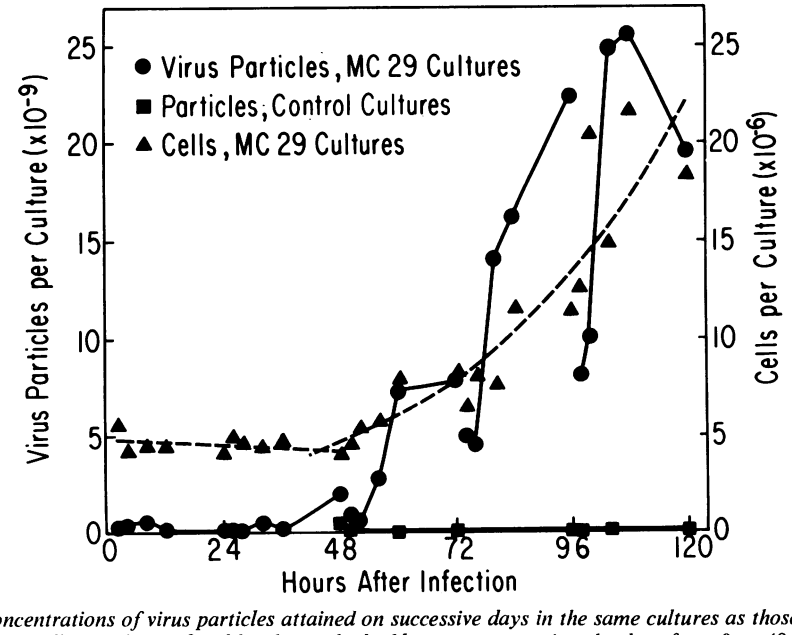

FIG. 3. *Concentrations of virus particles attained on successive days in the same cultures as those of Fig. 2. The line representing cell growth was fitted by the method of least squares, using the data from 0 to 48 hr and from 48 to 120 hr separately.*

first plotted on a probit scale, and a straight line was fitted by inspection. The values from this straight line were then transferred to the linear scale of Fig. 4 to yield the sigmoid curve fitted to the points. The good agreement of the experimental values with the curve derived in this way indicates a normal distribution of individual cell response manifested by morphological alteration.

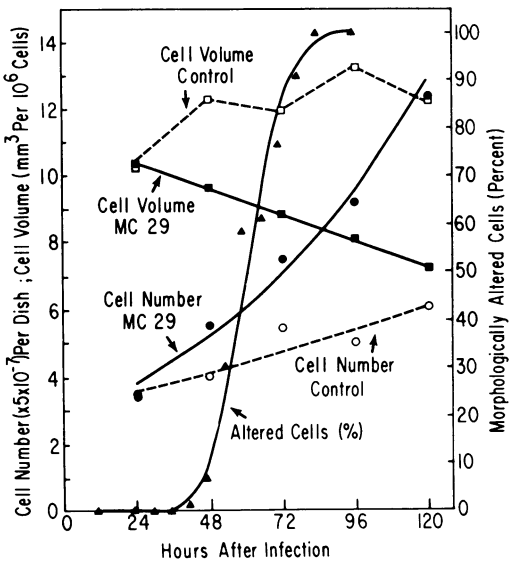

FIG. 4. *Rate of morphological conversion and changes in volume and growth rate of CEC exposed to strain MC29 at a multiplicity of 33 virus particles per cell. Estimates of percentage of converted cells were made in triplicate, and all other points are the means of estimates on two cultures. Culture fluid was changed at 24-hr intervals without transfer of cells.*

In this study, morphological change was first noted at about 42 hr and was uniformly complete at about 80 hr, as estimated by this method of examination.

Alteration of cell volume. Inspection of the converted cells had suggested that the altered elements were smaller than the nontreated cells. This was investigated in the experiment just described with a series of 10 cultures exposed to virus and 10 kept as controls. Medium was changed at 24-hr intervals, and two cultures for each group, virus-treated and not treated, were trypsinized for cell count and estimate of cell volume. As suspected (Fig. 4), there was a marked decline in the mean volume of the virus-treated cells which was closely related chronologically to the increase in the rate of cell growth. Control cells and those exposed to virus exhibited a PDT of about 112 hr for about 24 hr after introduction of virus, but thereafter, in the 4-day period, the PDT of the infected cultures decreased to about 54 hr. In this interval, decline in cell volume was inversely related to increase in cell number. Observations were extended for 24 days with cultures of cells derived from the same embryo stock as those used in the study of Fig. 4, but trypsinized and transferred as necessary. In this series (Fig. 5), the mean volume of the infected cells continued to decline for 9 days, and at this time the value was 4.5 $mm^3/10^6$ cells as compared to 10.2 $mm^3/10^6$ control cells. Little further change occurred in the remaining 15 days of observation.

Changes in nucleic acid and protein. The marked changes in cell growth rate, size, and morphology indicated that substantial changes in cell metabolism were occurring. It was of interest to examine the time course of such changes, to detect those which might be associated with the various events of infection, virus elaboration, and cell transformation. Exploratory experiments have revealed changes associated with morphological alteration but not with the processes of virus infection.

Analyses of nucleic acids and related components in three experiments yielded consistent results. Some of the data obtained in studies on the cultures of Fig. 1 are shown in Fig. 6 and 7. Throughout the 5-day study, DNA amounts remained essentially constant at the average level of about 3.3 $\mu g/10^6$ cells without evident differences between infected and normal cells. RNA content, in contrast to DNA, showed marked change in both control and virus-exposed cells during the course of the study. RNA amount, in comparison with that of DNA (Fig. 7), increased to approximately 70% above the initial value in 3 days and then declined somewhat. The close parallelism of the data from the virus-treated and control cultures indicated that the changes were

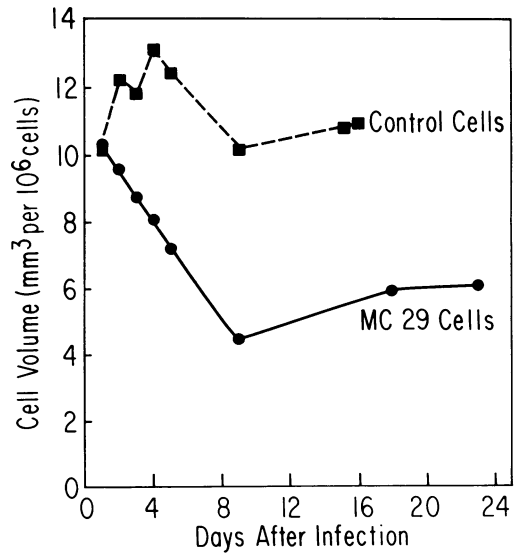

FIG. 5. *Mean cell volume of infected and control cells. The cell volume studies on the cultures (Fig. 4) were extended by trypsinizing and transferring the cells as necessary.*

related to the processes of adaptation of the cells to conditions in vitro without any influence of infection or morphological alteration.

Acid-soluble nucleotides, however, behaved differently. As in the case of RNA, the nucleotide pool increased sharply in both control and virus-treated cells for about 3 days (Fig. 6). In the MC29 cells, however, there was a marked decline between 3 and 5 days, whereas, in contrast, the high level was maintained in the control cells. The fraction containing nucleotides, which was not analyzed further, presumably contained nucleosides and bases as well. Nevertheless, nucleotides probably constituted the bulk of the total material measured by ultraviolet absorption (17).

Incorporation of ^{3}H-thymidine into DNA. The studies showed no increase in the cell content of DNA in either control or virus-treated cultures during the 5-day period of examination. To investigate further the metabolism of DNA, cultures were prepared from the same material and at the same time as those of Fig. 1 and 6, as indicated in Fig. 7. The rates of ^{3}H-thymidine incorporation increased slightly in both control and virus-treated cultures for approximately 2 days. In the interval between about 2 and 3 days, however, the specific activity of the DNA rose sharply and then leveled off after 80 hr. There was

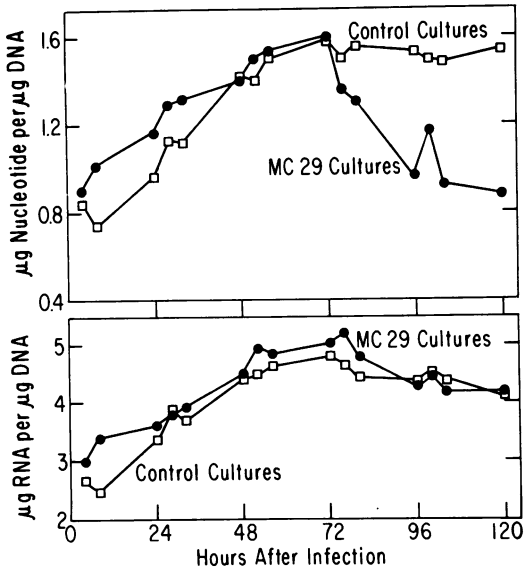

FIG. 6. *Acid-soluble nucleotide and RNA concentrations in MC29-treated and control cells in terms of the constant average value of 3.3 μg of DNA/10^6 cells. RNA and DNA analyses were made on the same cultures yielding the data of Fig. 1. Each point is the average of two single determinations on the respective duplicate virus-treated and control cultures.*

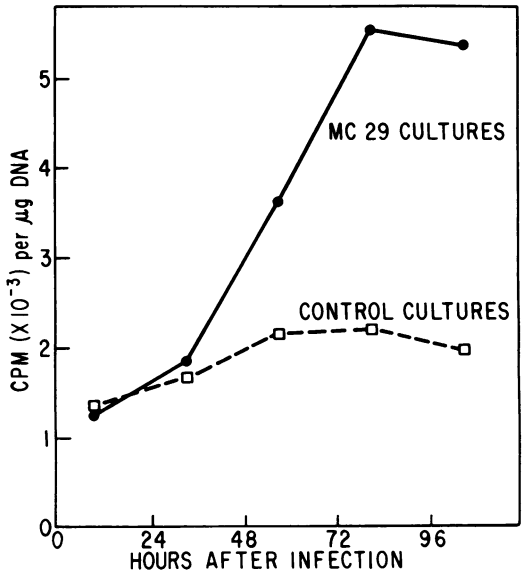

FIG. 7. *Incorporation of ^{3}H-thymidine into DNA of virus-treated and control cultures. The cultures were prepared in duplicate with the same materials and at the same time as those of Fig. 1 and 6. Culture fluids were changed, and the cells were exposed to 2 μc (per ml) of tritiated 5-methyl thymidine for 8 hr. Values for specific radioactivity are the average of the data from two cultures.*

but slight increase in the specific activity of the DNA of the control cultures in the 5-day period. At 80 and 104 hr, the specific activity of MC29 cell DNA was approximately 2.7-fold that of the control cells, which was in fair agreement with the relative rates of growth in the respective cultures (Fig. 1).

Disposition of ^{3}H-uridine added to cultures. Analyses of ^{3}H-uridine removal from the culture medium revealed major differences between the behavior of the control and that of the virus-treated cultures. Figure 8 discloses the results of studies with the same cultures yielding the results of Fig. 2 and 3. Measurements of radioactivity remaining in the culture fluid at intervals after addition of ^{3}H-uridine on the different days showed a nearly constant rate of label disappearance in the control cultures. Beginning on the 3rd day, however, there was a much increased initial rate of disappearance of label in the virus-treated cultures. This initial rate was then progressively greater from 3 to 5 days, and the time required for maximal removal of label declined from 12 to 8 to 4 hr. Nevertheless, the proportion of label disappearing from the medium on any day did not exceed 50% of that added in this study. By centrifugation of the used medium, it

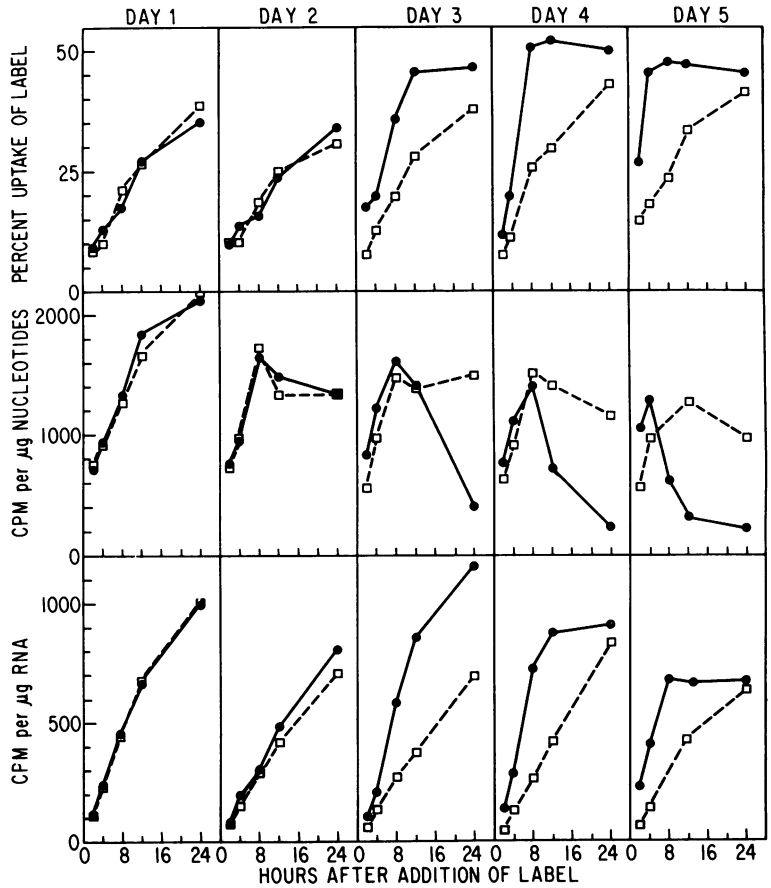

FIG. 8. *Analysis of the disappearance of tritium label from the extracellular medium and the incorporation of the label in the cellular nucleotide pool and RNA. The cultures were the same as those of Fig. 2 and 3 consisting of pairs, and the exposure multiplicity was 33 virus particles per cell. Medium was changed each day without cell transfer. Pairs of control and virus cultures were treated each day with 1 μc (per ml) of ^{3}H-uridine and terminated 2, 4, 8, 12, and 24 hr later. Samples of the media were analyzed for radioactivity, and uptake (upper curves) was calculated as the difference between the amount added and that found. The middle series of curves represents the radioactivity of the acid-soluble nucleotides extracted from the cells, and the lower series shows RNA radioactivity. Each point is the average of measurements on duplicate cultures. Symbols: □, normal cells; ●, virus-infected cells.*

was determined that less than 5% of the residual radioactivity could be accounted for in virus particles or sedimentable cell debris.

Marked differences were likewise observed in the specific radioactivity of the nucleotide pools of normal and MC29 cells (Fig. 8). In the control cultures, the specific activity of the pool increased throughout the initial 24-hr period of study. Beginning with the 2nd day and continuing throughout the remaining 4 days, however, the pattern changed somewhat, the activity increasing until 8 hr and then declining slightly. The virus-containing cultures behaved like the controls for the first 2 days in this respect, but later the increasing rate of label removal from the medium was accompanied by an increasingly rapid rise and fall of specific activity of the nucleotide pool, the fall beginning with the cessation of uptake. The term specific activity is applied somewhat loosely to the acid-soluble nucleotide pool, since the values are based not on the amount of uridine or uracil compounds alone, but on the total of all nucleotides, nucleosides, and bases, as measured by ultraviolet absorption.

The specific activity of RNA in the infected cells also began to exceed that of control cell RNA by the end of the 2nd day (Fig. 8). The initial rate of labeling of RNA from the 3rd through the 5th day was about two to three times more rapid in the infected cells than in the controls, but the

maximal level of specific activity reached was progressively lower. In relation to the cessation of uptake of labeled uridine and the rapid decline in the specific activity of the acid-soluble pool, which occurred at about the same time, the points at which specific activity of the RNA stopped increasing were delayed by about 4 hr.

Similar experiments, in which ^{3}H-cytidine was the labeled precursor or in which different cell concentrations were used, yielded qualitatively similar patterns of isotope uptake from the medium, as well as labeling of the acid-soluble pool and of RNA. The changes in the specific radioactivity of the RNA are dependent on several factors, including the rate of RNA synthesis and the specific activity of the uridine nucleotide pool. The latter depends upon the rates of uptake of ^{3}H-uridine from the medium and de novo synthesis of uridine, as well as incorporation of the ^{3}H-uridine into RNA and metabolism through other pathways. Further studies on the detailed composition of the nucleotide pool, as well as its metabolism, will be necessary for a better understanding of the marked differences between control and altered cells.

Additional studies were made to clarify the striking apparent limitations in uptake of ^{3}H-uridine from the medium by the MC29 cultures (Fig. 8). The amount of ^{3}H-uridine made available to identical cultures of fully altered cells made no difference in the proportion of label removed from the medium. When a portion of used medium was mixed with fresh unlabeled medium and offered to similar cell cultures, almost none of the residual radioactivity was taken up. Similar results were obtained when a control culture was used containing the same number of cells as the MC29 culture. It appears, therefore, that the radioactivity remaining in the medium probably represented metabolic products of a portion of uridine which was not utilized for RNA synthesis. About one-half of the residual radioactivity of the spent medium was volatile (40 C, atmospheric pressure) and probably was largely in the form of tritiated water.

Uptake and incorporation of ^{14}C-labeled amino acids. Little difference between the MC29 and control cultures was noted in the quantitative or qualitative aspects of the uptake of a ^{14}C-labeled amino acid mixture. The maximal amount of label removed from the medium was about 20% during any day in the period under study. The specific activity of the protein was similar in the control and virus-treated cells for the first 32 hr, but divergence was observed at 48 hr with the protein of the MC29 cells showing a greater incorporation of label. This time corresponded to the beginning of increased growth.

DISCUSSION

Investigation of response of primary CEC cultures to MC29 strain avian leukosis virus has disclosed cultural changes of two principal kinds: (i) behavior of the virus-exposed cells paralleling that of the control cultures related presumably to environmental effects on all cells newly introduced to in vitro conditions; and (ii) activities of the virus-exposed cells differing from those of the controls and thus related to virus-cell interaction.

A variety of minor changes occurred in all cultures in the first few days of each study. It should be emphasized that the experiments were made with primary cultures exposed to virus only 24 hr after preparation from the embryo. In the initial interval of approximately 24 to 48 hr after introduction of virus into the cultures, there was slow growth of both control and virus-treated cultures at rates of about 130-hr PDT. This was continued by the control cells for the 5-day study period in contrast to the change to rapid growth of virus-exposed cells occurring at about 24 to 48 hr. In both infected and control cultures, the amount of DNA per cell remained essentially constant during the 5-day study, while some increase in RNA per cell was observed in both cultures.

Among the various aspects of response to MC29 strain, the most striking was the alteration in cell morphology which differed markedly in its rapidity and uniformity from that of CEC responding to other avian leukosis virus strains (4, 12; S. Sankaran, Ph.D Thesis, Duke University, Durham, N.C.; A. J. Langlois, S. Sankaran, and J. W. Beard, *in preparation*). A characteristic feature of the morphological response to exposure to a sufficient number of virus particles was the speed of onset and, of more importance, the speed of alteration of most or all of the cells in the cultures. Under suitable conditions, such as those in the experiment of Fig. 4, alteration began at about 40 hr, and the increase in percentage of altered cells to about 80 hr followed a sigmoid curve, indicating a normal distribution of response of individual cells in the total population. The relative rapidity and completeness of morphological alteration of the culture, requiring an interval corresponding only to about one PDT, suggested that most or all of the cells of the culture were infected initially and were altered as a direct consequence of that infection. For additional evidence on this point, a few experiments were done with cells exposed to virus, washed, and plated immediately on irradiated feeder CEC

(A. J. Langlois and D. P. Bolognesi, *unpublished data*). Although the plating efficiency was only 8 to 10%, every clone of about 1,000 examined consisted entirely of altered cells. Further study is necessary, however, to establish unequivocally the proportion of cells initially exposed to virus which become infected and respond directly by morphological alteration.

It was notable that all other aspects of change not paralleling the behavior of the control cultures were remarkably closely related to the onset of morphological alteration. Most noticeable was the decrease in cell size. That the change in size was not fully related to other aspects of alteration was indicated by continued decline in cell volume for several days after the other characteristic changes in cell morphology were apparently complete. Decrease in size was considerable, reaching levels less than 50% of the volume of the control cells. There were also minor changes in the size of the control cells.

Attending these morphological alterations was the simultaneous major increase in growth rate corresponding to a PDT of approximately 30 to 50 hr, as compared with about 130 hr for the control cells. The higher rate was attained abruptly but continued at a fairly constant level of exponential growth for as long as 2 months (12), whereas the control cells did not survive for more than 2 to 3 weeks under the conditions of the experiments.

Concurrent with the increase in growth rate were increases in the rates of DNA and RNA synthesis and changes in uridine uptake and specific activity of the nucleotide pool. There was a smaller increase in the rate of protein synthesis. Amounts of DNA and RNA per cell remained essentially constant.

Virus liberated and suspended in the culture medium reached concentrations sufficient for electron microscopic count at about 48 hr. Thereafter, the rate of output was essentially constant at the level (Fig. 1) of about 140 particles per cell per hr. That this did not represent the total output has been evident from electron micrographs showing virus lying among the cells after withdrawal of the fluid and by examination of fluids from trypsinized cultures. Although attainment of the particle concentration requisite for electron microscopic study coincided approximately with beginning morphological change, it is unlikely that the high output of virus was related to cell alteration. Virus is liberated at similar intervals after exposure to BAI A, R, ES4, and RPL 12 leukosis virus strains long before morphological alteration is recognizable (12; A. J. Langlois, S. Sankaran, and J. W. Beard, *in preparation*). The relationship in MC29 cultures would appear to be entirely fortuitious and caused only by the speed of morphological change. Preliminary studies (A. J. Langlois and D. P. Bolognesi, *unpublished data*) indicated that measurable infectious MC29 virus began to appear within 18 hr after exposure to virus, which was about 30 hr before the onset of alteration.

The rate of virus output by the MC29 cultures was in the same range as that observed with CEC cultures infected with the other leukosis viruses. Rates as high as 500 to 800 particles per cell per hr have been observed (12) in occasional experiments with strain R and as low as 2 to 10 particles per cell per hr with strains ES4 and RPL 12. The rate of virus liberation appeared to be strongly dependent on the particular embryo from which the host cells were derived, as well as on the infecting virus strain. Variation in virus-synthesizing capacity among cells from different birds had been observed earlier in studies on infection of bone marrow by BAI strain A (1).

The response of CEC to strain MC29 is of particular interest, on the one hand, because of the rapid continuity of the processes of infection and morphological alteration. In addition, the behavior of the cultures is the more remarkable, since no other avian leukosis strain has been observed to induce changes in this manner in chick embryo cell cultures. Although the strain MC29 may be complex, none of the studies thus far, either in the chicken or in tissue culture, has given evidence of a sarcoma virus component. It is thus evident that the influence of this virus, responsible for neoplastic response principally of hematopoietic tissue in the intact host, does not differ in principle from that of sarcoma-inducing agents under in vitro conditions. The effects of MC29 on CEC exhibited similarities to those exerted by RSV, but differences were also evident. MC29-altered cells were smaller and grew much more rapidly than control cells, in contrast to RSV-altered cells which may be larger and grow more slowly than uninfected cells (8, 20–22). The rapid morphological alteration of all the cells in a culture by MC29 occurred within an interval corresponding essentially to a single PDT.

Exploratory biochemical studies have revealed differences between the metabolism of MC29-altered cells and that of the control cells. Analogous investigations with RSV systems have yielded conflicting results (8, 21) which preclude comprehensive comparison of the respective activities of the RSV and MC29 cultures with present information. The studies of DNA and RNA in MC29-infected cells served to outline the relationship of nucleic acid metabolism to morpho-

logical alteration, to locate the changes in time, and, thus, to serve as a basis for more detailed investigations of this important phenomenon. The fact that no differences in nucleic acid amount or synthesis were seen in relation to the establishment of infection or particle liberation was to be expected. The amount of RNA and protein required for virus production is extremely small compared to that used in continuing cell growth (30).

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