Component of Strain MC29 Avian Leukosis Virus with the Property of Defectiveness

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Three clones of morphologically altered cells (L⁻MC29) of singular properties were isolated from MC29 (subgroup A) leukosis virus-infected chick embryo cells. Supernatant fluids from cultures of the cloned cells produced no transforming or interfering activity on chick embryo cells susceptible to known avian leukosis-sarcoma viruses. No virus associated with the cells was demonstrable by fluorescent-antibody staining or by electron microscopy. All L⁻MC29 clone cells were activated, however, by four strains of Rous-associated viruses (RAV) representative of A, B, C, and D subgroup avian leukosis viruses and by two strains of MC29 virus. Virus L⁻MC29 cells activated by superinfection with RAV-1 and RAV-2 was characterized by helper-dependent and helper-independent properties. These findings suggest that the strain MC29 leukosis virus, or a component thereof, possesses properties of defectiveness similar to those of the Bryan high-titer Rous sarcoma virus.

Strain MC29 leukosis virus (12) induces neoplasms (18) in the chicken involving myeloid, prythroid, renal, and hepatic tissues. MC29 strain likewise produces marked, rapid morphological alterations in chick embryo cell (CEC) monolayers (16) and foci (14) similar to those caused by Rous sarcoma virus (RSV) and proportional to the input virus particle number (15).

Complexity of strain MC29 was demonstrated more recently, however, by isolation from MC29 virus preparations of a component (MC29-AV; reference 17) which does not produce foci in CEC monolayers. Furthermore, the non-focus-forming agent does not cause myeloid or other MC29-induced neoplasms with the exception of erythroblastosis and occasional lymphomatosis (17). This suggested further possible relationships of virus types in strain MC29 such as those between the defective Bryan high-titer RSV and its associated helper agents. Thus far, no component with the properties ascribed to defectiveness has been recognized in leukosis virus preparations. Application of techniques like those used with RSV in this respect, however, have yielded an agent with the behavior of defectivenss as characterized by the induction of cells lacking the capacity to produce infectious virus. This report describes isolation and characterization of the agent.

MATERIALS AND METHODS

Viruses. Standard strain MC29 contains avian virus of subgroups designated as A and B (28). Specimens of pure MC29-A (R. E. Smith, *personal communication*) and MC29-B subgroups cloned by end point dilution in phenotype C/A and C/B CEC cultures were generously provided by R. E. Smith. Subgroup MC29-A was used for isolation of L⁻MC29 cells. Two nontransforming or noncytopathogenic agents, MC29-AV(L) isolated previously by A. J. Langlois (17) and MC29-AV(i) isolated from the standard MC29 strain in the present work, were also used. The subgroup aspects of MC29-AV(L) were not determined, but MC29-AV(i) was identified as an A subgroup agent (R. Ishizaki, *unpublished data*). Rous-associated viruses (RAV), RAV-1, RAV-2, RAV-7, and RAV-50, representative of A, B, C, and D subgroup RAV, respectively (2), were kindly supplied by P. K. Vogt and H. Hanafusa. Three pseudotypes of Bryan high-titer RSV(RAV-1), RSV(RAV-2), and RSV(RAV-50) were used as indicator viruses. The transforming agent B77 of subgroup C was obtained from P. K. Vogt (2). All virus stocks were prepared as described previously (11).

Cell culture and virus assay. Cultures of C/O cells were prepared from 10-day-old chick embryos free of Rous resistance-inducing factor (RIF; reference 19). RSV (19), B77 (2), and MC29 viruses (17) were assayed by focus formation in CEC monolayers. The other cell phenotypes (C/A, C/B, and C/AB) were prepared from eggs of line 7 and line 16 chickens supplied by the Regional Poultry Research Labora-

tory, East Lansing, Mich. Cell cultures of Japanese quail (Shamrock Poultry Farm, North Brunswick, N.J.) were prepared from 10-day-old embryos (6), and secondary cultures served for assay of RSV (0) (26) and test for the possible presence of an MC29(0) virus.

Leukosis virus-negative transformed cells. Beta type L⁻R (leukosis virus-negative) Rous sarcoma cells (6) were obtained from H. Hanafusa. Leukosis virus-negative MC29 transformed cells (L⁻MC29) were isolated by the methods of Hanafusa (5) as modified by Vogt (24) from single foci produced in C/O cultures infected with one or two focus-forming units of strain MC29-A sonically treated for 3 min to disperse possible virus aggregates. The cultures were overlaid 6 hr later with 0.8% agar medium containing 0.5% high-titer MC29-A chicken antiserum no. 999 (3). After 7 days, transformed cells from single MC29-A foci aspirated under a microscope into a capillary pipette inserted through the agar were inoculated onto fresh RIF-free C/O secondary CEC cultures containing 1.2×10^6 cells. From these cultures, transferred serially at 2- to 3-day intervals, L⁻MC29 cell lines designated as clones a, b, and f were obtained. Although these lines grew more readily than L⁻R cells without addition of normal feeder CEC, transfer after the 23rd passage was difficult, and fresh RIF-free C/O CEC were added once at the 23rd passage in the course of the study.

Antisera. Immune serum no. 999 was produced by initial intramuscular injection into the chicken of Formalin-inactivated MC29 virus followed by active agent given intravenously after 7 weeks. Serum was taken 10 days later (3). RAV-1 (no. 399) and RAV-2 (no. 295) immune sera were produced by the intravenous inoculation of 4-week-old chickens with appropriate active virus (11).

Interference. Interference capacity was tested by infecting CEC with RAV-1, RAV-2, or other materials and challenging with appropriate pseudotypes of RSV or with B77 and MC29 viruses after successive transfers (19).

Neutralization. A constant amount of virus was incubated with 10-fold dilutions of serum for 40 min at 37 C. Virus not inactivated was assayed on CEC by focus count (11).

CF test. For group-specific (gs) antigen detection, micro-complement fixation (CF) tests were used. For the antigen preparation, suspensions containing 1.2×10^7 of a, b, and f L⁻MC29 cells in 0.25 ml of phosphate-buffered saline were treated with three cycles of freeze-thawing and extracted with ether. The aqueous phase was used as gs antigen. The tests were made with two units of anti-gs antigen pigeon serum derived from pigeons with tumors induced by Schmidt-Ruppin strain Rous sarcoma virus (SR-RSV; reference 21). The antibody titer was determined in preliminary checkerboard experiments with an extract of SR-RSV chicken tumor as the antigen. The results are cited as the last dilution of antigen fixing two units of complement in the presence of two units of hemolysin.

FA staining. For fluorescent-antibody (FA) staining, chicken immune serum was conjugated (1) with fluorescein isothiocyanate (BBL). Infected cultures and L⁻MC29 cells were stained on cover slips without prior fixation which, as shown in analogous work by others with RSV (27), reveals only surface antigens or extracellular, cell-associated virus.

Morphology. Cells grown on cover slips placed in the culture dishes were fixed in Carnoy's solution and stained with May-Grünwald-Giemsa. Cultures were examined also by phase-contrast microscopy without fixation and staining.

For electron microscopy, cells grown in monolayers in Leighton tubes were fixed in situ by diluting the culture fluid with an equal volume of 5% glutaraldehyde and prepared further for examination as described (10).

Virus particle enumeration. Culture fluids were diluted with glutaraldehyde to 1% concentration of the fixative and stored in the cold. All counts were made in this laboratory by the method of electron microscopy of virus particles sedimented on agar (22).

RESULTS

Morphology of L⁻MC29 cells. Morphologically altered cells cloned from single foci and inoculated onto normal C/O CEC proliferated in three dishes (a, b, and f) but showed no evidence of liberation of infectious MC29 virus or MC29-AV in subsequent test experiments. For purposes of description, the cells of these clones and their derivatives were designated as L⁻MC29 as representative of leukosis virus-negative MC29-A-infected cells somewhat in accord with the terminology, L⁻R, employed relative to leukosis virus-negative RSV cells (6).

Despite apparent absence of virus liberation, the L⁻MC29 cells differed from uninfected CEC (Fig. 1) in the same principal features (Fig. 1) of basophilic, vacuolated cytoplasm and deeply stained, rounded nuclei with one or two large, very prominent nucleoli like those characteristic of MC29 virus-altered CEC (10). Nevertheless, the a, b, and f clones varied from one another in some respects. Cells of the f clone were most uniformly transformed and sometimes were piled up in clumps. The b clone produced fewer transformed cells scattered about the normal cell population. The a clone (Fig. 2) resembled the f clone, but the transformed-cell growth was not as prolific as that of the f clone cells. Because of these attributes, stained altered cells were easily distinguished from the normal feeder cells. Differential counts of the stained cells showed proportions of the a, b, and f L⁻MC29 cells of 78, 43, and 92% of the total respective populations of transformed and normal feeder cells.

As with L⁻R cells (8), L⁻MC29-altered CEC could be assayed by focus formation on normal secondary cells. Cultures (60-mm dishes) con-
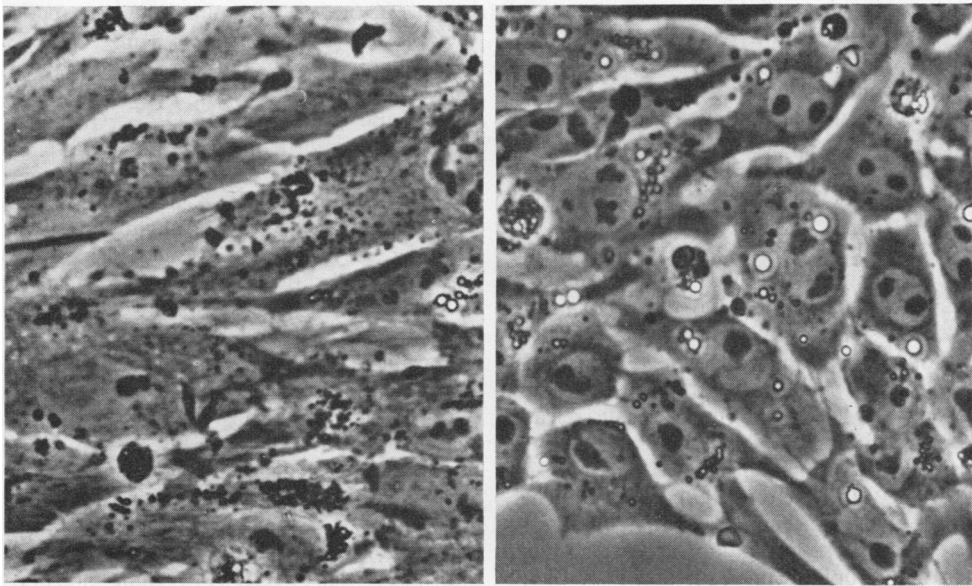
FIG. 1. *Phase-contrast micrographs of uninfected chick embryo cells (left) in culture and of chick embryo cells of clone a (L⁻MC29) morphologically altered (right) after infection with strain MC29. Altered cells, L⁻MC29, showing angular but smooth peripheral contours and large, clear nuclei with one or more greatly enlarged nucleoli did not produce detectable virus.* $\times$ *600.*
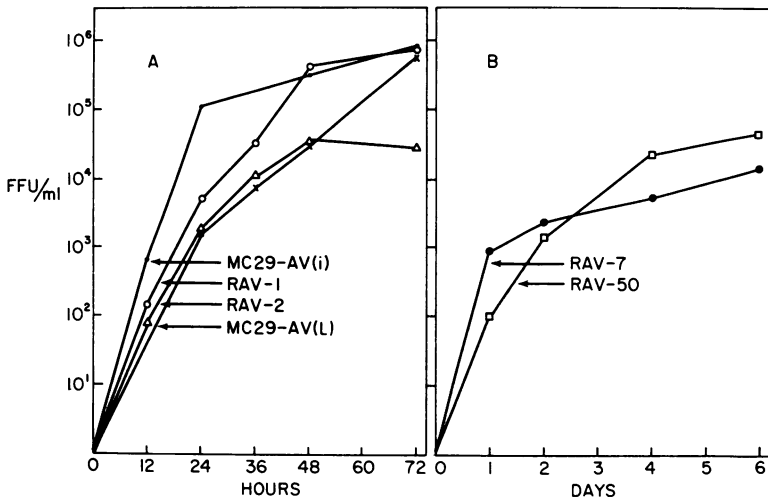

FIG. 2. *Virus liberation from L⁻MC29 cells activated (A) by RAV-1, RAV-2, MC29-AV(i), and MC29-AV(L) and (B) by RAV-7 and RAV-50.*

taining about 5×10^5 CEC inoculated with 0.1 ml of appropriately diluted altered-cell suspensions were overlaid with agar medium, and, after the 7 days, altered-cell colonies were counted as foci. The results showed that about 1:50 (2.6, 1.76, and 2.1%) of the total number of a, b, and f clone cells, respectively, responded as foci.

Failure to detect leukosis virus by interference test. Despite cell morphological alteration, no

focus-forming MC29 virus was demonstrable in the culture fluid from the various clones. The possible presence of a nontransforming agent like, or behaving as, MC29-AV (17) was investigated by interference tests (19) with challenge agents: RSV(RAV-1), RSV(RAV-2), B77 sarcoma virus, and RSV(RAV-50), representative, respectively, of A, B, C, and D subgroup viruses (2). No significant positive interference was observed in cultures inoculated with 1 ml of

the respective culture fluids from a, b, and f clones taken at the 20th transfer.

Physical virus particle count. Counts of virus particles in fluids from 13 samples of beta type L⁻R (6) cell cultures revealed an average of 1.5×10^8 particles per ml. However, counts of particles in the approximate size range of the virus in samples from L⁻MC29 cells varied from 2.4×10^6 to 5.9×10^7 per ml which were at or below the limits (17) of the counting method. Comparable cultures of CEC infected with standard MC29 virus and morphologically altered to the same extent as the cells of the a, b, and f clones would have contained about 10^9 particles per ml (15). It was thus evident that the particles observed did not indicate release of virus from the L⁻MC29 cell cultures.

Electron microscope observations. L⁻MC29 cells differed distinctly from the normal elements by high content of free cytoplasmic ribosomes, poorly developed rough endoplasmic reticulum, frequent fat droplets, and smooth cell membranes with few pseudopodia or membrane protrusions. The greatly enlarged nucleolus exhibited the characteristic distributions of component A dense granules in relatively high concentration. A well-defined fibrillar component B and an amorphous component C were also observed. These aspects of ultrastructural morphology of all a-, b-, and f-clone L⁻MC29 cells were indistinguishable from those of MC29 virus-altered CEC described previously (10).

Nevertheless, examinations of many sections and fields about the altered cells of all L⁻MC29 clones, both in Leighton tubes and in pellets from standard cultures, revealed no virus particles outside the cells, on the cell membranes, or in cytoplasmic vacuoles where virus is profuse in usual CEC cultures infected with MC29 virus.

FA staining. Nonfixed leukosis virus-infected CEC are much more sensitive (13, 23) than fixed cells to FA staining for detection of virus envelope antigen at the cell surface. The same occurred, also, with fluorescein-conjugated MC29 immune chicken serum no. 999 in the detection of MC29-A virus antigen. However, there was no evidence in the present work that FA was bound to the surface or any other aspect of L⁻MC29 cells of the a, b, or f clones. This negative result obtained with L⁻MC29 cells like those observed by others (24) with L⁻R cells emphasized the parallelism with the behavior of RSV NP cells.

Group-specific gs antigen. With only negative results thus far described (infectivity, interference, FA-staining, and electron microscopy) for evidence of cell infection other than morpho-

logical alteration, examination was made for the possible presence of gs antigen like that occurring in MC29-altered CEC. Such antigen can be demonstrated in L⁻R CEC by CF test with serum from hamsters (30) or pigeons (21) bearing Schmidt-Ruppin virus tumors. With such immune pigeon serum, the antigen prepared from L⁻MC29 a, b, and f clone cells as described above gave very slightly positive CF tests for gs antigen in dilutions of only 1:2, whereas preparations from control cells used as feeder CEC for isolation of L⁻MC29 cells yielded entirely negative results. In comparison, preparations of gs antigen from a-cloned L⁻MC29 cells superinfected with MC29-AV(i) or beta type L⁻R cells gave positive results in dilutions of 1:64 and 1:8, respectively, in similar tests. The findings suggested that L⁻MC29 CEC might be positive for gs antigen but only in very low titer.

Activation of L⁻MC29 cells by superinfection with RAV and MC29-AV. Superinfection of L⁻R cells with RAV or other avian leukosis RSV helper viruses activates their capacity to synthesize RSV (7) with envelope properties controlled by the helper virus (4). Potentials for comparable activation of L⁻MC29 cells were tested with RAV of different subgroups and with MC29-AV(L) and MC29-AV(i) isolated from MC29 stock virus. L⁻MC29 CEC seeded (10^6 per plate) in 60-mm dishes were inoculated with 0.1 ml of respective undiluted virus stocks containing about 10^7 to 10^8 infectious units assayed by interference test. After 3 hr of incubation, the medium was removed, the cells were washed twice gently with 5 ml of cold complete medium, and incubation was resumed after addition of 5 ml of pre-warmed complete medium. Samples of fluid taken at intervals were replaced with equal volumes of fresh medium.

Because of the somewhat slow elaboration of the RAV of the C and D subgroups, however, treatment of these agents was different from that of the other viruses. Three hours after inoculation of 10^6 L⁻MC29 cells with approximately 10^5 infectious units of the agents, the medium was changed. These cultures were then transferred on alternate days with daily medium changes. The culture medium removed was stored at −85 C for activated-virus assay and other studies.

Superinfection of L⁻MC29 cells of a-clone with these RAV and MC29-AV resulted in liberation of virus (Fig. 2) which was infectious for CEC and induced the same morphological alteration and focus formation in cell monolayers as did the original MC29 strain. As observed in analogous studies of L⁻R cells (20), this influence of the newly synthesized virus on the infected cell was

independent of the activating agent. These and similar findings with CEC of clones b and f demonstrated the consistent release of infectious virus by L⁻MC29 cells superinfected with the A, B, C, and D RAV subgroups and MC29-AV. Continuing studies both in tissue culture and in chicken cells indicate that the activated virus is pathogenically indistinguishable from strain MC29.

Envelope properties of virus liberated by activated L⁻MC29 cells. As already reported, the envelope properties of RSV are determined by the helper virus (4) shown (29) by intraspecies host range, interference patterns, and antigenic specificity. Tests were thus made of the corresponding behavior of agent liberated by L⁻MC29 cells activated with RAV-1 and RAV-2.

In host range studies, C/O, C/A, C/B, and C/AB CEC were inoculated with RSV(RAV-1), RSV(RAV-2), B77, MC29(RAV-1), and MC29 (RAV-2). Virus from cells activated with RAV-1 and RAV-2 showed the same host range as RSV(RAV-1) and RSV(RAV-2), respectively.

Interference tests were made with C/O CEC preinoculated with RAV-1 and RAV-2 and transferred twice. Challenge of these cells with RSV (RAV-1), RSV(RAV-2), B77, and virus from activated L⁻MC29 cultures showed (Table 1) that the virus yielded by activation with RAV-1 and RAV-2 exhibited the same behavior as RSV(RAV-1) and RSV(RAV-2), respectively.

In the neutralization study, anti-RAV-1 and anti-RAV-2 chicken immune sera were used in cross tests against RSV(RAV-1) and RSV(RAV-2) and, as well, newly activated MC29(RAV-1) and MC29(RAV-2). Table 2 shows that MC29 virus from the three clones (a, b, and f) activated with RAV-1 and RAV-2 were neutralized with anti-RAV-1 and anti-RAV-2 immune sera, respectively. It is thus evident from these results and those of the host range and interference studies that the envelope properties of MC29 virions derived from L⁻MC29 cells activated by superinfection with RAV-1 and RAV-2 were controlled by helper virus in the same manner as RSV under similar conditions.

Noninfectivity of virus for quail embryo cells. Infective RSVβ(0) virus prepared from L⁻Rβ

TABLE 1. *Patterns of interference between RAV-1 and RAV-2 and virus from L⁻MC29 clone a activated by RAV-1 and RAV-2*

Interfering virus	Challenge viruses and dilutions							
	RSV(RAV-1)		RSV(RAV-2)		B77 10^{-1}	aMC29(RAV-1)		aMC29 (RAV-2) 10^0
	10^{-1}	10^{-2}	10^0	10^{-2}		10^0	10^{-1}	
None	TNTC[a]	1,045[b]	TNTC	300	688	NT	205	150
RAV-1	0	NT[c]	NT	600	533	0	NT	469
RAV-2	NT	885	0	NT	460	NT	211	0

[a] Too numerous to count.
[b] Foci per plate (average of two plates).
[c] Not tested.

TABLE 2. *Cross-neutralization tests with L⁻MC29 virus from a, b, and f clones activated by RAV-1 and RAV-2 against anti-RAV-1 and RAV-2 immune sera*

Virus	Immune serum[a]					
	Anti RAV-1 No. 399			Anti RSV-2 No. 295		
	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
RSV (RAV-1)	<0.001	<0.001	0.23	1.10	1.08	1.02
aMC29 (RAV-1)	<0.01	0.02	0.08	0.88	0.86	0.84
bMC29 (RAV-1)	<0.01	0.04	0.22	1.08	1.13	0.91
fMC29 (RAV-1)	<0.01	<0.01	0.07	0.92	0.94	0.97
RSV (RAV-2)	1.03	1.01	1.07	<0.001	0.05	0.35
aMC29 (RAV-2)	0.95	0.93	1.11	<0.01	0.03	0.17
bMC29 (RAV-2)	1.01	0.91	0.99	<0.01	<0.01	0.29
fMC29 (RAV-2)	1.24	1.02	1.11	<0.01	<0.01	0.21

[a] Values expressed as fraction of virus survivors at the serum dilutions indicated.

cells can be consistently assayed on Japanese quail embryo cells (5, 26). Whether a similar infectious MC29(0) virus might be produced by L⁻MC29 cells was tested by inoculation on quail embryo cells. Eight quail embryo cell cultures susceptible to RSVβ(0), RSV (RAV-1), and MC29 (RAV-1) showed no focus formation by possible MC29(0) materials obtained from any of the L⁻MC29 cells derived from the a, b, and f clones.

DISCUSSION

Virus behavior defined as defectiveness has been observed with a variety of agents including viruses responsible for some avian and murine neoplastic diseases. Much work with RSV has resulted in the isolation of the virus-infected, morphologically altered cells, L⁻R, which produce virus either of very low or no infectivity. Analogous studies of a subgroup of MC29 avian leukosis virus yielded cells, L⁻MC29, or properties resembling those of L⁻R. The presence of virus associated with these cells could not be detected either by infectivity studies in various systems, interference assays, FA staining, or by electron microscopic analysis. The results of the studies thus far suggest that strain MC29 may be defective in the same manner as RSV. It has not yet been determined, however, whether a nondefective MC-29 component also exists in the population.

More recent investigations of RSV have indicated the existence of two types of CEC distinguishable by the respective capacities to produce infectious (C/O) or noninfectious RSV (0) (C/O′). Therefore, the distinction between C/O and C/O′ CEC is of much importance for complete characterization of L⁻R cells based on the criterion of production of infectious RSV(0) (9). Such study was not made in the present work and, in consequence, the L⁻MC29 CEC were not as fully characterized as L⁻R cells. Nevertheless, the results described may be interpreted to suggest that these L⁻MC29 cells resemble more closely the L⁻Rβ′ or the L⁻Rα rather than those of the L⁻Rβ type as indicated by the failure to detect an infectious MC29(0) virus on quail embryo cells.

It is characteristic of RSV that the sarcomagenic potential is preserved in L⁻R cells in the defective virus state (8) and the nontransforming or nontumorigenic RAV can effect synthesis of the complete, infectious RSV in these cells (4). Such infectious RSV is characterized, as noted, by both helper-dependent and helper-independent properties (25). Although entrance of RSV into the host cells is determined by the helper-controlled character, its cell-altering or tumorigenic activity is determined by the helper-independent character (29). The results with MC29 virus from activated cells indicate that the agent is of biphasic character as manifested by helper-independent or helper-dependent behavior. Morphology of the altered cells infected by activated MC29 virus is of helper-independent character, whereas the envelope properties are helper-dependent in parallel with the corresponding behavior of RSV (25).

The finding that the same manifestations with respect to RAV are paralleled by the MC29 leukosis virus investigations involving the defective state might provide opportunities for analyses of tumorigenic or leukemogenic attributes of this leukosis virus by application of methods useful with RSV. Studies of this aspect of the problem are underway, and the results will be described in another report.

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