Enhanced Growth of a Murine Coronavirus in Transformed Mouse Cells

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Plaque formation by A59 virus, a murine coronavirus, was facilitated in AL/N and Balb mouse cells transformed by polyoma virus, simian virus 40, murine sarcoma virus, or mammary tumor virus. In these virus-transformed cells, A59 virus plaques were larger, they appeared earlier, and plaquing efficiencies were higher than in normal, untransformed cells. "Spontaneously" transformed AL/N cells behaved similarly to untransformed cells, whereas "spontaneously" transformed Balb cells resembled virus-transformed cell hosts. Both untransformed and transformed AL/N and Balb cells were permissive hosts for A59 virus. However, multiplication of A59 virus was enhanced at least fivefold in the virus-transformed AL/N cell hosts. Larger differences (100-fold or greater) in A59 virus production were obtained during the first cycle of infection in Balb cells at low multiplicities and in AL/N cells after multiple cycles of virus growth. In virus-transformed and "spontaneously" transformed Balb cells, A59 virus induced extensive syncytia formation.

Coronaviruses are medium-sized, enveloped, ribonucleic acid viruses which, in negatively stained preparations, appear round and bear a corona of irregular, petal-shaped surface projections (5). Biologically, coronaviruses display marked host specificity. Most of the coronaviruses of man were first identified by the use of human, embryonic tracheal and nasal organ cultures (11, 15, 20). Several of these viruses have not yet been adapted to a cell culture system (7, 8, 12). Coronaviruses of avian origin are influenced by phenotypic differences in the avian cell host (13, 14) and undergo very limited growth in cell cultures derived from nonavian species (9, 10). Murine coronaviruses, which include mouse hepatitis viruses, have been cultivated only in cells derived from mouse tissues (16). In some cases, the growth of these viruses is markedly affected by the genetic background of the host (6, 17).

During an investigation employing transformed cells, we observed pronounced host-dependent differences in the plaquing behavior of several murine coronaviruses.

In this communication the interaction of A59 virus, a murine coronavirus, with normal (untransformed), virus-transformed, and "spontaneously" transformed derivative AL/N and Balb mouse cell lines is documented. (A preliminary report of this work was presented at the 70th Annual Meeting of the American Society for Microbiology, Boston, Mass., 26 April to 1 May 1970.) Plaque formation by this virus is shown to be facilitated in virus-transformed and certain "spontaneously" transformed cell types. In these transformed cell hosts, A59 virus yields are increased.

MATERIALS AND METHODS

Cell cultures and media. The following prefixes have been employed to identify the origin of each cell type: N, normal (untransformed); ST, "spontaneously" transformed; Py, polyoma virus-transformed; SV, simian virus 40 (SV40)-transformed; MSV, murine sarcoma virus (MSV)-transformed.

Many cell lines of mouse origin were examined as hosts for A59 virus growth, cytopathogenicity, and plaque production. The N-AL/N cell line and its derivatives were developed in this laboratory (19). N-AL/N is a nontumorigenic cell line established from mouse embryos of the AL/N strain. ST-AL/N is a "spontaneously" transformed (tumorigenic) derivative of N-AL/N obtained after 30 to 40 passages of N-AL/N in culture. It was negative for murine leukemia virus when tested by the complement-fixation (CF) test. (For CF [COMUL] tests, 20% cell extracts were tested against "broad reactive" rat serum obtained from rats bearing transplantable Moloney sarcoma
virus-induced tumors. The tests were kindly performed by H. C. Turner.) ST-AL/N-T is a cell line derived from a tumor induced in a syngeneic mouse with ST-AL/N cells. Py-AL/N (clone 3) is a derivative of N-AL/N cells transformed in vitro by polyoma virus. SV-AL/N was derived from N-AL/N cells transformed in vitro by the small plaque-forming variant of SV40. The N-Balb-3T3 and Balb-3T12 cell lines and derivatives were obtained from S. Aaronson, M. Martin, H. Smith, and G. Todaro. N-Balb-3T3 is a nontumorigenic cell line established from mouse embryos of the Balb/c strain (3). ST-Balb-3T3-TA31 C1 1 and 17 Cl 1 are “spontaneously” transformed derivatives of N-Balb-3T3. Balb-3T12 and Balb-3T12-4 were also derived from Balb mouse embryos. They grow to higher saturation densities than N-Balb-3T3 and both are tumorigenic. Balb-3T12-4 releases murine leukemia virus spontaneously (1). SV-Balb-3T3-N1-8, T2, and 10AC1 7-34 were derived from N-Balb-3T3 transformed in vitro by SV40. MSV-Balb-3T3-24 and MSV-Balb-3T3-K were derived from N-Balb-3T3 transformed in vitro by MSV. MSV-Balb-3T3-24 releases MSV and murine leukemia virus. MSV-Balb-3T3-K shows no evidence of production of either virus. However, addition of murine leukemia virus results in rescue of the MSV genome (2). Balb mouse embryo cell cultures were prepared in this laboratory and used after one or two passages. MT-Balb is a cell line established in this laboratory from a tumor induced in a Balb mouse by murine mammary tumor virus. Py-Ts-a-3T3, obtained from M. Vogt, was derived from the Swiss-3T3 line transformed by the Ts-a mutant of polyoma virus described by M. Fried. Polyoma virions are produced by these cells at the permissive temperature (31 C). L2, a subline of NCTC Cl 929 of Earle's C3H mouse L-cell strain, was obtained from S. Dales. Vero cells were purchased from the American Type Culture Collection. The L929 (CCL) cell line employed for interferon assay was obtained from S. Baron. All of the cell lines were tested frequently for mycoplasmas and reinitiated from frozen stocks when contamination was detected.

The growth medium for all cells was Dulbecco medium supplemented with unheated fetal bovine serum at a concentration of 10%. All media contained penicillin (100 to 250 units/ml) and streptomycin (100 ug/ml).

**Viruses.** The A59 strain of mouse hepatitis virus prepared in NCTC-1469 cells was supplied by J. Parker. It was triply plaque-purified before use. Murine leukemia virus (Moloney strain) was obtained from J. Hartley and W. Rowe. This stock preparation did not contain MSV. Mengo virus was supplied by L. Caliguri; reovirus type 3 by P. Gomatos; and vesicular stomatitis virus (VSV), Sindbis, and Chikungunya viruses by C. Buckler and S. Baron. Stocks of these viruses were prepared in L2 and Vero cell types. Preparation and assay of GDVII virus were performed as described previously (18).

**Plaque titrations.** Plaque assays were performed by inoculating virus which was diluted in 10- or 5-fold steps in Dulbecco medium with 10% fetal bovine serum, onto cell monolayers in 60-mm tissue culture dishes, 0.5 ml per dish. Virus was allowed to adsorb for 1 hr (2 hr for reovirus) at 37 C in 5% CO2 in humidified air with gentle tilting every 10 to 20 min. Three plates were used for each dilution. After the adsorption period, the inoculum was removed, and each plate received 8 ml of an overlay medium consisting of 0.95% Noble agar in Eagle minimal essential medium with 5% unheated fetal bovine serum. A59 virus plaques on L2 cells were counted after incubation for 2 days at 37 C. With other virus-cell systems, 4 ml of a second overlay containing 0.005 to 0.01% neutral red was added 12 to 24 hr before plaques were enumerated. A59 virus plaques were counted at 4 or 5 days in Py-AL/N; VSV, at 3 or 4 days in L2; Mengo virus and reovirus, at 7 to 10 days in L2; and Sindbis, at 7 to 10 days in Vero cell types. Plaques of A59 virus in other mouse cell systems were counted between 3 and 14 days after infection (see Results).

**RESULTS**

**Host-dependent differences in plaque production by A59 virus.** Plaque production by the A59 strain of mouse hepatitis virus has been investigated with related normal (untransformed), “spontaneously” transformed, and virus-transformed cell types of murine origin. The results obtained with AL/N cell lines indicate that plaque formation by A59 virus is facilitated in virus-transformed cells.

A59 virus produced minute, indistinct plaques in normal, nontumorigenic cells (N-AL/N) and in “spontaneously” transformed, tumorigenic cells (ST-AL/N and ST-AL/N-T). Virus plaques were not detected until 7 or more days after infection. These plaques were less than 4 mm in diameter at 12 to 14 days.

Plaque formation was facilitated on cells transformed by polyoma virus (Py-AL/N) or SV40 (SV-AL/N). A59 virus produced plaques at least 4 mm in diameter by 3 to 5 days, and the titers were 2- to 10-fold higher in virus-transformed than in untransformed and “spontaneously” transformed AL/N cells.

A59 virus plaques on virus-transformed AL/N cells have a distinctive appearance. They are red and distinguished by a light halo (Fig. 1, left). These plaques are turbid in character. The central region of each plaque contains both dead cells and infected cells which accumulate increased amounts of neutral red. The diminished accumulation of neutral red at the periphery by cells most recently infected is transient (Fig. 1, right).

Three other murine coronavirus, MHV-1, MHV-3, and MHV-S, exhibited plaquing behavior identical to A59 virus when tested in N-AL/N and Py-AL/N cell types (L. S. Sturman and K. T. Takemoto, Bacteriol. Proc., p. 180, 1970).

Host-dependent differences in virus plaque production may result from changes in the thermal stability of the virus, from differences in virus ad-
The rates of adsorption of A59 virus measured by plaque production on Py-AL/N cells were the same for virus produced in N-AL/N and Py-AL/N cells. By 1 hr at 37°C, the level of A59 virus adsorbed by Py-AL/N cells was about 60% of that obtained at 3 hr. The rates of virus adsorption by plaque production on N-AL/N cells were not measured because the plaques were indistinct and could not be enumerated accurately.

As a measure of the rate and efficiency of the early events of infection, the accumulation of A59 virus-specific antigen was determined in N-AL/N and Py-AL/N cells by indirect fluorescent-antibody staining. In both cell types, discrete foci of cytoplasmic fluorescence were observed 5 hr after infection. At 6 to 7 hr, a large mass of fluorescent material appeared in the perinuclear region. Bright, diffuse cytoplasmic fluorescence developed by 7 hr and persisted. Small numbers of polykaryocytes with virus-specific fluorescence were observed by 12 hr. With virus multiplicities of 0.01 to 10 plaque-forming units (PFU; L2)/cell, no significant differences were noted between N-AL/N and Py-AL/N cells in the rates of appearance of virus-specific antigen or in the proportion of cells which developed specific fluorescence during the first cycle of infection. With an input multiplicity of 50 to 100 PFU (L2)/cell, 15 to 30% of the cells of both types developed specific fluorescence by 12 hr.

Growth kinetics of A59 virus in N-AL/N, Py-AL/N, and ST-AL/N-T cells. Because of the great thermolability of A59 virus, virus production was determined by repeated collection and readdition of media at 2-hr intervals. Growth kinetics have been determined from the cumulative yields of released virus.

Monolayers of N-AL/N, Py-AL/N, and ST-AL/N-T cell types were inoculated with A59 virus at multiplicities of 70, 7, 0.7, and 0.07. After adsorption for 1 hr at 37°C, the monolayers were washed three times and reincubated at 37°C with Dulbecco medium supplemented with 10% fetal bovine serum. At 2-hr intervals, from 4 to 36 hr after infection, media were collected from cultures of each type which had been infected at each
multiplicity; samples were frozen rapidly and stored at \(-90^\circ\) C for assay.

The first increase in A59 virus was detected in the interval between 6 and 8 hr after infection (Fig. 2). The kinetics of A59 virus production were similar in N-AL/N, Py-AL/N, and ST-AL/N-T cell types. At multiplicities from 0.07 to 70, A59 virus production during the first 16 hr after infection was five- to sixfold higher in Py-AL/N cells than in N-AL/N cells. During the same interval, virus production in ST-AL/N-T cells was 35- to 45-fold lower than in N-AL/N cells.

Cell-associated A59 virus was also measured at frequent intervals. Differences of similar magnitude were found between titers of both intracellular and released virus from N-AL/N, Py-AL/N, and ST-AL/N cells.

The differences between A59 virus yields from cultures infected at lower multiplicities became larger at later times. By 36 hr in cultures infected at a multiplicity of 0.07, the yield of A59 virus from Py-AL/N cells was 40 times that from N-AL/N cells. The cumulative yield from ST-AL/N-T cultures infected at the same multiplicity was more than 120-fold lower than that from N-AL/N cells by 36 hr. At 48 hr after infection of cell cultures at a multiplicity of 0.07, A59 virus-specific fluorescence could be detected in 100% of Py-AL/N cells, compared with less than 1% of N-AL/N cells.

Except for small numbers of syncytia, virus-induced cytopathological changes did not become apparent in all AL/N cell types until 12 to 24 hr after maximal levels of A59 virus production were reached.

Interferon production and interference induced by A59 virus in N-AL/N and Py-AL/N cells. Confluent monolayers were infected with A59 virus at a multiplicity of 5 PFU/cell. Uninfected cultures and cultures exposed to heat-inactivated Chikungunya virus served as controls. Media were collected at 24 hr, incubated for 5 days at 4°C at pH 3, neutralized, and assayed for interferon by the method of GDVII virus yield reduction in L929 cells. The cell monolayers 24 hr after infection were challenged with VSV or Sindbis virus. Media were collected 18 hr later and assayed for these viruses.

Not more than 2 units of interferon per ml were detected at 24 hr in the culture media of both

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**Fig. 2. Growth of A59 virus in AL/N cell types.** Symbols represent cumulative yields of released virus from (A) N-AL/N cells, (B) Py-AL/N cells, and (C) ST-AL/N-T cells after infection at multiplicities of 70, 7, 0.7, and 0.07.
N-AL/N and Py-AL/N cells infected with A59 virus. In the same cell types, heat-inactivated Chikungunya virus induced 40 to 100 or more units of interferon per ml. Interference induced by A59 virus infection caused 10- to 100-fold reductions in VSV and Sindbis virus production, respectively, in both cell types. Interference induced with heated Chikungunya virus resulted in 100- to 1,000-fold reductions in VSV and Sindbis virus production. These data indicate that A59 virus is a poor inducer of interferon and interference alike in both N-AL/N and Py-AL/N cells.

Growth of other ribonucleic acid viruses in N-AL/N, Py-AL/N, and ST-AL/N-T cells. To determine whether increased virus production occurs generally in Py-AL/N cells, the growth of Mengo virus, reovirus, Sindbis virus, and VSV was measured in normal and transformed AL/N cell types. Each virus was employed at multiplicities of 0.1, 1, and 10. Virus production was measured at intervals from 16 to 72 hr after infection.

Similar virus yields were obtained from normal and transformed AL/N cell types in all cases but one. The growth of VSV was 3- to 10-fold lower in N-AL/N cells than in the Py-AL/N and ST-AL/N-T cell types.

With several viruses, cytopathological changes were more marked in one of the cell types. This was the case with Sindbis and VSV in N-AL/N and with reovirus in Py-AL/N cells.

A59 virus infection of normal and transformed Balb cells. The specificity of facilitation of A59 virus plaque formation and virus multiplication were further tested with normal and transformed Balb-3T3 and 3T12 cell lines. A59 virus plaques were obtained on normal Balb-3T3 (N-Balb-3T3) cells only irregularly, even 10 or more days after infection, whereas, on both "spontaneously" transformed (ST-Balb-3T3-17C1 1, TA31C1 1, Balb-3T12, and Balb-3T12-4) and virus-transformed (SV-Balb-3T3-10A CI 7-4J, MSV-Balb-3T3-24, and MSV-Balb-3T3-K) Balb cells, A59 virus plaques were evident by 2 to 3 days. Some SV40-transformed Balb-3T3 cell lines (SV-Balb-3T3-11A-8 and T2) could not be maintained adequately with an agar overlay. Under liquid medium, however, virus-induced cytopathogenicity and the rate of spread of infection determined by immunofluorescence were significantly greater in these cells than in N-Balb-3T3.

A59 virus plaque formation was no different on murine leukemia and sarcoma virus-producing and -nonproducing MSV-transformed Balb-3T3 cells; neither were there any differences between murine leukemia virus-releasing and -nonreleasing "spontaneously" transformed Balb-3T3 cell lines. Large A59 virus plaques were obtained with a cell line of Balb origin derived from a mammary tumor virus-induced tumor (MT-Balb). Plaques of A59 virus on most transformed Balb cells were syncytial in character.

Murine leukemia virus infection of normal Balb mouse embryo cells did not facilitate A59 virus plaque formation. Untransformed, COMUL-positive, Balb mouse embryo cell cultures infected for 9 to 14 days with Moloney leukemia virus did not differ from uninfected cells as hosts for A59 virus plaque production. Both were similar to the N-Balb-3T3 cell line.

Growth kinetics of A59 virus in N-Balb-3T3, SV-Balb-3T3, and ST-Balb-3T3 cells. A59 virus production was measured in N-Balb-3T3, SV-Balb-3T3 (1IA-8), and ST-Balb-3T3 (17C1) cell types. Cell cultures were infected with A59 virus at multiplicities of 70, 7.0, 0.7, and 0.07. After adsorption for 1 hr, cell monolayers were washed and reincubated with Dulbecco medium supplemented with 10% fetal bovine serum. A59 released virus was determined between 4 and 24 hr after infection by collection and readdution of new media at 2-hr intervals. The data in Fig. 3 represent cumulative yields of released virus.

Pronounced syncytia formation occurred in A59 virus-infected 1IA-8 and 17C1 cell types. Syncytia were detected as early as 6 hr. 17C1 cell monolayers infected with a multiplicity of 0.07 formed a single syncytium by 14 to 16 hr after infection. In contrast, in N-Balb-3T3 cells, A59 virus displayed very slight syncytia-inducing effect.

In all three cell types, the first increase in A59 virus was detected between 6 and 8 hr after infection. A59 virus production was considerably higher in both SV40-transformed and "spontaneously" transformed Balb-3T3 cells. The extent of the difference was related to the multiplicity of infection. By 12 hr after infection at a multiplicity of 70, A59 virus production was three- to sixfold lower in N-Balb-3T3 cells. With infection at a multiplicity of 0.07, the differences in A59 production measured at 12 hr were 100-fold or greater between the normal and transformed Balb-3T3 cell types.

These data indicate that the disparity between A59 virus multiplication in normal and virus-transformed cells is independent of strain differences between cells of AL/N and Balb mouse genotypes.

DISCUSSION

The normal and transformed cells tested were all susceptible to A59 virus infection. This is shown by the progressive nature of virus-induced cytopathological changes, the spread of virus antigen synthesis detected by immunofluorescence, and the confluence of plaque formation at high multi-
plicities of infection. The growth of A59 virus, however, has been found to be host-dependent. Approximately fivefold greater virus yields per cell were obtained during the first cycle of infection of virus-transformed AL/N cell hosts.

In Balb-3T3 cells, larger differences, 100-fold or greater, were found during the first cycle of infection at low multiplicities. This is probably the result of the rapid spread of infection among transformed Balb cells through syncytia formation. The syncytia-inducing capacity of A59 virus in these cells facilitates the production of virus plaques.

As revealed by the growth curves and by immunofluorescence, the rate of spread of A59 virus infection differs also in the normal and virus-transformed AL/N cells. A59 virus titers at later intervals from AL/N cells cannot be directly correlated with virus yields per cell, since the number of cells participating in the later cycle and the duration of A59 virus production by cells infected earlier are not known.

Marked differences have been noted between "spontaneously" transformed AL/N and Balb-3T3 cells as hosts of A59 virus. The ST-AL/N-T cell is a poorer host for A59 virus than even the N-AL/N cell type. In contrast, ST-Balb-3T3 cells resemble SV40-transformed Balb-3T3 cells in supporting high levels of A59 virus production and syncytia formation. The Balb-3T3 cell line has been shown to contain the genetic information for an oncornavirus (4). The presence of this genome may play a role in determining the phenotypic character of the "spontaneously" transformed Balb-3T3 derivatives.

A59 virus growth is presumed to be independent of the production of infectious transforming virus for the following reasons: (i) no infective SV40 or polyoma virus virions are produced by mouse cells transformed by these viruses, (ii) A59 virus plaque size and virus growth are not affected by the state of MSV production in MSV-transformed cells, and (iii) A59 virus plaques of similar large size are obtained at both permissive and nonpermissive temperatures in a cell line transformed by the TS-a mutant of polyoma virus (Py-Ts-a-3T3).

The role of the transforming viruses in the response of the transformed cell hosts is not known. Investigations are in progress on the chemistry and biosynthesis of coronaviruses which may provide the basis for understanding the mechanism responsible for these differences in A59 virus growth.

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