Endogenous C-Type Virus of a Mouse Cell Line and Its Defectiveness

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MLg cells originating from a newborn ddY mouse began to release C-type virus by treatment with 5-iodo-2'-deoxyuridine (5 μ g/ml) for 48 h, or spontaneously after prolonged cultivation. The data indicated the presence of three categories of the viruses spontaneously induced, i.e., virus equipped with the abilities of infecting and transforming the S+L- C182 cells, virus which can infect C182 cells but is unable to transform C182 cells, and virus which cannot infect nor transform the C182 cells, but is positive for the XC test. The type of the virus produced depended upon the cell clone. With the advance in the passage level, the cell population was dominated by the cells producing, uniquely, the last category of the virus.

MLg cells originating from the lung tissues of a newborn ddY mouse were susceptible to Friend leukemia virus (FLV). Curiously, however, MLg cells of the advanced passage levels (more than about 60 to 70 passages) were resistant to the virus (17). The cause of the cellular resistance to FLV at higher passage levels was recently found to be due to the spontaneous induction of C-type virus. The spontaneously-induced viruses were heterogeneous in population, and some were devoid of certain viral functions. This information is detailed in this report.

MATERIALS AND METHODS

The MLg cell line was established from the lung tissues of a newborn ddY mouse. The origin of the ddY mouse is the same as that of DDD mouse strain characterized by Odaka and Yamamoto (10). MLg cells infected with FLV at the 42nd passage continued producing FLV (designated as MLg₄₂-FLV) (16, 17). MLg cells infected with FLV at the 65th passage (MLg₆₅-FLV) released "attenuated" virus (16, 17).

The XC cell line and S+L- C182 cell line were obtained from W. P. Rowe of the National Institute of Allergy and Infectious Diseases, and Y. Ikawa of the National Cancer Institute. The C3H2K and YH-7 cell lines were established by Yasuko Hirokawa, and found to be N and B types, respectively, according to the classification by Hartley et al. (6, 14).

The method of virus infection, plaque titration in mouse cells by the UV-XC procedures (XC test) (12) and in the S+L- C182 cells by direct counting of the

"cytopathic plaques" (5) were the same as described before (15).

RESULTS

Type of MLg cells. MLg cells at the 38th passage were infected with either murine sarcoma virus (MSV) (FLV), or MSV (WN1802B) or NR-tropic Moloney MSV (M-MSV); the former was N-tropic and the latter was B-tropic MSV pseudotype (14). As controls, N-type C3H2K and B-type YH-7 cells were infected also with these viruses. MLg cells, like N-type C3H2K cells, were susceptible to N-tropic MSV (FLV) but resistant to MSV (WN1802B) (Table 1). Thus, MLg cells were N type.

Testing the presence of C-type virus in MLg cells. MLg cells at the 38th and 80th passages were infected with M-MSV. MLg cells at the 38th passage developed foci, whereas those at the 80th passage did not. The XC test developed for detection of murine leukemia virus (MLV) (12) was positive for MLg cells at the 80th passage, but negative at the 38th passage. Thus, MLV present in the MLg cells at advanced passages must have interfered with the M-MSV or FLV infection of the cells.

Induction of MLV by IUdR. As the C-type virus was induced spontaneously from the MLg cells, the virus may also be induced by 5-iodo-2'-deoxyuridine (IUdR) as reported in AKR and other mouse cells (2, 8, 11).

MLg cells at the 38th passage inoculated at a density of 10^5 cells per 60-mm petri dish were treated with IUdR (Sigma Chemical Co.) at a

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TABLE 1. *Titration of MSVs in MLg, YH-7, and C3H2K cells^a*

Cells	No. of foci/dish		
	N-tropic MSV (FLV)	B-tropic MSV (WN1802B)	NB-tropic M-MSV
C3H2K (N type)	Confl ^b	0 0	confl
YH-7 (B type)	20 19	confl	confl
MLg, 38th passage	Confl	0 0	confl

^a N-type C3H2K cells, B type YH-7 cells, and unknown MLg cells at 38th passage were infected with MSV (FLV), MSV (WN1802B), or M-MSV. For the inoculum, undiluted preparations were used. Foci were counted 5 to 7 days after infection.

^b Confluent.

concentration of 5 μ g/ml for 48 h. After the medium was changed, the cells were overlaid with 10^5 untreated MLg cells per dish. Six days later, some of the cultures were submitted to the XC test. Six to ten XC plaques appeared per dish. The other cultures were subcultured twice more at 5-day intervals, and then submitted to the XC test. The cultures developed polykaryons confluent. The untreated MLg cells were negative for the XC test.

Titration of the spontaneously-induced MLV. The culture fluid of MLg cells at the 80th passage was titrated in the N-type MLg cells, B-type YH-7 cells, and the S+L- C182 cells. The virus produced far more XC plaque in MLg cells than in YH-7 cells (Table 2). Thus, the induced MLV was N-tropic virus. Curiously, in C182 cells, no "cytopathic plaques" (4) appeared. However, when further submitted to the XC test, these cultures developed many XC plaques. It should be noted that with FLV the number of the cytopathic plaques and that of XC plaques were nearly equal (cf. Table 2).

To avoid confusion in the terminology, in the rest of this paper the cytopathic plaques (4) will be called rescue foci.

Infectious center assay of the virus-producing MLg cells, MLg₄₂-FLV cells and MLg₆₅-FLV cells. Endogenous virus-producing MLg cells at various passages, and MLg cells induced to produce the virus by the IUdR treatment (vide supra) were assayed in the S+L- C182 cells as the infectious center for rescue foci and also as XC plaques. The cells were trypsinized, and a known number of the cells were plated onto the S+L- C182 cells. The rescue foci were counted on the fifth day, and the cultures were further submitted to the XC test.

The data are summarized in Fig. 1. The number of rescue foci were plotted against the number of XC plaques. The F/P ratio was lower than 0.1 for the endogenous virus-producing MLg cells, and was slightly higher than 0.1 for MLg cells which were treated by IUdR (5 μ g/ml) at the 38th passage for virus induction and subcultured twice. On the other hand, for FV131 cells which were assayed similarly, the ratio was nearly 1.0. The plaquing efficiency of MLg cells became lower and lower, as the passage level of the cells increased; the plaquing efficiency was 10 to 30% for MLg₄₂-FLV cells, while for MLg cells at the 101st passage it was 0.1 to 0.2%. The plaquing efficiency of IUdR-induced MLg cells was comparable to that of MLg₄₂-FLV cells.

The "attenuated" FLV-producing MLg₆₅-FLV cell line, tested similarly, showed an extremely low F/P ratio.

The data indicate that in MLg₄₂-FLV cells almost all the infectious centers for XC plaques were also infectious centers for the rescue foci. In other words, only a few percent of the MLg cells release virions which produce both rescue foci and XC plaques in C182 cells, whereas the rest of the cells release virions which cannot produce foci but can produce XC plaques in C182 cells, or virions whose capacities of rescue focus- and XC plaque-formations are undetectable (vide infra).

Infectious center assay of the cloned MLg cells. MLg cells at the 70th passage spontane-

TABLE 2. *Titration of the culture fluids of MLg cells at the 82nd passage and of FV131 cells^a*

Virus source	Dilution	MLg cells (N) (no. of XC plaques)	YH-7 cells (B) (no. of XC plaques)	C182	
				No. of rescue foci ^b	No. of XC plaques
MLg, 82nd passage	1	97	2	0	54
		126	3	0	56
	$1/2$	NC ^c	NC	NC	26
					12
	$1/4$	42	NC	NC	10
MLg ₄₂ -FLV		20			14
	$1/10$	ND ^d	ND	154	146
				168	118
	$1/100$	ND	ND	10	10
				10	14

^a The culture fluids of MLg cells at the 82nd passage and those of MLg₄₂-FLV cells were titrated in MLg, YH-7, and C182 cells. In the C182 cells, both the rescue focus titer and XC plaque titer were measured. The figures indicate the number of rescue foci or XC plaques per dish. MLg cells were about 100-fold more sensitive to FLV than YH-7 cells.

^b Equivalent to "cytopathic plaques" of Bassin et al. (4).

^c Not counted.

^d Not done.

FIG. 1. Infectious center assay of IUDR-treated MLg cells at 38th passage, MLg cells spontaneously releasing endogenous MLV, MLg₄₂-FLV cells, and MLg₆₅-FLV cells, and also of the cells in a picked rescue focus from C182 cells inoculated with MLg cells at the 94th passage. (i) MLg cells which came to develop polykaryons confluent two passages after treatment with IUDR at the 38th passage, MLg cells at various passages spontaneously producing endogenous virus, MLg₄₂-FLV cells, and MLg₆₅-FLV cells were trypsinized, and suspended in the culture medium. The cells were inoculated onto C182 cells in amounts of 10^4 and 10^3 cells per dish. After rescue foci were counted at 5 days, the cultures were submitted to the XC test. The number of rescue foci was plotted against that of XC plaques. The straight lines indicate the number of rescue foci divided by that of the XC plaques (the F/P ratio) to give 1.0, 0.1 and 0.01. Symbols: □, MLg₄₂-FLV; ●, endogenous virus-producing MLg cells. The cells at different passage levels are indicated by the different size symbols together with the figures indicating the passage level of the cells: ○, MLg cells treated with IUDR at the 38th passage and then subcultured twice; ■, MLg₆₅-FLV. (ii) MLg cells at 94th passage were plated onto the C182 cells in an amount of 2×10^4 cells per dish. One of the rescue foci thus obtained was picked under the agar. The picked cells were successively transferred to the C182 cells. The cells at each transfer level were plated onto C182 cells and the efficiencies of rescue focus and XC plaque formations were assayed. The data are plotted with different size triangular symbols with the figures indicating the transfer number. The data are discussed in the last section of the results.

ously producing endogenous viruses were cultured at a concentration of 500 cells per dish. After incubation for 2 weeks, about 10 visible

colonies appeared per dish. The soft agar (0.6%) was overlaid onto the cultures after the liquid medium was removed. When the agar became solid, each colony was scraped and aspirated by a Pasteur pipette through the agar layer, and the aspirated cells were transferred to the petri dishes containing liquid medium. In this way, 15 colonies were isolated. After a subculture, the clones were tested for the infectious center of rescue foci as well as of XC plaques in C182 cells in the same manner as described in the previous section.

The data are summarized in Fig. 2. Five of 13 clones, clones 4, 8, 12, 16, and 17, produced both rescue foci and XC plaques relatively in high efficiency. They were called F+P+ clones (focus plus, plaque plus). Two clones, clones 1 and 2, produced XC plaques in high efficiency but an undetectable number of rescue foci (less than $1/10^4$). They were called F-P+ clones (focus minus, plaque plus). The clone 3 was similar to F-P+ clones but produced very few but a detectable number of rescue foci ($3/10^4$). The other seven clones were unable to produce rescue foci or XC plaques in a detectable amount (less than $1/10^4$), but, when cocultivated with XC cells, they produced the polykaryons abundantly (F-P- clones).

In the same figure, similar analysis of the MLg cells cloned at the 101st passage is shown. Here, the majority of the clones (12 out of 15) were F-P-. The remaining three clones were similar to F-P+, but the plating efficiency as XC plaques was extremely low. There were no F+P+ clones.

These data indicate the following. (i) There were three types of viruses, i.e., those which infect and transform C182 cells (named F+P+ virus), those which infect but do not transform C182 cells (named F-P+ virus), and those whose infectious and transforming capacities were undetectable, but positive for the XC test (F-P- virus). (ii) The character of the virus produced was determined by the cell clone. It is not known, however, whether one cell type produces only one virus type or several. In other words, the F+P+ clone may produce F+P+ virus plus F-P+ and/or F-P- virus. On the other hand, F-P- clones only produce F-P- virus. (iii) With the advance in the passage level, the F-P- clones became dominant over F+P+ or F-P+ clones in the population.

Properties of the C182 cells infected with the "F-P+" virus. The culture fluid of MLg cells at the 80th passage was diluted serially fivefold, and was used to infect C182 cells. The cells infected with the undiluted or fivefold-diluted preparation developed transformed cells after one to two subcultures, but those infected

FIG. 2. Infectious center assay of MLg cells cloned at the 70th and 101st passages. The cloned MLg cells were plated onto the C182 cells in amounts of 10^3 and 10^4 cells per dish. The rescue foci were counted 5 days later, and then the cultures were further submitted to the XC test. The straight lines indicate the F/P ratio to give 1.0, 0.1, 0.01, and 0.001. The number of rescue foci was plotted against the number of XC plaques. Symbols: ○, MLg cells cloned at the 70th passage; △, MLg cells cloned at the 101st passage. The figures beside the symbols indicate the clone number. Seven clones of 15 and 12 of 15 were negative for both rescue foci and XC plaques for MLg cells at the 70th and 101st passages, respectively. All the clones, however, produced polykaryons when the clones cells were cocultivated with the XC cells.

with 25-fold-diluted preparation did not develop foci within a further 20 subcultures, and remained quite similar to the uninfected C182 cells in morphology. The cells were positive by the XC test. This cell line was designated as NT-MLg/C182 cell line.

At the same time, C182 cells were infected with the culture fluid of FV131 cells. The cells were confluent transformed. After eight to nine passages, however, the cells became indistinguishable from the uninfected MLg. This FLV-infected C182 cell line was designated as FV/C182.

The infectious center assay in C182 cells as rescue focus and XC plaque was performed with the NT-MLg/C182 and FV/C182 cells in the manner described in the previous section.

The F/P ratio was about 1.0 for FV-C182 cells both at 38 and 79 days after infection with FLV, whereas the ratio was lower than 0.1 for NT-MLg/C182 cells both at 38 and 79 days after infection (Fig. 3). Here, FV/C182 cells showed normal morphology at 79 days, whereas at 38 days transformed morphology was demonstrated.

The culture fluids of NT-MLg/C182 and that of FV/C182 were assayed in C182 and C3H2K cells. The culture fluid of the FV/C182 produced

as many, or even more foci than plaques in C3H2K cells, and in C182 cells the number of rescue foci and that of XC plaques were almost the same. On the other hand, in the case of NT-MLg/C182, although the XC plaque titer was fairly high (except for titration of the culture fluid at 35 days in C3H2K cells), the focus or rescue focus-forming activity was extremely low, but not zero. These experiments suggest the following: (i) The "F-P+" virus from MLg cells retains its character of low F/P ratio even when propagated in C182 cells. (ii) The culture fluid of NT-MLg/C182 contained transforming virus although in a very low titer. This may indicate that F-P+ virus can rescue MSV though in a low efficiency, or that F-P+ virus existed in NT-MLg/C182 cell cultures in an undetectable amount, i.e., in an amount insufficient to produce transformed cells in the culture.

Demonstrating the presence of endogenous virus whose rescue focus formation is equally efficient as XC plaque formation. In the previous section, it was shown that the properties of FLV or endogenous viruses with respect to the rescue focus-forming ability and XC plaque-forming ability were retained even if the virus was transferred from MLg cells to C182

FIG. 3. Comparison of FV/C182 and NT-MLg/C182. (i) FV/C182 or NT-MLg/C182 cells at 38 or 79 days after infection were trypsinized, and 10^5 or 10^3 cells were plated per C182 cell culture. The rescue foci and XC plaques were counted in the manner described already. The straight lines indicate the F/P ratio to give 1.0, 0.1, and 0.01. Symbols: ●, FV/C182; ○, NT-MLg/C182. The figures beside the symbols indicate the days after infection. (ii) The culture fluids of FV/C182 and NT-MLg/C182 were titrated on C3H2K or C182 cells. The number of foci or rescue foci were counted 5 days after infection and then the cultures were submitted to the XC test. The focus or rescue focus titer and the XC titer were obtained with the same dishes, and the titers were calculated simply by multiplying the number of foci or plaques by the dilution factor. Symbols: ■, FV/C182 culture fluid titrated in C3H2K cells; □, NT-MLg/C182 culture fluid titrated in C3H2K cells; ▲, FV/C182 culture fluid titrated in C182 cells; △, NT-MLg/C182 culture fluid titrated in C182 cells. The figures beside the symbols indicate the days after infection.

cells. So, it is possible to isolate endogenous virus-infected C182 cells, whose F/P ratio is 1, it may indicate that there existed in the culture fluid of MLg cells the virions whose capacities of inducing rescue foci and XC plaques are the same.

MLg cells at the 94th passage were plated onto a C182 cell monolayer in an amount of 2×10^4 cells per dish. Two to three rescue foci appeared per dish. Two rescue foci were picked with a Pasteur pipette under the agar in the manner described above. The picked cells were

dispersed as much as possible by vigorous pipetting and then plated onto a C182 cell monolayer. A C182 culture inoculated with one of the picked foci developed 30 rescue foci and the other developed 38. The latter culture was trypsinized, and 10^4 or 10^5 cells were plated onto C182 cells (second transfer). The cultures inoculated with 10^4 cells were used for estimating the efficiencies of the rescue focus as well as XC plaque formation. The cultures inoculated with 10^5 cells were trypsinized, and 10^4 or 10^5 cells were plated onto C182 cells (third transfer).

The same procedures were repeated. The number of rescue foci was plotted against the number of XC plaques as shown in Fig. 1. At the second transfer, the F/P ratio was already 1.0, and with the increased transfer the plating efficiency as XC plaques as well as rescue foci gradually increased, reaching the level of that of FV131 cells. Thus, it was possible to obtain endogenous virus-infected C182 cells whose F/P ratio was about 1.0.

DISCUSSION

MLg cells began to release endogenous MLV after prolonged cultivation in vitro. The endogenous virus was able to infect the homologous MLg cells which are not yet producing the virus. The tropism of the virus (6) was N. However, when the virus was assayed in the S+L- clonal cells, the virus produced no rescue foci, but produced XC plaques, after the cells were further submitted to the XC test. This indicated that the endogenous virus of MLg cells was inefficient in forming rescue foci, but was able to replicate in the C182 cells.

Cloning experiments of the MLg cells releasing the endogenous virus revealed the existence of the three categories of the cells, i.e., F+P+, F-P+, and F-P- clones. On the infectious center assay in C182 cells, F+P+ clones produced both rescue foci and XC plaques efficiently; F-P+ clones produced undetectable amounts of rescue foci, but were efficient in XC plaque formation; F-P- clones did not produce rescue foci nor XC plaques in detectable amounts. This seems to indicate that there were three corresponding types of the endogenous viruses, i.e., F+P+, F-P+, and F-P- viruses. The type of the virus produced was determined by the cell clone.

As the cloning was performed only once, it was possible that F+P+ clones were contaminated by F-P+ or F-P- cells, and F-P+ clones by F-P- cells. This may be suggested by the low F/P ratio of F+P+ clones, and low plating efficiency as XC plaques of F+P+ or F-P+ clones both relative to the FV131 cells. It

is not known, however, if a single clone produces only one type of the virus or several, although at least F-P- clones seem to produce only F-P-virus.

The number of physical particles produced by each clone was not determined. The difference in the clones with respect to the F/P ratio may simply be interpreted in terms of the different amount of particles produced by the different clonal cells. But, this is hardly probable firstly because the infectious center assay efficiently scores the cells producing infectious virions even in extremely low amounts, and secondly because the F+P+ and F-P+ clones, which are greatly different with respect to the rescue focus formation, produced nearly equal amounts of XC plaques.

The "attenuated" virus-producing MLg₆₅-FLV cells behaved in a similar manner to the endogenous virus-producing MLg cells. This may indicate that the attenuation of MLV in vitro (3, 9, 13, 16) may be brought about by the induction of the endogenous viruses in the MLV-infected cells, which is followed by the exclusion of the MLV by the endogenous virus.

In connection with the attenuation, it was observed that, with the increase in the passage level, the endogenous virus-producing MLg cells seemed to become, as a whole population, attenuated virus-producing cells, as the plating efficiency of the cells as XC plaque infectious center gradually decreased due to the gradual dominance of F-P- cells in the population. As there seemed to be no difference in the growth rate in F+P+ and F-P- cells, there would have been no selective growth of any one of the clonal cells; it is quite possible that F+P+ cells were converted to F-P+ cells, and that F-P+ cells were converted to F-P- cells. Therefore, the diversity of the cells with respect to the properties of the virus induced may be brought about by this attenuation process, i.e., all the cells may have been F+P+ just after induction, and then gradually, one by one, the F+P+ cells may have turned into F-P+ and then to F-P- cells.

Concerning the diversity of the cell clones with respect to the endogenous virus, there remain two other possibilities. Firstly, the virogenes (viral genomes endogenously present in the cells) which should be induced were predetermined in each cell before the induction, i.e., the virogenes of the cells became mutated in such a way as to give rise to the F+P+, F-P+, and F-P- clones after the induction. Secondly, although the virogenes are identical from one cell to another, the number of the virogenes induced may not always be the same, and vary from one cell to another somehow by chance.

Endogenous MLVs which are unable to replicate in mouse cells but do replicate in heterologous rat cells (at least in a form of MSV pseudotype) have been isolated from NZB (7) and BALB-c mice (1). The F-P- virus resembles these viruses in that infectivity in the mouse cells was undetectable. However, although the F-P- virus-producing cells were positive for the XC test, at least one of these previously reported endogenous viruses was unable to be detected by the XC test (7). The infectivity of the F-P- virus to the rat cells has not been tested.

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