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Antigenic Properties of Endogenous Type-C Viruses from Spontaneously Transformed Clones of BALB/3T3

(immunoelectronmicroscopy/cell-surface antigens)

TADAO AOKI AND GEORGE J. TODARO

Viral Leukemia and Lymphoma Branch, National Cancer Institute, Bethesda, Maryland 20014

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ABSTRACT **During long-term tissue culture of spontaneously transformed clones derived from BALB/c 3T3 mouse-embryo cells, some clones spontaneously begin to produce high titers of endogenous murine type-C viruses. The antigenic properties of these viruses have been analyzed by indirect immunoelectronmicroscopy and can be classified into two distinguishable populations: (*a*) BALB/c murine myeloma-associated extracellular viruses that carry a specific envelope antigen, xVEA, different from the typical murine leukemia viral envelope antigens; and (*b*) previously uncharacterized type-C viruses that have neither xVEA nor the murine leukemia viral envelope antigens. The former produces PC1 antigen and the latter might induce a new cell-surface antigen. Neither of these two populations of BALB/3T3 endogenous type-C viruses was able to infect BALB/c cells but both could infect NIH Swiss cells. A single BALB/3T3 clone, then, can release infectious endogenous type-C viruses with at least two different antigenic properties. We conclude that BALB/c somatic cells contain preexisting genetic information for production of at least two closely related but, nevertheless, distinct type-C viruses.**

Mouse cell lines spontaneously transform in cell culture and show malignant properties when tested in a susceptible host (1, 2). In addition, many such cell lines release murine type-C viruses (3, 4). One of the most extensively studied sets of mouse cell lines was derived from random-bred Swiss mice. These cell lines include 3T3, 3T6, and 3T12 (5). Under identical culture conditions, a similar series of cell lines was subsequently developed from an inbred BALB/c embryo culture (6). The 3T3 cells of both the random-bred and BALB/c series remained strongly contact-inhibited and nonmalignant, while the 3T12 cultures, which were always transferred under conditions that favor loss of contact inhibition of cell division, spontaneously transformed, were able to grow on top of one another, and were malignant when tested in suitable hosts (6). The type-C viruses appeared in certain of the cell cultures (e.g., 3T12) that had spontaneously transformed and not in the cell cultures that had retained normal growth properties (e.g., 3T3) (7). The spontaneous appearance of type-C viruses in 3T12 cultures of both the random-bred Swiss and the BALB/c series suggested that genetic information for virus production was present in the original cell cultures and became expressed as complete viruses in the spontaneously transformed 3T12 cultures, but was somehow maintained in an unexpressed form in the 3T3 cultures (7, 8).

The studies of Weiss *et al.* (9) and Rowe *et al.* (10) have shown that chick-embryo cells and certain clones of AKR cells could remain virus free and then spontaneously begin producing virus. Production of type-C virus was shown by Lowy *et al.* (11) to be greatly accelerated by the use of the thymidine analogs, bromodeoxyuridine (BrdU) and iododeoxyuridine (IdU). The AKR cells are readily reinfected by the virus induced from them, and the animals are highly susceptible to the development of leukemia. With BrdU and IdU, virus could also be induced from the 3T12 and 3T3 cells of the BALB/c series (12). Type-C viruses, however, would also appear spontaneously in clonal lines of spontaneously transformed BALB/3T3 cells without added inducer (13). The spontaneous variants of BALB/3T3 resemble virus transformants produced by exogenous virus; they grow to high saturation densities in factor-free medium, on monolayers of normal cells, and in agar.

Murine type-C RNA viruses have been categorized based on their morphology in the electronmicroscope (14) and on certain biochemical properties (15), regardless of their function *in vivo* or their envelope antigen properties. It has become evident by immunoelectronmicroscopy that the murine type-C viruses can be classified into at least two major groups; murine leukemia virus (MuLV) and murine myeloma-associated viruses (MuMAV) (16, 17)*. These type-C viruses all contain a common gs-1 (or intraspecies) antigen (18, 19) and antigenically similar RNA-directed DNA polymerases (20). Certain isolates of MuLV and MuSV are oncogenic by animal inoculation, but the oncogenicity of MuMAV has not yet been demonstrated. MuLV has been found in a variety of tissues from a variety of mouse strains while MuMAV has so far only been described in BALB/c myeloma cells, such as MOPC-70A and MPC-113 (16, 21).

In the present report,we describe the antigenic properties of the endogenous virus released by three different clonal lines of spontaneously transformed BALB/3T3 cells. We have found by immunoelectronmicroscopy the presence of both MuMAV and another as yet uncharacterized type-C virus being produced by each of these clones. There is no evidence, however, that the endogenous type-C virus these cells are producing is of the most commonly described MuLV type.

MATERIALS AND METHODS

Animals. 2-Month-old BALB/c, C57BL/6, and (C57BL/6 × DBA/2)F_1 mice were obtained from the National

Abbreviations: MuLV, murine leukemia virus; MuSV, murine sarcoma virus; MuMAV, murine myeloma-associated virus; SBMV, southern bean mosaic virus; LVEA, MuLV envelope antigen; xVEA, MuMAV envelope antigen; G, Gross.

* Murine sarcoma virus (MSV) could be classified as a third group of murine type-C viruses by biological characteristics, e.g., sarcoma production. The specificity of MSV envelope antigens, however, cannot be distinguished from the other groups by immunoelectronmicroscopy, for the release of mature MSV appears to require the coat of the helper type-C virus.

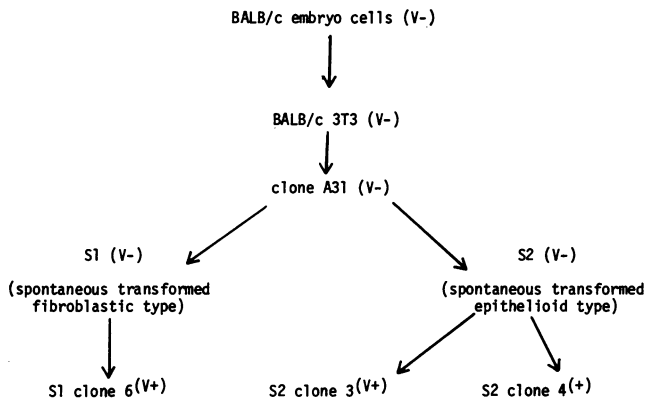

FIG. 1. Genealogy of various long-term cultured clones derived from BALB/c 3T3 mouse embryo cells.

Institutes of Health (NIH) breeding colony, Bethesda, Md. 4-Month-old (W/Fu × BN)F_1 rats were gifts from Dr. E. A. Boyse, Division of Immunology, Sloan-Kettering Institute for Cancer Research, New York, N.Y.

Cells. Cultured cells were grown in Dulbecco's modification of Eagle's medium supplemented with 10% calf serum as described (22). In addition, two transplanted murine tumors were used as the reference tumors. The origin of individual cell lines is presented in Table 1.

Two clones, S1 and S2, are spontaneous transformants that have appeared in BALB/3T3 clone A31 cultures; they generally appear as small areas of multilayered cells in confluent A31 cultures that have been left at confluence for several weeks. They have been recloned several times, and the most extreme cell type among the clones, based on altered morphology and the degree of loss of contact inhibition of cell division, was then selected for further propagation. Clone S1 is a transformed fibroblastic cell that reaches saturation densities 10- to 15-times greater than that of A31, but tends to remain in parallel orientation; it shows the *in vitro* properties of transformed cells and produces tumors upon inoculation into newborn BALB/c mice (13, 23). Clone S2 is a spontaneous epithelioid variant that differs drastically in its morphology from A31. It reaches saturation densities as great as have been seen with any of the cells transformed by exogenously added virus. Fig. 1 summarizes the genealogy of the cell lines used in the studies to be described.

Antisera. The standard anti-PC1 typing serum (*C57BL/6 x DBA/2*)F_1 *anti-MOPC-70A* contains at least three different antibodies directed against (*i*) a cell surface differentiation antigen, PCI (24), (*ii*) a viral envelope antigen, xVEA, of myeloma extracellular type-C particles, and (*iii*) a G (Gross) cell-surface antigen, GCSA (16). The G-typing *rat* serum (*W/Fu x BN*)F_1 *anti-W/Fu(C58NT)D* contains multiple antibodies directed against a variety of G-antigens as well as xVEA (16, 18, 25).

Absorption of Antiserum. Two different absorptions were performed: (*i*) For removal of natural xenoantibody against normal mouse components from the G-typing *rat* serum, *in vivo* absorption was always employed before use. 1 ml of undiluted antiserum was injected intraperitoneally into normal 2-month-old C57BL/6 mice. The serum was recovered 2 hr later. The absorbed serum was examined for its specificity by immunoelectron microscopy with the transplanted C57BL leukemia EL4(G^-) to confirm that natural xenoantibody against viable mouse cells had been absorbed out, and with the transplanted G^+C57BL/6 Gross leukemia E♂G2 (26) and the transplanted BALB/c myeloma MOPC-70A (24), to show that antibodies against different classes of G-antigens and xVEA still remained. (*ii*) For examination of whether the specific reactivity of the anti-PC1 typing serum and the G-typing *rat* serum was absorbed out with test cells, *in vitro* absorption was done by incubation of undiluted antiserum with equal volumes of well-washed, packed appropriate cells at room temperature for 30 min and then at 4° for 30 min with periodic shaking. The same procedure was then repeated with fresh cells.

Immunoelectronmicroscopy. Briefly, 3–5 million viable target cells, either gently scraped from monolayers with a rubber policeman or collected from leukemic spleen (E♂G2) and ascites (MOPC-70A), were well washed in medium 199 by centrifugation at 0° and then incubated with 0.05 ml of undiluted serum. After two washes, the cells were incubated with hybrid antibodies with dual specificity for (*a*) mouse-IgG or rat-IgG and (*b*) southern bean mosaic virus (SBMV)

TABLE 1. *Derivation of cell lines tested for properties of the endogenous BALB/c type-C viruses*

Original mouse strain	Designation of cell lines	Description of cell lines	Refs
BALB/c	BALB/3T3	A clonal line of embryonic fibroblasts, free of type-C viruses, morphologically untransformed and nontumorigenic	22
	S1-6	A clonal line of spontaneously transformed fibroblastic cells derived from A31, type-C virus-producing, tumor-producing	13, 23
	S2-3; S2-4	Two sublines cloned from a spontaneous epithelioid variant of A31, type-C virus-producing	13
	S2-3(R)	A S2 clone 3 subline superinfected with Rauscher strain of murine leukemia virus, type-C virus positive	13
	MOPC-70A	The reference transplanted BALB/c myeloma induced by mineral-oil, type-C virus-producing	24
C57BL/6	E♂G2	The reference transplanted Gross leukemia induced infection by Passage A Gross virus, type-C virus-producing	26

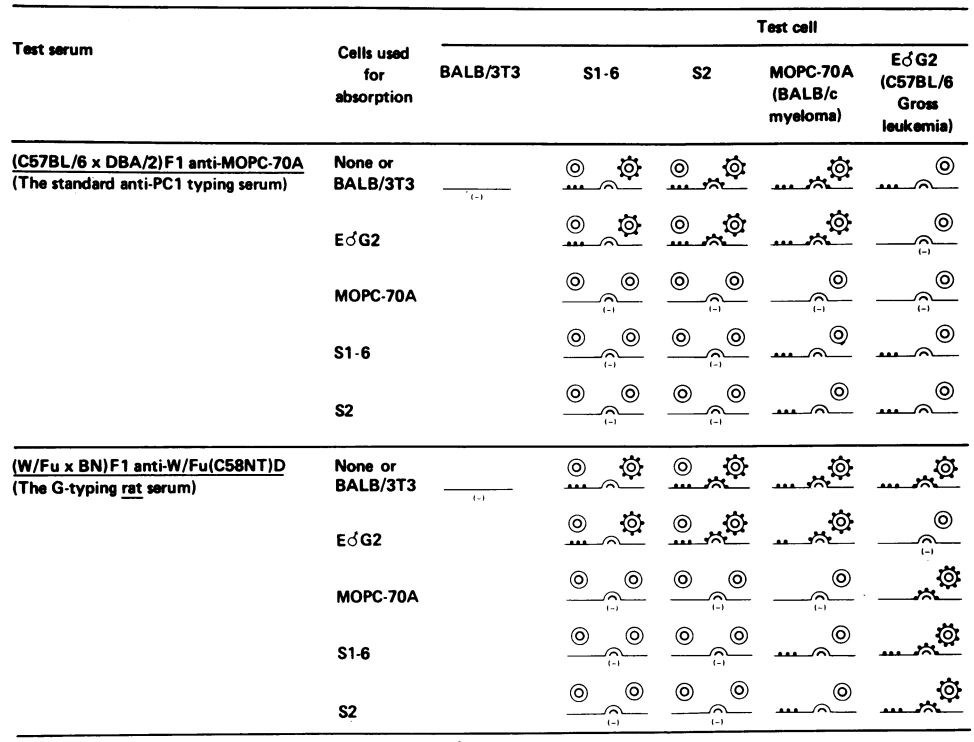

FIG. 2. Summary analysis of BALB/3T3 endogenous type-C virus-associated surface antigens (see *text*).

or ferritin (16). All incubations were done on ice for 30 min with periodic shaking. The final pellets of viable cells were fixed with glutaraldehyde and osmium tetroxide, dehydrated, and embedded in Epon (16). After double staining with uranyl acetate and lead citrate, thin sections were examined under a Siemens microscope Elmiskop 1A.

RESULTS

The results obtained by immunoelectronmicroscopy are presented in Fig. 2 and may be summarized as follows:

(*1*) *Normal BALB/3T3 cells showed no detectable phenotypic expression of endogenous type-C virus-associated antigens on the cell surface.* These cells reacted with neither the standard anti-PC1 typing serum (*C57BL/6* x *DBA/2*)F_1 *anti-MOPC-70A* nor the G-typing *rat* serum (*W/Fu* x *BN*)F_1 *anti-W/Fu-(C58NT)D*. The viral genome must then remain as latent information in these cells, since they have the ability to spontaneously release complete type-C viruses after they have become spontaneously transformed.

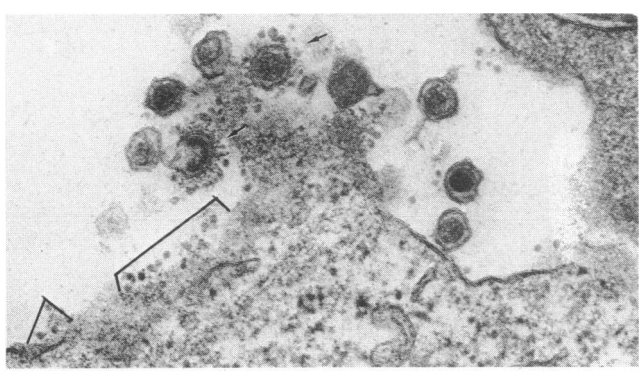

FIG 3. S2-3 cells were reacted with the standard anti-PC1 typing serum (*C57BL/6* x *DBA/2*)F_1*anti-MOPC-70A* and labeled with SBMV. Small sectors on the cell surface (*brackets*) and the entire envelope of some virions (*arrows*) were labeled, but other virions were not labeled. ×32,900.

(*2*) *One population of endogenous type-C viruses from S1 and S2 cell lines was MuMAV.* The standard anti-PC1 typing serum has always reacted with the cell membranes of both of these transformed cells, and it also reacts with some of the extracellular endogenous type-C particles, labeling small sectors on the cell surface and the entire viral envelope (Fig. 3). The ratio of extracellular particles showing positive reaction was different from cell line to cell line, being about 50% in clones S1-6 and S2-3 cells and about 80–90% in clones S2-4. Even in the same cell line, however, this ratio could differ to some extent from passage to passage. The antigenic specificity of these positively reacting particles was demonstrated by the complete absorption of this activity from the anti-PC1 typing serum with BALB/c myeloma MOPC-70A cells, indicating that the antigenic property of the endogenous BALB/c type-C envelope is quite similar to that of the MuMAV envelope, xVEA. The immunologic identity of positive reactions between the endogenous particles from S2-3, S2-4, and S1-6 cells was proven by crossabsorption studies. Each virus-producing clone was able to absorb out this activity. The results described above also indicate the possibility that xVEA$^+$MuMAV induces the cell-surface antigen PC1, because of changing PC$^-$ BALB/3T3 cells or their PC$^-$ transformed cells to PC$^+$ cells associated with the spontaneous production of xVEA$^+$MuMAV virions.

(*3*) *Another population of endogenous particles remains unclassified.* A previous study demonstrated two different antibodies in the G-typing *rat* serum (*W/Fu* x *BN*)F_1 *anti-W-Fu(C58NT)D* against both the envelope antigens on MuLV

TABLE 2. *Populations of type-C viruses in different transformed cell lines and transplanted tumors*

Population of type-C virus	Cell and tumor line							
	Nontransformed cells			Spontaneously transformed clones of BALB/3T3			Transplanted tumor	
	BALB/3T3	BALB/3T3(R)	NIH/3T3 infected with type-C viruses from S lines	S1-6	S2	S2(R)	MOPC-70A	E♂G2
MuLV	-	+	-	-	-	+	-	+
MuMAV	-	-	+	+	+	+	+	-
Uncharacterized virus	-	-	+	+	+	+	-	-

(R) = infected with Rauscher strain of MuLV.

(LVEA) and MuMAV (xVEA) (25). Particles of the S2 lines can be classified into two groups; first, positive with the G-typing *rat* serum, and second, negative, indicating the presence of a previously undescribed population of particles (Fig. 4). However, the passage A Gross virus of E♂G2 did not absorb any activity of the G-typing *rat* serum with the S2-3 particles. S2-3 cells containing a large number of type-C particles also did not absorb any activity of the G-typing *rat* serum tested with the E♂G2 cells and viruses. Similar findings by immunoelectronmicroscopy were obtained when virus-negative S2 subclones were treated with iododeoxyuridine (IdU) so as to induce endogenous type-C viruses. These results indicate that endogenous type-C particles of S2 lines contain two distinguishable populations, xVEA$^+$MuMAV and uncharacterized particles, but either lack MuLV or contain too few MuLV to absorb a detectable amount of antibody against MuLV. Furthermore, it seems most likely that the cell-surface antigen that reacted with the G-typing *rat* serum is PC1 or a modified antigen similar to PC1.

(4) Both populations of viruses are capable of infecting NIH Swiss/3T3 cells. After infection of NIH/3T3 cells with the endogenous type-C viruses of these BALB/c spontaneously transformed lines, the NIH/3T3 cells also produced two distinguishable populations of type-C viruses, MuMAV and the previously uncharacterized virus. The type-C viruses produced after infection of NIH/3T3 cells with S1-6 or S2-3 supernatant fluids possessed the same specificities of envelope antigens as those of the S1-6 and S2-3 lines themselves (Table 2). In other words, two populations of endogenous type-C viruses of S lines are both N-tropic and NIH/3T3 cells, which have endogenous type-C virus information (27) but produce no complete endogenous virus, appear to be able to replicate both of the endogenous BALB/3T3 viruses. S2 clone 3 cells superinfected with Rauscher murine leukemia virus (R-MuLV) produced complete MuLV. The G-typing *rat* serum preabsorbed with MOPC-70A cells reacted to label the entire viral envelope (Fig. 5). This finding demonstrates that the S2 cells have the ability to replicate MuLV if they are exogenously infected with MuLV. Whether different experimental conditions will result in the release from these cells of an endogenous type-C virus with MuLV antigenic properties remains to be determined. We conclude, therefore, that the endogenous type-C particles of spontaneously transformed BALB/3T3 have at least two distinguishable populations, MuMAV and a type-C virus, that have not previously been characterized, having the envelope antigen properties of neither MuLV nor MuMAV.

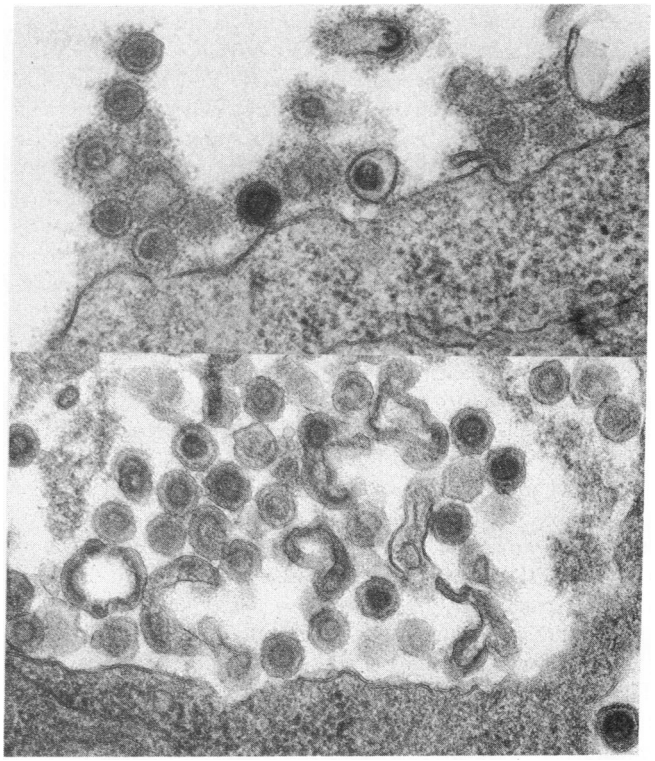

FIG. 4. S2-3 cells were reacted with the G-typing *rat* serum *(W/Fu* x *BN)F$_1$ anti-W/Fu(C58NT)D* and labeled with ferritin. Sectors on the cell surface and the entire envelope of one virus population (*top*) were labeled, while another virus population(s) (*bottom*) was not labeled at all. ×49,350.

DISCUSSION

The recent immunological studies of BALB/c myeloma (MOPC-70A, MPC-113) cells induced by mineral oil and extracellular type-C viruses (MuMAV) that they produce

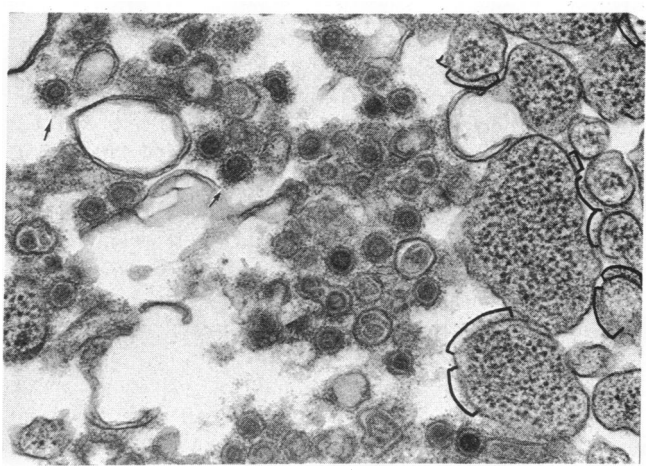

FIG. 5. S2-3 cells superinfected with R-MuLV were reacted with the G-typing *rat* serum *(W/Fu* x *BN)F$_1$ anti-W/Fu(C58-NT)D*, which was preabsorbed with MOPC-70A cells and labeled with ferritin. Sectors on the cell surface (*brackets*) and the entire envelope of all virions were labeled with ferritin. Typical positive virions are indicated by *arrows*. ×32,900.

have shown that natural antibodies against the differentiation antigen PC1 and MuMAV-envelope antigen xVEA occur in normal mice of PC^+ and PC^- strains (21). Thus, it has been strongly indicated that PC1 antigen may be induced by MuMAV, and these results support the postulate that most, if not all, cells of BALB/c mice carry the MuMAV genome that induces PC1 antigen on the cell surface as well as $xVEA^+$ type-C extracellular particles. This postulate is further substantiated by the results described here, which show that nonmalignant BALB/3T3 cells that have spontaneously transformed and that are releasing endogenous type-C viruses have changed from PC^- to PC^+. No induction of PC1 antigen occurred in the cell lines which transformed but did not produce endogenous type-C viruses. The virus-producing cells simultaneously acquired another cell-surface antigen in changing from PC^- to PC^+. This cell-surface antigen is recognized by the G-typing *rat* serum preabsorbed with G^+ C57BL/6 Gross leukemia E♂G2 cells that contain all known G antigens. Since the G-typing *rat* serum cannot recognize PC1 antigen (16), the antigen could be a new antigen different from PC1. Its appearance might be related to the production of the uncharacterized type-C viruses.

Some viral antigens on the envelope of G (Gross) and FMR (Friend, Moloney, and Rauscher) MuLV have common specificities, since the G-typing *rat* serum recognizes both of them (18). This finding has been confirmed by use of the serum *rabbit anti-various MuLV* (28). In this paper, therefore, MuLV of G- and FMR-types were considered as one group of type-C particles (see *Introduction*). The findings that none of the spontaneously transformed BALB/3T3 clones absorbed out the activity of the G-typing *rat* serum with antigens on the envelope of MuLV indicate that the clones are *not* producing MuLV of either G or FMR type and rule out the possibility of exogenous MuLV contamination as an explanation for the appearance of type-C viruses in these clones. In addition, the type-C viruses derived from the BALB/c continuous lines are exclusively N-tropic (able to infect NIH Swiss but not BALB/c cells) (7, 12, 13, 23). These facts also eliminate the possibility of cell-to-cell infection by the endogenous BALB/c type-C viruses.

It is not known whether MuMAV and/or the other type-C virus has oncogenic potential or whether spontaneous transformation in cell culture and by mineral oil in the animal "switches on" both the phenotypic expression of MuMAV and the tumor-producing potential of the cells without these alterations being causally related to one another. The fact that endogenous viruses can have different antigenic properties and the finding of a previously undescribed type-C virus in these clones raises the possibility that several related, but somewhat different, type-C genomes may be contained in normal mouse cells. In support of this hypothesis, recent studies with an antiserum that was prepared in (BALB/c x C57BL/6)F_1 mice by immunization with an x-radiation-induced BALB/c transplanted leukemia (Sato, H. *et al.*, in preparation) have shown that this antiserum did not react with MuMAV but clearly reacted to label the entire viral envelope of some of the S2-3 and S2-4 virions under conditions where the standard anti-PC1 typing serum absorbed with MOPC-70A cells showed no positive reaction (unpublished observations). These observations indicate that the (BALB/c x C57BL/6)F_1 serum contains antibodies to a previously undescribed type-C viral envelope antigen produced by the spontaneously transformed BALB/3T3 cells. The availability, then, of two different antisera, each reacting separately with a different population of BALB/c endogenous type-C viruses, may open a way to study the biologic properties of these viruses by isolation of individual virus populations after their neutralization with one or the other of these antisera.

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