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Demonstration of a Cell-Surface Antigen Associated with Murine Sarcoma Virus by Immunoelectron Microscopy

(Kirsten and Maloney strains/MuLV/viral envelope antigens)

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ABSTRACT **Cells transformed by murine sarcoma virus have been examined for the presence of a new virus-associated cell-surface antigen by immunoelectron microscopy. A common antigen has been detected on the surface of nonproductively transformed cells that were induced by two different strains of murine sarcoma virus, Kirsten and Moloney. This antigen shows crossreaction with cell lines transformed by murine sarcoma virus that were produced in two different mammalian species, rats and mice. Further, this antigen is distinct from previously described antigens on the surfaces of cells infected by murine leukemia virus, on the viral envelope, and on the surfaces of spontaneously transformed cell lines or cell lines transformed by x-irradiation.**

Several tumor viruses have been shown to induce the formation of virus-associated antigens in nonproductively transformed mammalian cells. Transformation with DNA-containing viruses such as simian virus 40 and polyoma virus results in the production of an intranuclear T antigen (1) and a strong transplantation antigen (2). Avian RNA-containing sarcoma viruses that transform mammalian cells cause production of virus-associated antigens including the avian C-type group-specific (gs) antigen (3) and a recently described transplantation antigen (4).

Murine sarcoma virus (MSV) also induces transformation of cells in the absence of detectable virus production (5, 6). MSV-transformed nonproducer cells have been shown to contain virus-specific RNA (7). In addition, the sarcoma viral genome can be rescued from MSV-transformed nonproducer cells by infection with a helper leukemia virus (5, 6). However, the production of any virus-associated antigens has yet to be demonstrated by either serological methods or by transplantation immunity techniques (8, 9). It has been shown that immunoelectron microscopy provides a sensitive and specific method for detection and location of antigens on cell surfaces (10). The availability of clonal lines of nontransformed cells and of sublines that have been transformed either spontaneously or by oncogenic viruses (11–13) has led us to search for MSV-associated antigens on the surfaces of MSV-transformed nonproducer cells by immunoelectron microscopy.

MATERIALS AND METHODS

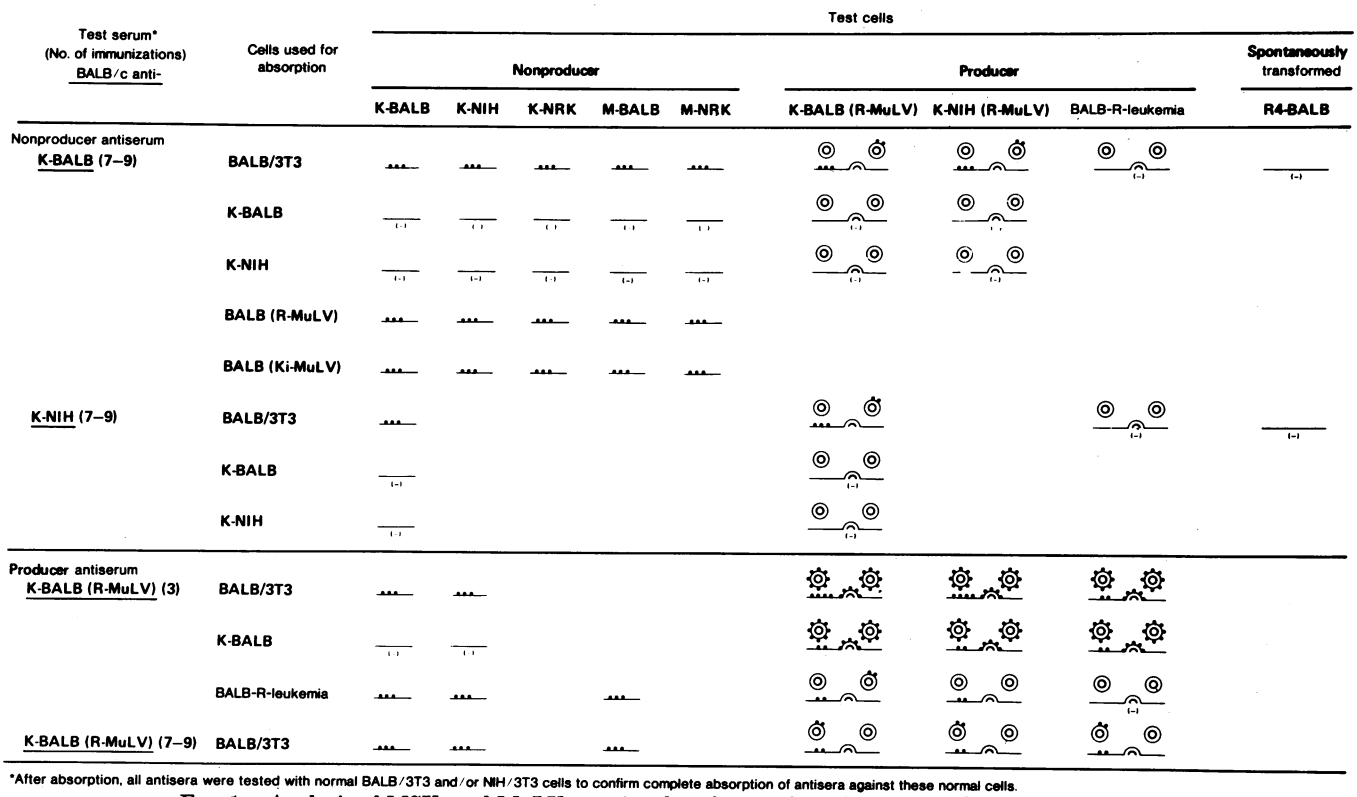
Cells and Viruses. Cells were grown in Dulbecco's modification of Eagle's medium supplemented with 10% calf serum. The individual cell lines used are described in Table 1. In addition to the above tissue-culture lines, Rauscher murine leukemia virus (MuLV)-induced BALB/c leukemia was maintained in syngeneic mice and used as a source of MuLV-induced surface antigen. The sources of murine sarcoma and murine leukemia viruses used have been reported (9, 12).

Antisera. All antisera were prepared in 6- to 8-week-old female BALB/c mice obtained from the National Institutes of Health breeding colony, Bethesda, Md. Tissue culture cells

TABLE 1. *Summary of cell lines used for immunizations, absorptions, and as target cells for immunoelectron microscopy*

Designation of cell lines	Description	Refs.
BALB/3T3	A clonal line of BALB/c embryo cells	11
BALB(R-MuLV)	Same clonal line infected with Rauscher (R)-MuLV	
BALB(Ki-MuLV)	Same clonal line infected with Kirsten (Ki)-MuLV	
K-BALB	A Ki-MSV-transformed nonproducer subclone of BALB/3T3	9, 12
K-BALB(R-MuLV)	A line of MSV- and MuLV-producing transformed cells, obtained by superinfecting K-BALB cells with R-MuLV	9
M-BALB	A Moloney (M)-MSV transformed nonproducer subclone of BALB/3T3	6
R4-BALB	A tumorigenic subclone isolated after x-irradiation of BALB/3T3 (spontaneous or x-irradiation induced)	13
NIH/3T3	A clonal line of NIH Swiss embryo cells	14
K-NIH	A Ki-MSV-transformed nonproducer subclone of NIH-3T3	12
K-NIH(R-MuLV)	A line of MSV- and MuLV-producing transformed cells, obtained by superinfecting K-NIH cells with R-MuLV	
NRK	A clonal line of normal rat-kidney cells	15
K-NRK	A Ki-MSV-transformed nonproducer subclone of NRK	12
M-NRK	An M-MSV-transformed nonproducer subclone of NRK	

Abbreviations: MSV, murine sarcoma virus; MuLV, murine leukemia virus.



*After absorption, all antisera were tested with normal BALB/3T3 and/or NIH/3T3 cells to confirm complete absorption of antisera against these normal cells.

FIG. 1. Analysis of MSV- and MuLV-associated surface antigens by immunoelectron microscopy.

were harvested by trypsinization, washed twice, and resuspended at 10^7 cells/ml in serum-free medium 199. Groups of five BALB/c mice were immunized nine times at weekly or biweekly intervals by intraperitoneal inoculation of 1.0 ml of cell suspension per animal. Before injection, the K-BALB and K-BALB (R-MuLV) cell lines (9) were treated with mitomycin C (20 μg/ml) for 20 min at 37° to block cell division and prevent tumor development in the inoculated syngeneic mice. Mitomycin C pretreatment was omitted in the case of the allogeneic immunization with K-NIH cells. Antisera were obtained 10–14 days after the last immunization. This allogeneic antiserum was tested exclusively against BALB/c-derived cell lines, K-BALB, K-BALB(R-MuLV), R4-BALB, and BALB(R-MuLV), to avoid reaction with alloantigens. The antisera against nonproducer cell lines, *BALB/c anti-K-BALB* and *BALB/c anti-K-NIH*, are designated as "nonproducer antisera" whereas the antiserum against the virus-producing cell line, *BALB/c anti-K-BALB (R-MuLV)*, has been designated as "producer antiserum" in this study.

Absorption of Antisera. Absorption was performed by incubation of serum with an equal volume of well-washed, packed cells at 4° for 60 min with periodic agitation. Cell suspensions were centrifuged at 1500 rpm (300 × *g*) for 15 min and the absorption procedure was repeated with fresh cell packs. In each experiment, antisera were tested for activity against BALB/3T3 and NIH/3T3 cells, and then only completely absorbed antisera were used.

Immunoelectron Microscopy. The methods for detection of cell-surface antigens by immunoelectron microscopy have been described (10). Briefly, viable cells were gently scraped from monolayers with a rubber policeman, washed twice in serum-free medium 199 by centrifugation at 4°, and distributed to small centrifuge tubes at about 3×10^6 cells per tube. Cells were incubated with 0.05 ml of undiluted antiserum for 30 min, washed twice with serum-free medium 199, and then incubated with hybrid antibody with dual specificity for mouse-IgG and for southern bean mosaic virus or ferritin. After two further washes, the cells were incubated with southern bean mosaic virus or ferritin. All incubations were on ice for 30 min with periodic agitation. Washed cell pellets were fixed with glutaraldehyde and osmium tetraoxide, dehydrated, and embedded in Epon as usual. After double staining with uranyl acetate and lead citrate (southern bean mosaic virus was used as marker) or single staining with lead citrate (to add contrast to ferritin), thin sections were examined under a Siemens microscope Elmiskop 1A.

RESULTS

A summary of the data is given in Fig. 1 and Table 2.

Presence of a new cell-surface antigen on MSV-transformed nonproducer cells

Studies were first performed to determine whether any new antigens were present on the surface of MSV-transformed nonproducer cells. Antisera prepared from BALB/c mice after either three immunizations or hyperimmunization (7–9 immunizations) with the nonproducer cell line, K-BALB, were first doubly absorbed with normal BALB/3T3 cells to remove any activity with normal BALB/c antigens and then tested by immunoelectron microscopy against K-BALB cells. The early serum showed a relatively weak reaction with K-BALB cells. However, the hyperimmune serum clearly labeled small sectors on the cell surface of the MSV-transformed cells (Fig. 2). The activity of the serum *BALB/c anti-K-BALB* was eliminated by double absorption with K-BALB cells.

TABLE 2. *Summary of immunoelectron microscopy studies on MSV- and MuLV-associated cell-surface and viral envelope antigens*

		Test cells				
		Nonproducer	MuLV-superinfected nonproducer		Murine leukemia (Rauscher)	
Test serum	Cells used for absorption	Cell surface	Cell surface	Viral envelope	Cell surface	Viral envelope
Nonproducer antiserum*	Nontransformed- & non-virus-infected cells	+	+	−	−	−
	Nonproducer	−	−	−	−	−
	Murine leukemia (Rauscher)	+	+	−	−	−
Producer antiserum*	Nontransformed- & non-virus-infected cells	+	+	+	+	+
	Nonproducer	−	+	+	+	+
	Murine leukemia (Rauscher)	+	+	−	−	−

* See *Methods*.

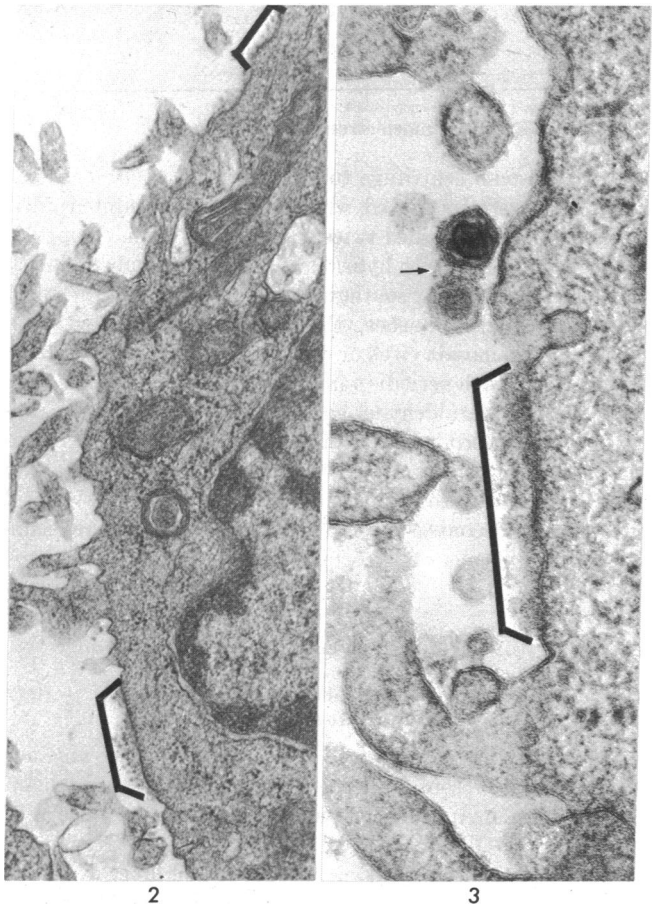


FIG. 2. K-BALB cells were reacted with the serum *BALB/c anti-K-BALB* absorbed with BALB/3T3 cells and labeled with southern bean mosaic virus. Small areas on the cell surface are labeled with the virus. ×29,700.

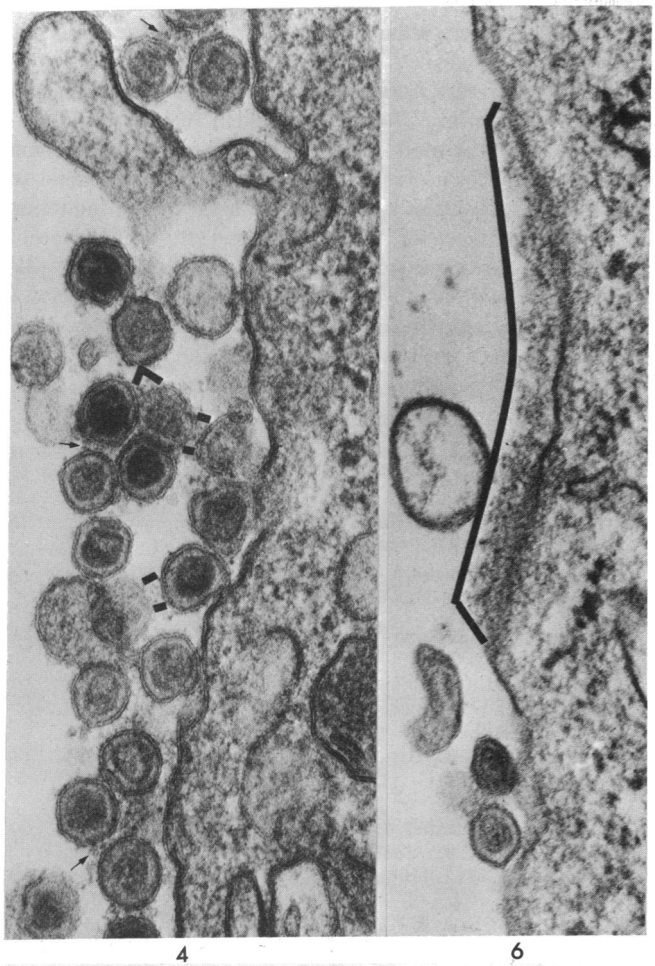
FIG. 3. K-BALB(R-MuLV) cells were reacted with the serum *BALB/c anti-K-BALB*, absorbed with *BALB/3T3* cells, and labeled with ferritin. Small areas on the cell surface and a tiny spot on the viral envelope are labeled with ferritin (see Fig. 4). ×71,280.

Crossreactivity of surface antigens on Ki-MSV-transformed nonproducer cells of different species

Studies were performed to establish whether the newly detected surface antigen on MSV-nonproducer cells was common to MSV-transformed cells derived from different strains of mice. The two nonproducer antisera, *BALB/c anti-K-BALB* and *BALB/c anti-K-NIH*, reacted with both nonproducer K-BALB and K-NIH lines, labeling small sectors on the cell surfaces; the same sera were completely negative when tested against normal BALB/3T3 and/or NIH/3T3 cells. The specificity of these reactions was shown by the fact that the activities could be eliminated from each serum by cross-absorption with either nonproducer cell line. A subclone of BALB/3T3 cells, obtained after exposure to a high dosage of x-irradiation, R4-BALB, was also tested for antigens that crossreacted with those of the MSV-nonproducer cells. There was, however, no evidence of MSV-associated antigens on this cell line. The nonproducer antiserum did react with a Ki-MSV-transformed rat nonproducer line (K-NRK) but not with control normal rat-kidney cells (NRK), indicating crossreaction of the antigen between MSV-transformed cells of two different species.

Crossreaction between MSV-associated surface antigens of cell lines nonproductively transformed by different strains of MSV

The Kirsten strain of murine sarcoma virus (Ki-MSV) was originally isolated by passage of a mouse erythroblastosis virus in W/Fu rats (16). The virus could, therefore, be of rat origin. In contrast, the Moloney strain of MSV (M-MSV) was obtained after passage of Moloney MuLV in mice (17). Although similar in most of their tissue culture properties (7, 12), cells transformed by these viruses do show morphological differences (Aaronson, S. A., unpublished observations). It was of interest, therefore, to determine whether crossreaction could be detected between the MSV-associated surface antigens on cells nonproductively transformed by these two MSV strains. The nonproducer antiserum, *BALB/c anti-K-BALB*, when tested with both M-BALB and M-NRK cell lines, was



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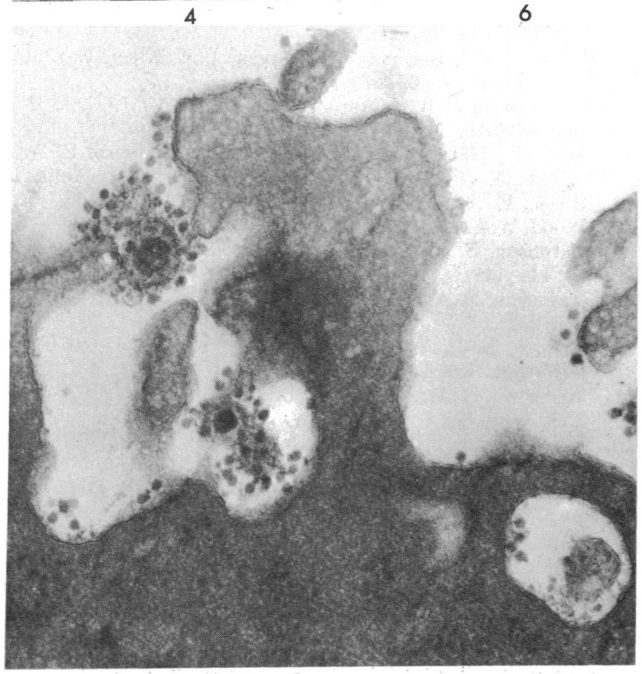

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FIG. 4. Same as the Fig. 3, but tiny spots of labeling on the R-MuLV are shown more clearly. ×75,600.

FIG. 5. K-BALB(R-MuLV) cells were reacted with the serum *BALB/c anti-K-BALB (R-MuLV)* (after the third immunization), absorbed with BALB/3T3 cells, and labeled with southern bean mosaic virus. Small areas on the cell surface and the entire viral envelope of R-MuLV are labeled with the virus. ×62,400.

FIG. 6. K-BALB(R-MuLV) cells were reacted with hyperimmune serum *BALB/c anti-K-BALB(R-MuLV)*, absorbed with BALB/3T3 cells, and labeled with ferritin. Small areas on the cell surface are labeled with ferritin, but the viral envelope is not labeled. ×70,000.

positive by immunoelectron microscopy, whereas, as described above, this serum was negative when tested with both nontransformed parent BALB/3T3 and NRK lines. On the basis of these findings, it is clear that the MSV-associated antigens of cells transformed by either M-MSV or Ki-MSV possess crossreacting antigenic determinants.

Relationship between MuLV- and MSV-associated surface antigens

When the two nonproducer antisera were reacted with R-MuLV-superinfected transformed cells, K-BALB(R-MuLV) and K-NIH (R-MuLV), small sectors on the cell surface were clearly labeled (Fig. 3) and a few virions were labeled on small areas of the envelope (Fig. 4). This latter labeling may have resulted from acquisition by virions of antigens previously existing on the cell surface during budding, as observed for some alloantigens that have been detected on the envelope of Gross-MuLV (18). To determine whether the surface antigen detected on MSV-transformed producer and nonproducer cell lines was related to the previously described MuLV-associated surface antigen (19), the nonproducer antisera, *BALB/c anti-K-BALB* and *BALB/c anti-K-NIH*, were tested with the Rauscher leukemia cell line, BALB-R-leukemia. No positive reactions were observed, indicating that the antigen common to MSV nonproducer cells was absent from the surface of leukemia virus-infected cells. Further evidence that MuLV- and MSV-associated antigens differed was obtained by absorption of the serum *BALB/c anti-K-BALB* with either BALB (R-MuLV), BALB (Ki-MuLV), or BALB-R-leukemias; absorption with either of these three MuLV-producing cell lines failed to absorb out the activity of this antiserum with the nonproducer cells. These findings lead to the conclusion that there is no crossreaction between MuLV-associated and MSV-associated cell-surface antigens.

Antigens of MSV-transformed virus-producing and nonproducing cells were next compared by use of sera made against producer cells. Antisera were obtained from BALB/c mice immunized with the R-MuLV-superinfected, transformed cell line, K-BALB(R-MuLV). The antisera were tested for activity against MSV-transformed producer [K-BALB(R-MuLV), K-NIH (R-MuLV)], nonproducer (K-BALB, K-NIH), and BALB-R-leukemia target cells. Sera obtained after the third immunization showed only very weak labeling of the surface of K-BALB target cells. K-BALB (R-MuLV) target cells showed a similar weak surface labeling and, in addition, there was a labeling of the entire envelope of mature and budding viruses (Fig. 5). Absorption of the producer antiserum *BALB/c anti-K-BALB(R-MuLV)* with BALB-R-leukemias resulted in loss of the activity with antigens on the BALB-R-leukemia cell surface and R-MuLV envelope. The absorbed sera, however, retained their activity against the cell-surface antigens of both the nonproducer and producer transformed cell lines. The hyperimmune serum *BALB/c anti-K-BALB(R-MuLV)* (7–9 immunizations) also labeled small sectors on the surface of both nonproducer (K-BALB, K-NIH) and producer [K-BALB (R-MuLV), K-NIH(R-MuLV)] target cells (Fig. 6). However, this hyperimmune serum only weakly labeled the viral envelope. The decreased level of antibody to viral envelope antigens in the hyperimmune serum demonstrated "immune paralysis" and suggests a much greater strength of the viral envelope anti-

gen(s) than the MSV-associated cell-surface antigen; the latter was only clearly detectable after repeated immunization. These findings also provide further evidence that the specificity of nonproducer cell-surface antigen differs from that of R-MuLV-associated antigens.

Although the producer antiserum *BALB/c anti-K-BALB (R-MuLV)* no longer reacted with K-BALB cells after absorption with this cell line, it still labeled the surface of virus-producing K-BALB(R-MuLV) cells. Since this absorbed serum also reacted with the surface of BALB(R-MuLV) cells, it is very likely that the residual activity with K-BALB(R-MuLV) cells was due to the presence of antibodies against R-MuLV-associated cell-surface and viral-envelope antigens.

DISCUSSION

The present studies were performed to determine whether transformation by MSV results in antigenic alteration of the cell surface. MSV-transformed cells, which do not release detectable C-type virus, were found to contain a cell-surface antigen not present on normal mouse cells. Previous studies of RNA and DNA oncogenic viruses have shown that the same antigenic specificities are demonstrable in all tumors induced by the same virus, even if they arise in different tissues, strains, and species, but are different in tumors induced by unrelated viruses (19). Thus, the crossreaction among MSV nonproducer cell lines induced from cells of rats and different strains of mice by Ki- and M-MSV strongly suggests that the antigen(s) detected in the present study are MSV-associated. This hypothesis is further substantiated by the fact that the cell-surface antigen detected in MSV-transformed nonproducer cells is absent from cells transformed spontaneously or by x-irradiation. The MSV-associated antigen does not crossreact serologically with the leukemia virion or with leukemia virus-associated cell-surface antigens. Furthermore, superinfection of nonproducer cells with helper murine leukemia virus leads to production of MuLV-associated cell-surface and virion antigen but does not alter the ability to detect the MSV-associated cell-surface antigen.

There is considerable evidence indicating that the newly described MSV-associated cell-surface antigen is only weakly antigenic. In previous studies, the antigenicity of MSV-nonproducer cells was not detectable by serologic techniques while those of MSV-producer cells were readily demonstrated (8, 9). More recently, in studies with the S^+L^- line of MSV-transformed cells that release only a very low amount of non-infectious virus (20), sarcoma virus-associated antigens were not demonstrable by either humoral or cellular cytotoxocity techniques (21). The present findings, that the MSV-associated cell-surface antigen was weak enough to require hyperimmunization for detection while MuLV-associated antigens produced immune paralysis after hyperimmunization of the animal, provide another line of evidence that the strength of these antigens differs greatly. In previous reports, detection of MSV-associated antigens in "nonproducer" transformed cell lines (22) is difficult to interpret due to lack of adequate control cell lines and in view of more recent findings showing each to be virus-productive (ref. 23; Ting, R. C., personal communication). The detection of the weak MSV-associated cell-surface antigen by immunoelectron microscopy clearly demonstrates greater sensitivity of this technique compared to other serologic methods.

The MSV-associated cell-surface antigen described in the present study could represent either a cellular genome-determined antigen induced by MSV or, alternatively, it could be virus-coded. This latter possibility would be of considerable interest since antigens coded for by MSV have not yet been described. Such a virus-coded antigen would provide an important marker for the presence of the sarcoma genome in transformed cells. In either case, it is of interest to determine whether this antigen is involved in the induction and/or maintenance of the transformed state. The recent isolation of temperature-sensitive mutants of MSV (24) should allow a direct test of this latter possibility, since a new antigen involved in maintenance of the transformed state should be present on the cell surface at the permissive, but be lacking at the nonpermissive, temperature.

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1. Pope, J. H. & Rowe, W. P. (1964) *J. Exp. Med.* **120,** 124–128.
2. Sjögren, H. O., Hellström, I. & Klein, G. (1961) *Cancer Res.* **21,** 329–337; Habel, K. (1961) *Proc. Soc. Exp. Biol. Med.* **106,** 722–725.
3. Huebner, R. J., Armstrong, D., Okuyan, M., Sarma, P. S. & Turner, H. C. (1964) *Proc. Nat. Acad. Sci. USA* **51,** 742–750.
4. Kurth, R. & Bauer, H. (1972) *Virology* **49,** 145–149.
5. Huebner, R. J., Hartley, J. W., Rowe, W. P., Lane, W. T. & Capps, W. I. (1966) *Proc. Nat. Acad. Sci. USA* **56,** 1164–1169.
6. Aaronson, S. A. & Rowe, W. P. (1970) *Virology* **42,** 9–10.
7. Green, M., Rokutanda, H. & Rokutanda, M. (1971) *Nature New Biol.* **230,** 229–232.
8. Chuay, J. C., Berman, L., Gunven, P. & Klein, E. (1969) *Int. J. Cancer* **4,** 465–479.
9. Stephenson, J. R. & Aaronson, S. A. (1972) *J. Exp. Med.* **135,** 503–515.
10. Aoki, T., Hämmerling, U., deHarven, E., Boyse, E. A. & Old, L. J. (1969) *J. Exp. Med.* **130,** 979–1001; Aoki, T., Boyse, E. A., Old, L. J., deHarven, E., Hämmerling, U. & Wood, H. A. (1970) *Proc. Nat. Acad. Sci. USA* **65,** 569–576.
11. Aaronson, S. A. & Todaro, G. J. (1968) *J. Cell. Physiol.* **72,** 141–148.
12. Aaronson, S. A. & Weaver, C. A. (1971) *J. Gen. Virol.* **13,** 245–252.
13. Pollock, E. J., Aaronson, S. A. & Todaro, G. J. (1970) *Int. J. Radiat. Biol.* **17,** 97–100.
14. Aaronson, S. A., Jainchill, J. L. & Todaro, G. J. (1970) *Proc. Nat. Acad. Sci. USA* **66,** 1236–1243.
15. Duc-Nguyen, J., Rosenblum, E. M. & Zeigel, R. F. (1966) *J. Bacteriol.* **92,** 1133–1140.
16. Kirsten, W. H. & Mayer, L. A. (1967) *J. Nat. Cancer Inst.* **39,** 311–315.
17. Moloney, J. B. (1966) *Nat. Cancer Inst. Monogr.* **22,** 139–142.
18. Aoki, T. & Takahashi, T. (1972) *J. Exp. Med.* **135,** 443–457.
19. Old, L. J. & Boyse, E. A. (1964) *Annu. Rev. Med.* **15,** 167–186; Old, L. J. & Boyse, E. A. (1965) *Fed. Proc.* **24,** 1009–1017.
20. Bassin, R. H., Phillips, L. A., Kramer, M. J., Haapala, D. K., Peebles, P. T., Nomura, S. & Fischinger, P. J. (1971) *Proc. Nat. Acad. Sci. USA* **68,** 1520–1524.
21. Strouk, V., Grundner, G., Fenyö, E. M., Lamon, E., Skurzak, H. & Klein, G. (1972) *J. Exp. Med.* **136,** 344–352.
22. Ting, R. C. (1967) *Proc. Soc. Exp. Biol. Med.* **126,** 778–781; Law, L. W. & Ting, R. C. (1970) *J. Nat. Cancer Inst.* **44,** 615–620; McCoy, J. L., Ting, R. C., Morton, D. L. & Law, L. W. (1972) *J. Nat. Cancer Inst.* **48,** 383–391.
23. Ting, R. C. (1968) *J. Virol.* **2,** 865–868.
24. Scolnick, E. M., Stephenson, J. R. & Aaronson, S. A. (1972) *J. Virol.* **10,** 653–657.