TUMOR INDUCTION BY MURINE SARCOMA VIRUS IN AKR AND C58 MICE

Reduction of Tumor Regression Associated with Appearance of Gross Leukemia Virus Pseudotypes*‡

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A common feature of avian and mammalian sarcoma viruses is the induction of soft tissue sarcomas which are characterized in the species of origin by a very short latent period and by a high frequency of complete spontaneous regressions. This unique property is not shared by any other oncogenic agents, either viral or chemical in nature, since the tumors they induce usually arise after weeks or months and show a fatal progressive growth.

Some strains of sarcoma virus are considered defective for replication in the sense that they are apparently unable to synthetize an outer envelope, and consequently cannot produce an infectious virus progeny. As shown first in the avian (1), and subsequently confirmed in the murine (2) and in the feline systems (3), type C leukemia viruses can act as helpers for sarcoma virus replication by providing envelope components necessary for infection. Infectious sarcoma particles then possess the surface specificities of the helper virus as to host range and antigenicity.

Virus preparations commonly used to produce tumors in animals contain a dual population of sarcoma and leukemia viruses mixed in variable ratios. In the mouse, the Moloney murine sarcoma virus (M-MSV)[1] induces tumors which release infectious virus whose envelope antigens are specified by the associated helper Moloney murine leukemia virus (M-MuLV) (4). Furthermore, the new surface antigens acquired by neoplastic cells appear to be largely similar to those determined by M-MuLV (5). The vigorous host

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[1] *Abbreviations used in this paper:* ALS, antimouse lymphocyte serum; B, BALB; FFU, focus-forming units; FIU, focus-inducing units; G,Gross; GCSA, G-MuLV-determined cell surface antigens; Gi, Graffi; M-MuLV, Moloney murine leukemia virus; MCSA, M-MuLV-determined cell surface antigens; M-MSV, Moloney murine sarcoma virus; N, NIH Swiss; PFC, plaque-forming cells; RSV, Rous sarcoma virus; VEA, viral envelope antigen.

immune response evoked by tumor tissue is known to be directed mostly against M-MuLV-coded antigens. It is believed that the regression of tumors observed in adult mice injected with M-MSV is largely dependent on the humoral and cellular immune reactions elicited by the strongly antigenic sarcomas. Suckling mice or adults immunosuppressed by various treatments develop progressively growing tumors following injection with M-MSV (5). Nonspecific stimulation of the immune system, on the other hand, significantly inhibits M-MSV tumorigenesis (6).

Potentiation of M-MSV tumor induction has also been observed in mice preinfected with MuLVs (7, 8). In previous experiments (9 and footnote 2), we have found that BALB/c, CBA, and (C58BL/6 × AKR)F_1 mice neonatally infected with Graffi (Gi−) or passage A Gross (G−) MuLV showed an increased tumor incidence after inoculation of M-MSV. More significantly, the great majority of tumors grew progressively and eventually killed the host. The MSV recovered from these tumors was readily neutralized by antisera specific against Gi- or G-MuLV, but not by an antiserum directed against M-MSV. It was concluded that the tumors were actually caused by a new MSV pseudotype possessing the antigenic specificity of the coinfecting Gi- or G-MuLV. Additionally, a state of partial tolerance (see Discussion) present in MuLV neonatally infected mice could account for the lack of regression of these MSV-induced sarcomas.

In the present experiments, we investigated the in vivo interaction between MuLV and MSV in a more natural setting. Mice of the high leukemia strains AKR and C58, commonly considered as natural carriers of wild type G-MuLV, have been examined for their oncogenic response to M-MSV. In addition, the biology of tumors, the host immune reactivity, and the antigenic properties of retrieved MSV have been studied.

Materials and Methods

Mice. AKR, C58, BALB/c, and C57BL/6 (hereafter called BALB and B6, respectively) mice were obtained from the colony of the Laboratory of Experimental Oncology, Padova University, and from the Animal Production Branch, Division of Research Services, National Institutes of Health, Bethesda, Md. Since a similar tumor response to M-MSV was observed in mice of a given strain from the two sources, no distinction has been made in reporting the experimental results.

Viruses. The M-MSV has been maintained by serial in vivo passages in 1- to 2-week old BALB mice. A cell-free extract from pooled neoplastic tissues was prepared by homogenization and differential centrifugation according to the Moloney procedure (10). In the experiments performed at the National Cancer Institute, Bethesda, Md., the M-MSV preparation no. 240, obtained through the courtesy of Dr. A. F. Gazdar, was employed. M-MSV (3 to 5 × 10^4 focus-forming units [FFU]/0.05 ml) was injected intramuscularly (i.m.) in the thigh region into adult mice.

The M-MuLV was obtained from University Laboratories, Inc., Highland Park, N.J., as a 10% cell-free extract from the spleen of leukemic BALB mice (lot no. MLS 15). When titered on sarcoma-positive leukemia-negative (S^+L^-) cells (11) this preparation contained about 5.6 × 10^5 focus-inducing units (FIU)/ml. The extract was further diluted 1:10, and 0.05 ml was injected subcutaneously (s.c.) into AKR mice within 24 h after birth. M-MSV-injected mice were inspected twice a week for tumor development and growth. Tumor size was evaluated by measuring the major diameter of tumor mass with a caliper and deducting the diameter of the opposite normal thigh.

Histology. Some mice were killed when moribund or at selected time intervals. Representative fragments of tumor, spleen, and lymph nodes were removed, fixed in Bouin's or Carnoy's fluid and

[2]Chieco-Bianchi, L., A. Colombatti, D. Collavo, and G. Biasi. 1974. Manuscript submitted for publication.

processed for histological examination. Paraffin sections were stained with hematoxylin-eosin and methyl green pyronin.

Hemolytic Plaque Forming Cell (PFC) Assay. M-MSV injected and control AKR mice were immunized intraperitoneally (i.p.) with 4×10^8 sheep red blood cells (SRBC) (Sclavo-Siena, Siena, Italy). The mice were killed on the 5th day after immunization and the number of direct spleen PFCs was determined by the method of Jerne et al. (12) with minor modifications.

Virus Neutralization. Virus neutralization was performed by a focus reduction method (2). In experiments to detect virus neutralizing antibody in the serum of M-MSV-injected mice, blood was collected from the retroorbital sinus at intervals after M-MSV injection. Pooled sera, inactivated at 56°C for 30 min, were incubated at appropriate dilutions with approximately 40 FFU of M-MSV for 60 min at 22°C and then maintained at 0°C for an additional 30 min. The mixtures were assayed for focus formation on DEAE-dextran-pretreated 3T3FL mouse cells, plated the day before at a concentration of 10^5 in 60-mm plastic petri dishes. The cultures, in duplicate, were grown in Dulbecco's medium (Grand Island Biological Company, Grand Island, N. Y.), supplemented with 10% heat-inactivated and filtered calf serum and antibiotics. Foci were scored on the 5th day under a microscope. A similar procedure was employed to characterize the MSV pseudotype recovered from AKR and C58-induced tumors (for details and typing antisera, see Tables IV, V, and X).

Humoral Cytotoxicity. Humoral cytotoxicity was measured by the dye-exclusion method with trypan blue (13). Fresh nontoxic rabbit serum at 1:3 dilution was used as the complement source. The reaction mixture, consisting of 0.05 ml each of target cell suspension (5×10^6 cells/ml), antiserum and complement, was incubated at 37°C for 45 min with constant shaking. A qualitative absorption was carried out as follows: packed absorbing cells, obtained from primary tumors by teasing the tumor mass and pressing it through a 40-mesh stainless steel sieve, were mixed with an equal vol of antiserum at 2–3 twofold dilutions below the 50% end point, and incubated for 60 min on ice. The residual cytotoxic activity in the supernate was tested as described above using target B6 MBL-2 (M-MuLV-induced) or E♂G2 (G-MuLV-induced) transplanted leukemia cells. Antiserum against M-MuLV-determined cell surface antigen (MCSA) was obtained from B6 mice immunized i.p. by seven weekly injections with increasing doses (5×10^6 to 5×10^7) of BALB-LSTRA (M-MuLV-induced) transplanted leukemia cells. Antiserum against G-MuLV-determined cell surface antigens (GCSAs) was obtained by immunizing B6 mice with AKR-K36 spontaneous leukemia, as previously reported (14).

Antilymphocyte Serum Treatment. Rabbit antimouse lymphocyte serum (ALS) was obtained from Microbiological Associates, Bethesda, Md. (lot no. 13120). AKR mice received 0.20 ml ALS i.p. the day before M-MSV injection; 0.10 ml was subsequently given on days 1, 3, 5, 7, 9, 11, and 13 after M-MSV injection.

Results

Oncogenic Response of AKR and C58 Mice to M-MSV. Since both immune reactivity and endogenous MuLV titer might change with age, two age groups of AKR and C58 mice were studied. As seen in Table I, the age of mice at M-MSV injection did not significantly affect tumor incidence: 46–69% of AKR mice and 92–94% of C58 mice developed sarcomas at the injection site. The mean latent period of tumor appearance was considerably more prolonged in AKR than in C58 mice. However, in both strains the majority of sarcomas grew progressively and ultimately killed the host.

The pattern of tumor growth is schematically represented in Fig. 1; again, no differences were observed in relation to the age at the time of virus inoculation. C58 mice exhibited a rapid increase in size of tumor mass, and almost all died within 4 wk after tumor appearance. Sarcomas induced in AKR mice showed variable behavior. Considering the two age groups together, 18% of tumors regressed shortly after appearance, 55% grew slowly but constantly, while the

TABLE I
Development of Sarcomas in AKR and C58 Mice Injected with M-MSV

Strain	Age	Total no. mice	Mice with sarcoma* (mean latent period; days)	Mice with regressed sarcoma‡	Mice dead from sarcoma‡
	wk		%	%	%
AKR	6–12	80	69 (41)	20	80
	24–38	24	46 (54)	9	91
C58	6–12	35	94 (16)	3	97
	24–38	25	92 (17)	0	100

* Percentage of mice calculated from total number of mice.
‡ Percentage of mice calculated from number of mice with sarcoma.

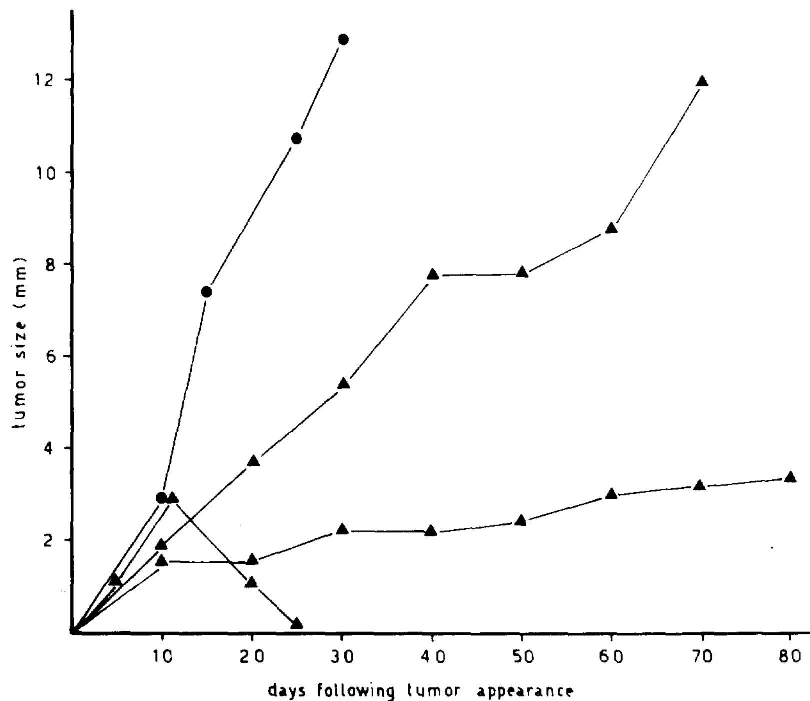

FIG. 1. Different growth behavior of AKR and C58 tumors. ▲, AKR tumors: 18% regressing, 55% progressing, 27% persisting; ●, C58 tumors: 98% progressing.

remaining 27% persisted for long periods, sometimes for several months without obvious enlargement. In the last group of mice death usually occurred from the concomitant leukemia. It is also noteworthy that the tumors which eventually regressed had a shorter latency (10–15 days).

Briefly, AKR mice showed a considerable degree of resistance to tumor induction following M-MSV inoculation as indicated by the very long latency. However, most of the sarcomas that subsequently arose did not regress. On the other hand, C58 mice developed sarcomas with higher incidence, shorter latency, and rapidly progressive fatal growth.

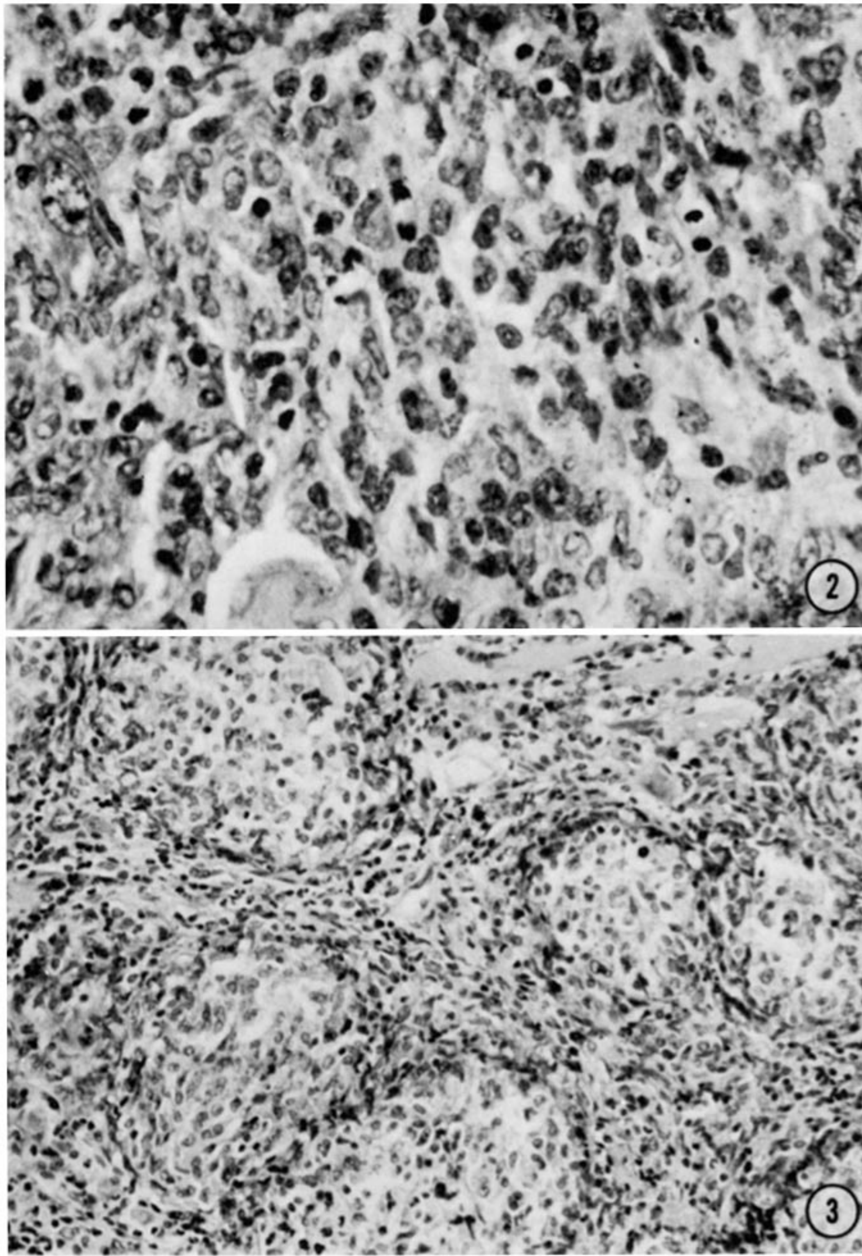

FIG. 2. C58 tumor. Note the remarkable pleomorphism of the neoplastic tissue. Hematoxylin and eosin. × 625.
FIG. 3. C58 tumor. Neoplastic tissue mainly composed of proliferating cells arranged in a nodular pattern. Hematoxylin and eosin. × 250.

Histology. Histological examination of AKR and C58 tumors revealed the typical pleomorphism and granulomatous appearance of M-MSV-induced sarcomas (5, 15). Stellate, spindle, giant straplike or racket-shaped cells with irregular and often multiple nuclei were seen along the muscle fibers which

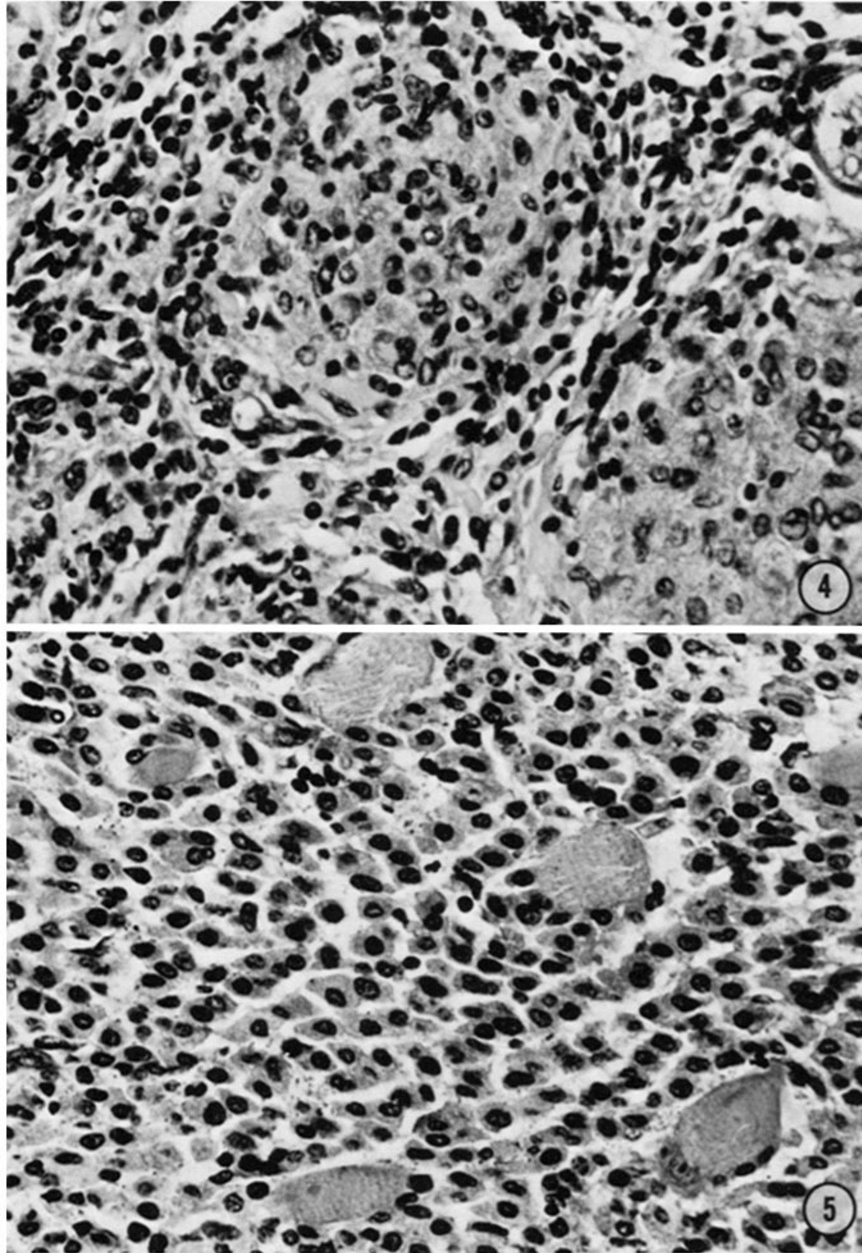

FIG. 4. AKR tumor. Nodular aggregates of large eosinophilic neoplastic cells surrounded by lymphocytes and neutrophils. Hematoxylin and eosin. × 435.

FIG. 5. AKR tumor. Myoblast-like neoplastic cells and scattered residual muscle fibers in a tumor field. Hematoxylin and eosin. × 435.

appeared dissociated, showing signs of atrophy and regeneration (Fig. 2). Vascular proliferation as well as cells undergoing degenerative changes and more or less marked infiltration by neutrophils, lymphocytes, and macrophages were also frequent findings.

In some cases, however, in addition to the pleomorphic areas described above, the neoplastic tissue showed an unusual nodular pattern. Multiple small nodular foci, composed of polygonal tumor cells possessing abundant pale eosinophilic cytoplasm with indistinct boundaries and large vesicular nuclei were observed and were often surrounded by leukocytes (Figs. 3 and 4). In other fields, diffuse proliferation of myoblastic-like cells was also present (Fig. 5). Thus in these M-MSV induced mouse sarcomas, two kinds of tumor growth patterns seemed to co-exist: one consists of simultaneous proliferation (probably through continuous viral recruitment) of different cell types of mesenchymal origin with a high rate of cell death and inflammatory reaction; the other shows a nodular or diffuse growth of monomorphic cells, most likely myoblastic in origin, reminiscent of clonal aggregates.

It is interesting to note that in all cases examined, even in C58 mice bearing rapidly growing progressive tumors, the spleen was increased in volume and presented follicles with large germinal centers and plasmocytoid cells in the red pulp. Similarly, the lymph nodes, mainly homolateral inguinal and axillary, exhibited in the outer cortex follicles with prominent germinal centers and conspicuous plasmocytosis in the medullary cords. The paracortical area also appeared enlarged and contained numerous scattered pyroninophilic cells.

Immune Reactivity of AKR and C58 Mice Injected with M-MSV. To test whether the progression of tumors was dependent on a decreased immune reactivity caused by M-MSV, groups of AKR mice were immunized with SRBC at 15, 30, or 60 days after M-MSV injection. As shown in Table II, no differences were observed in the number of direct spleen PFC between control and M-MSV-injected mice. To correlate further the immune reactivity with the oncogenic response, the PFC assay was performed using surgically removed spleens from a group of 13 mice (not included in Table II), which were immunized with SRBC 15 days after M-MSV injection. The follow-up of the individual mice did not reveal any variations in the PFC values in regard to sarcoma induction, latency and growth, or leukemia development.

Since the progression of sarcomas could also be due to a specific immunologic unresponsiveness, M-MSV injected mice were tested for the presence of virus neutralizing antibodies. Groups of eight to ten 6- to 8-wk old AKR and C58 mice were bled before and after M-MSV injection. Their sera were pooled and assayed in vitro for neutralizing activity against M-MSV. Groups of 6 to 10 BALB and B6 mice, similarly treated, were used as donors of positive control sera. The results are summarized in Table III. 20 days after M-MSV injection, the AKR and C58 mice produced virus neutralizing antibody titers which were even higher than those observed in BALB and B6 mice. It should be noted that by day 40 the sarcomas in 8 out of 10 BALB and in 5 out of 6 B6 mice regressed completely. On the other hand, the AKR and C58 mice showed the usual tumor response, i.e., 10 AKR mice were negative on day 20 and only two of them had small palpable masses on day 40; all eight C58 mice had 2–3 mm tumors on day 20, and 8–10 mm tumors on day 40. Thus, no immune depression was detected in M-MSV injected AKR and C58 mice by comparing either the anti-SRBC response to that of uninjected controls, and, more specifically, the virus neutralizing antibody levels to those observed in BALB and B6 mice with M-MSV tumors which regressed.

Antigenic Characterization of MSV Retrieved from AKR and C58 Tumors.

TABLE II
*PFC in AKR Mice after M-MSV Injection**

Days after M-MSV injection	No. mice tested	PFC/10^6 cells‡	PFC/spleen‡
15	10	1.841 ± 0.118	3.895 ± 0.113
30	8	1.796 ± 0.030	3.938 ± 0.062
60	9	1.787 ± 0.092	3.984 ± 0.131
—	6	1.727 ± 0.099	3.848 ± 0.130

* 6–8-wk old mice injected with M-MSV were immunized with SRBC, and the number of PFC was determined on the 5th day.
‡ Log_{10} ± standard error of the means of the number of direct PFC.

TABLE III
Neutralization of M-MSV by Sera from M-MSV Injected Mice

Serum donors*	Neutralization titer‡			
	Days after M-MSV injection			
	-1	10	20	40
AKR	4	4	64	≥64
C58	4	4	64	≥64
BALB	≥4	16	32	32
B6	≥4	≥4	16	≥64

* 6–8-wk old mice were injected with M-MSV.
‡ Around 40 FFU of M-MSV were mixed with appropriate dilutions of antiserum and the mixtures after incubation were assayed in 3T3FL cells. Neutralization titer is expressed as the reciprocal of serum dilution that resulted in a 50% reduction of focus formation.

The possibility was then considered that the high level of endogenous G-MuLV in AKR and C58 tissues could influence the growth of tumors through an in vivo helper effect. The MSV recovered from the induced sarcomas was therefore analyzed antigenically by a neutralization assay.

To determine the specificity of the antisera to be used, a previous neutralization test was carried out on cloned M-MuLV and G-MuLV, using the FIU reduction method on S^+L^- cells (11). As shown in Table IV, the reaction was quite specific.

Two AKR and four C58 cell-free tumor extracts were assayed for MSV content and viral envelope antigenicity by a neutralization test on 3T3FL cells. All tumors examined yielded a considerable amount of MSV (Table V). In all cases, the focus-forming virus was not affected by the anti-Moloney serum but was completely neutralized by the anti-Gross serum. It was clear that M-MSV itself was neutralized and not only the attending MuLV, because in each neutralization assay helper virus was added which could not be neutralized by the antiserum used. Thus, a drop in FFU could not be attributed to the elimination of the helper virus necessary for the detection of two-hit M-MSV foci. These

TABLE IV
*Specificity of Neutralization by Anti-M-MuLV and Anti-G-MuLV Sera**

Test virus	Test antiserum	
	Anti-M-MuLV Vn/Vo	Anti-G-MuLV Vn/Vo
M-MuLV-IC (3T3-cell derived)	≤0.01	0.65
G-MuLV (3T3-cell derived)	0.72	0.009

*The assayed was performed with MuLVs in S^+L^- cells as previously described (11). The nonneutralized fraction, Vn/Vo, was calculated from the reduction in FIU. The identical proportion of virus was neutralized if the respective pseudotypes of MSV were assayed as foci in 3T3FL cells. Anti-M-MuLV serum was prepared in Swiss mice inoculated with the IC isolate of M-MuLV which was passed in 3T3 cells (4). The rat anti-G-MuLV serum was produced as described in (16). The sera were used at 1:50 and 1:250 dilutions, respectively.

TABLE V
*Neutralization of Tumor-Derived MSV by Anti-M-MuLV and Anti-G-MuLV Sera**

Cell-free tumor extract tested	Original titer	Test antiserum	
		Anti-M-MuLV Vn/Vo	Anti-G-MuLV Vn/Vo
	FFU/ml		
AKR	6.1×10^5	0.84	0.005
AKR	1.0×10^6	1.05	0.005
C58	7.0×10^5	0.95	0.004
C58	3.1×10^5	0.91	0.006
C58	5.1×10^5	0.95	0.005
C58	5.0×10^5	0.81	0.005

* Between 100–200 FFU of MSV were mixed with antiserum and the mixtures were assayed in NIH-3T3 cells to ensure correct tropism for potential AKR or C58 MuLV coats. In previous experiments the number of foci of M-MuLV (IC) was essentially identical in NIH-3T3 or 3T3FL cells. An optimal MuLV helper inoculation (2×10^4 FIU/0.1 ml) of the type opposite to, and not neutralized by the antiserum employed, was added to the cells about 1 h after the mixture of MSV and antiserum was added. The anti-M-MuLV and anti-G-MuLV sera were used at 1:50 and 1:250 dilutions, respectively.

results suggested that G-MuLV pseudotypes of MSV were formed in vivo, since the MSV recovered from AKR and C58 tumors had the envelope antigenicity of the endogenous G-MuLV.

Cell Surface Antigenicity of AKR and C58 MSV-Induced Tumors. The

recovery of M-MSV coated with G-MuLV from AKR and C58 tumors did not necessarily mean that the tumors were in fact induced by this MSV pseudotype. To obtain direct evidence for the etiological role of M-MSV coated with G-MuLV, the specificity of sarcoma cell surface antigens was determined. Cell suspensions from two AKR and two C58 sarcomas were incubated with typing anti-M- or anti-G-MuLV sera to absorb cytotoxic antibodies. After incubation, the residual cytotoxic activity was tested at various dilutions on M-MuLV induced MBL-2 or G-MuLV-induced E♂G2 leukemia target cells. As controls, the MBL-2 and E♂G2 reference cells, as well as cell suspensions from two BALB M-MSV-induced sarcomas were used for the qualitative absorption test. The results are shown in Table VI. AKR and C58 sarcoma cells absorbed out the cytotoxic antibody directed against GCSA, but did not affect the activity of the

TABLE VI

Absorption Tests with AKR, C58, and BALB Sarcomas Induced by M-MSV Inoculation: Humoral Cytotoxicity Technique by Trypan Blue Staining against B6 Moloney Leukemia MBL-2 and Gross Leukemia E♂G2 Cells, used with the Moloney- and Gross-Typing Sera Preabsorbed with Various Cells

Cells used for absorption	Test antiserum									
	B6 anti-LSTRA* (M-typing serum)					B6 anti-K36‡ (G-typing serum)				
	Serum dilution				Absorption	Serum dilution				Absorption
	1/2	1/4	1/8	1/16		1/3	1/6	1/12	1/24	
	% of trypan blue stained cells					*% of trypan blue stained cells*				
None	80	69	53	47	–	62	56	39	28	–
MBL-2	<10	<10	<10	<10	+	63	30	21	13	–
E♂G2	57	39	35	34	–	<10	<10	<10	<10	+
AKR-sarcoma	59	41	45	29	–	<10	<10	<10	<10	+
C58-sarcoma	54	47	34	34	–	<10	<10	<10	<10	+
BALB-sarcoma	<10	<10	<10	<10	+	28	23	<10	<10	±

* LSTRA, transplanted BALB M-MuLV-induced leukemia.
‡ K36, transplanted AKR spontaneous leukemia.

anti-MCSA serum. On the other hand, BALB sarcoma cells absorbed the cytotoxicity of the anti-MCSA serum. It is interesting that the latter cells could also absorb the activity of the anti-GCSA serum. These findings demonstrated that AKR and C58 sarcoma cells possessed surface antigens specified by the endogenous G-MuLV.

Effect of ALS Treatment or M-MuLV Neonatal Infection on Tumor Induction by M-MSV in AKR Mice. From the above studies, it seemed clear that MSV with new envelope characteristics was etiologically related to the induced sarcomas in AKR and C58 mice. The collateral finding of the unusual long latency of AKR tumors was, however, unexplained. Among others, the possibility that a strong immune reaction could prevent early tumor development was considered. To investigate this point, experiments were performed with the aim

of depressing the immune response of M-MSV injected mice. 6- to 8-wk old AKR mice were given ALS 1 day before and every second day for 2 wk after M-MSV injection. Sarcomas appeared with higher incidence and with a shorter latent period in ALS-treated mice than in controls (Table VII). This result would indicate that immunodepression by ALS was effective in decreasing the relative resistance of AKR mice to M-MSV. Alternatively, ALS could have raised the titer of endogenous G-MuLV, thus providing more helper for the new pseudotype formation. Actually, after such ALS treatment we have found in AKR mice a 10-fold increase in the G-MuLV titer of tail extracts (17) as assayed on S^+L^- cells.

Therefore, more substantial evidence was needed to support the immunologic basis of the relative resistance of AKR mice to M-MSV. With this purpose, AKR mice were given M-MuLV at birth, and subsequently were injected with M-MSV when 8 wk old. All mice developed sarcomas in about 2 wk, and seven of them died from concomitant leukemia (Table VIII). In the control group, consisting of

TABLE VII
Effect of ALS Treatment on Tumor Induction by M-MSV in AKR Mice

Inoculation with:	Total no. mice	Mice with sarcoma* (mean latent period; days)	Mice with regressed sarcoma‡	Mice dead from sarcoma‡
		%	%	%
M-MSV§	80	69 (41)	20	80
ALS + M-MSV	29	96.5 (16)	3.5	96.5

* Percentage of mice calculated from total number of mice.
‡ Percentage of mice calculated from number of mice with sarcoma.
§ Data from Table I.

mice neonatally injected with M-MuLV, all mice exhibited an earlier onset of leukemia and died in less than 4 mo.

It appeared likely that, in line with previous work (18), the neonatal injection of M-MuLV could have produced a state of partial immunological tolerance (see Discussion) to virus-specified antigens responsible for the accelerated appearance of M-MSV tumors. This interpretation was supported by the fact that ALS-treated mice could still synthetize considerable amounts of virus neutralizing antibody, whereas mice receiving M-MuLV neonatally and subsequently injected with M-MSV could not (Table IX).

Finally, the recovery and antigenic typing of MSV from sarcomas arising in two ALS-treated and in two M-MuLV neonatally injected mice was attempted. As shown in Table X, these early tumors yielded MSV which was neutralized by the anti-M-MuLV but not by the anti-G-MuLV serum. Thus, both treatments were able to circumvent the relative resistance of AKR mice, allowing the original M-MSV pseudotype to induce tumors with higher incidence and shorter latency.

TABLE VIII
Development of Sarcomas in AKR Mice Injected at Birth with M-MuLV and as Adults with M-MSV

Inoculation with:	Total no. mice	Mice with sarcoma* (mean latent period; days)	Mice with regressed sarcoma‡	Mice dead from sarcoma‡	Mice dead from leukemias§ (mean latent period; days)
		%	%	%	%
M-MuLV	7	—	—	—	100 (105)
M-MuLV + M-MSV	10	100 (16)	0	100	70 (87)

* Percentage of mice calculated from total number of mice.
‡ Percentage of mice calculated from number of mice with sarcoma.
§ Percentage of mice dead with leukemia calculated from total number of mice.

TABLE IX
Neutralization of M-MSV by Sera from ALS-Treated or M-MuLV Neonatally Infected AKR Mice, Injected with M-MSV

Serum donors inoculated with:*		Neutralization titer‡			
	Days after M-MSV injection:	−1	20	30	40
ALS + M-MSV		ND	16	32	32
M-MuLV + M-MSV		<8	<8	<8	<8
M-MSV		<8	32	64	64

* ALS treatment was started the day before and continued for 2 wk after M-MSV injection. M-MSV was injected in 6–8-wk old mice and M-MuLV in newborns.
‡ See Table III.

TABLE X
*Neutralization of AKR Tumor-Derived MSV by Anti-M-MSV and Anti-G-MuLV Sera**

Tumor extract donors inoculated with:	Original titer	Test antiserum	
		Anti-M-MSV Vn/Vo	Anti-G-MuLV Vn/Vo
	FFU/ml		
ALS + M-MSV	3.9×10^4	0.040	0.80
ALS + M-MSV	1.6×10^4	0.060	0.75
M-MuLV + M-MSV	1.8×10^5	0.060	0.80
M-MuLV + M-MSV	2.8×10^5	0.003	0.90

* The anti M-MSV serum was obtained from regressor (B6 × AKR)F1 mice bled 40 days after M-MSV injection. The anti G-MuLV was produced in W/Fu rats by eight weekly i.p. injections with increasing doses (10^6 to 10^7) of C58(NT)D transplanted syngeneic leukemia cells. Both sera were used at 1:50 dilution since this gave highly specific neutralization reactions in previous experiments. For additional details of the neutralization assay, See Table V.

Discussion

While the in vitro relationships between murine sarcoma and leukemia viruses are well established, relatively little information is available on their in vivo interactions. Chirigos et al. (7, 8) have reported that co-infection with R-, F-, or M-MuLV increases the oncogenicity of M-MSV. Our previous results have confirmed and extended these findings (9). Mice neonatally infected with Gi- or G-MuLV, and subsequently injected with M-MSV did show higher frequency of tumor induction and progressive growth.

The present studies were performed to determine whether endogenous MuLV, when expressed as infectious virus, could influence the oncogenicity by M-MSV. It was found that after M-MSV infection in mice of the high leukemia strains AKR and C58 which have considerable titers of endogenous G-MuLV early in life, the great majority of the induced tumors showed progressive fatal growth. Although M-MSV may nonspecifically depress the immune reactivity of the host (19), this effect seems to play no role in previous (9) as well as in present experiments. In fact, mice eventually developing progressive tumors reacted normally to SRBC immunization, and moreover, they could produce specific M-MSV neutralizing antibody even better than BALB and B6 mice with regressed tumors. In preliminary unpublished experiments using a ^{51}Cr-releasing cytotoxic test, we have also detected a considerable degree of cell-mediated immunity in M-MSV-injected AKR and C58 mice against target leukemia cells bearing M-MuLV-specified surface antigens. These observations, together with the morphological signs of marked reactivity in lymph nodes and spleens of tumor-bearing AKR and C58 mice, indicate that the failure of tumor regression cannot be due to immune depression caused by M-MSV.

The possibility that AKR mice may be utilized as an in vivo system to rescue the defective MSV genome was put forward after the results obtained by Huebner et al. (20) and by Ting and Law (21) using, respectively, hamster or rat MSV-transformed nonproducer cells. However, this was later questioned since the latter cell line (MSB-1) was shown to release both focus-forming virus (22) and a rat-tropic helper type C virus (23). In more recent experiments,[3] Kirsten (Ki)-MSV transformed, nonproducer K-NRK rat cells injected i.m. in adult AKR mice induced progressive tumors within 2–3 mo. The tumors exhibited the typical morphology of primary MSV-tumors and yielded infectious MSV with the usual host range of endogenous N-tropic MuLV. No tumors have been produced so far by injecting K-NRK cells in normal adult B6 mice.

The potential of endogenous G-MuLV to act as helper for the MSV oncogenic activity is further substantiated by our present data. These clearly show that the M-MSV injected in AKR and C58 mice may undergo a process of phenotypic mixing by acquiring envelope antigenicity of the endogenous helper G-MuLV. The complete virus neutralization by specific anti-G-MuLV serum, and the capability of the AKR and C58 sarcoma cells to absorb the cytotoxicity of the typing antiserum against GCSA makes it highly probable that the new MSV pseudotype is actually responsible for the tumor growth. Of interest is the behavior of the BALB-M-MSV-induced sarcoma cells in the absorption assay. These cells completely removed the cytotoxic activity of the anti-MCSA typing serum and at the

[3]Chieco-Bianchi, L., and M. M. Lieber. Unpublished observations.

same time, also showed absorption of the anti-GCSA serum. As recently found (24), some spontaneously transformed clones of BALB/3T3 and transplanted BALB radiation-induced leukemia BALB.RL♂1 release a type C virus population which shares type-specific viral envelope antigen (VEA) x 1-VEA, and subgroup-specific VEA. Type C virus with similar antigenic characteristics is also synthesized by transplanted AKR spontaneous leukemia K36 which was employed to produce the G-typing mouse serum. Consequently, this virus might be responsible for the induction of GCSA on M-MSV-induced BALB sarcoma cells, resulting in the absorption of cytotoxic activity to GCSA.

The spontaneous regression of M-MSV-induced sarcomas in mice as already outlined, is commonly explained in terms of host immune response. Although in vitro tests indicate that both bone marrow-derived (B) and thymus-derived (T) lymphocytes are cytotoxic, in vivo observations (25) suggest that the integrity of the T-lymphocyte population is crucial for the M-MSV tumor regression. Besides immunological manipulation of the host, the failure of tumors to regress may be due to a number of factors. MSV-transformed nonproducer cells do not possess surface antigens detectable by standard serological techniques (26). The same is true for the sarcoma-positive leukemia-negative (S^+L^-) line of MSV-transformed cells that release very low amounts of noninfectious virus (27). More recently, by using the sensitive procedure of immunoelectron microscopy, one of the authors (T. Aoki) has been able to demonstrate an MSV-associated cell surface antigen in nonproducer mouse and rat cells transformed by Ki- or M-MSV (28). This antigen seems, however, only weakly immunogenic since BALB mice preimmunized with Ki-MSV-transformed, nonproducer BALB/3T3 cells were not made resistant to subsequent challenge with the same cells. On the other hand, when helper leukemia virus was inoculated into the tumor mass, the transplanted Ki-MSV nonproducer tumors readily regressed (29). It thus appears that only tumor cells actively producing type C particles are strongly immunogenic, whereas nonproducer MSV-transformed cells are unable to evoke an allograft-like immune response. Since the above studies have been performed on transplanted tumor cells, it is difficult to envisage their relevance to the behavior of primary tumors.

In vivo studies in the avian system with Rous sarcoma virus (RSV) have shown that RSV-producing sarcoma cells have only a limited growth potential and perish after a finite number of divisions (30). Accordingly, it has been suggested that progressive tumor growth is largely dependent on release of infectious virus and continuous transformation of normal cells. This hypothesis has been supported by results of in vitro experiments on the proliferation dynamics of chicken embryo cells after transformation by RSV, Schmidt-Ruppin strain (31). RSV-transformed cells after a temporary phase of rapid growth failed to survive for any length of time. Added normal cells were rapidly transformed and were needed continuously in order to maintain a transformed proliferating cell pool.

There is indirect evidence that also in the mouse, under standard experimental conditions, the growth pattern of M-MSV-transformed cells may be essentially similar. Histologically, the sarcomatous tissue appears to be derived from a mixture of inflammation, cell lysis, and repair involving transformed mesenchymal cells. Although permanent cell lines such as 3T3 are easily transformed by M-MSV and may propagate indefinitely in vitro (32), it is difficult to establish permanent transformed cell lines by infecting with M-MSV primary mouse embryo cultures (33). Similarly, it is hard to culture cells from M-MSV-induced primary tumors (34). Thus, it may be possible that, as suggested for the avian Rous sarcoma system, development and growth of M-MSV tumors in the mouse follows a nonclonal kinetics which is dependent upon a constant high rate of virus replication and continuous recruitment of newly infected transformed cells. When virus synthesis is slowed down by a host reaction or antibody transfer, prevention of tumor formation as well as tumor regression may result (35). At variance with this line of

reasoning is the observation that some of the MSV-induced tumors revealed morphological features suggesting a proliferative pattern of the clonal type. Since only a limited number of tumors has been examined histologically, more studies are needed to determine if this represents a rare event or, more interestingly, another peculiar property of the progressive sarcomas arising in AKR and C58 mice. At present, however, a likely explanation for the lack of regression in AKR and C58 mice is that immunologic unresponsiveness, either apparent or actual, which is specific for the antigens coded by the endogenous G-MuLV, makes for a poor host immune response against the newly formed M-MSV coated with G-MuLV pseudotype. Both viral antigen released in the body fluids and antibodies bound to G-MuLV as immune complexes are known to occur in AKR mice (36) and may be responsible for mimicking a state of immunological tolerance. On the other hand, evidence of a real specific unresponsiveness selectively affecting the T lymphocytes present in mice neonatally infected with M-MuLV has been recently obtained (18). That the latter mechanism also holds for AKR and C58 mice remains to be elucidated.

As an alternative explanation for the failure of tumors to regress, the possibility should be considered that by growing in AKR or C58 host cells, MSV may also acquire from G-MuLV in addition to the viral envelope new genetic information which may modify its biologic and pathogenic properties. Differences in the immunogenicity and oncogenicity of different stocks of M-MSV (37) and Ki-MSV (29), as reflected in the pattern of tumor growth, are known. No indications are available, however, as to whether they depend on variation in the intrinsic characteristics of the virus or on different ratios between MSV and helper populations. In this regard, it is noteworthy that the Harvey isolate of MSV induces sarcomas which rarely regress and these tumor cells may be easily kept in cultures for long periods (5).

The fact that AKR mice had sarcomas after a long latent period has been interpreted to be a result of relative resistance to M-MSV infection. At first sight this might be due to an interference phenomenon between the endogenous G-MuLV growing in AKR cells and M-MSV. In vitro preinfection of mouse embryo fibroblasts with G-MuLV induced resistance to infection and transformation not only with the respective M-MSV coated with G-MuLV but also with M-MSV (M-MuLV) pseudotype (38). However, to our knowledge no clear demonstration for interference has been described as occurring in vivo, and more importantly, C58 mice, which show a similar high expression of endogenous G-MuLV, did not exhibit such a delayed sarcoma appearance. Therefore, it is likely that factors other than viral interference are responsible for the observed resistance. That a number of cellular genes control the expression of endogenous MuLVs as well as the host's susceptibility to infection and neoplastic transformation is firmly established (39). Specifically, the Fv-1 gene controls the relative resistance of MuLVs. NIH Swiss (N) cells carrying the Fv-1^n allele propagate "N-tropic" viruses more efficiently, whereas BALB (B) cells, with the Fv-1^b allele, are more susceptible to "B-tropic" viruses. M-MuLV, on the other hand, is NB tropic as are other passaged strains of MuLVs, and it can grow equally well in both cell types. Accordingly, no restriction on M-MSV infection should be exerted by AKR cells carrying the Fv-1^n genotype.

The influence of the immune response (Ir) genes on the capability of mice to react immunologically to a variety of natural and synthetic antigens is also well documented. For instance, the *Ir-1* gene, which has been mapped near the K end of the *H-2* region, is closely associated with or identical to the Rgv-1 gene which, in turn, seems to determine the strength of the immune response to MuLV-specified antigens (39, 40). Thus, it is conceivable that AKR resistance to M-MSV is sustained by immunologic phenomena. The results of ALS administration or M-MuLV neonatal infection on latency and incidence of M-MSV-induced tumors would support this hypothesis. Both treatments

shortened the latent period and increased the tumor yield, probably by rendering primarily the T-lymphocyte population unresponsive through nonspecific or specific mechanisms (18, 41). Moreover, the MSV recovered from these early tumors proved to be the original Moloney pseudotype.

In conclusion, the data reported indicate that AKR and C58 type C endogenous viruses can compete successfully with exogenous M-MuLV as helpers for in vivo MSV replication. The resulting MSV sarcomas interestingly enough lack the unique property of spontaneous regressions and behave very much like conventional tumors. Whether the helper effect is exerted by viruses with the usual N- or B-tropism, or by the newly isolated xenotropic, "S-tropic" viruses (42, 43) presents an interesting point for further investigation.

Summary

Adult AKR and C58 mice injected intramuscularly with murine sarcoma virus, Moloney isolate (M-MSV), developed high incidence of nonregressing local tumors. Histologically, these tumors revealed the typical pleomorphism of M-MSV sarcomas; in some cases, however, neoplastic tissue showed a nodular or diffuse growth of monomorphic myoblastlike cells, reminiscent of clonal aggregates. No depression of immune reactivity was found in M-MSV-injected mice as evaluated by direct hemolytic plaque-forming cells against SRBC and by virus-neutralizing antibody production.

The MSV recovered from the induced tumors proved to be, by neutralization assay, a Gross (G)-MSV pseudotype. Moreover, tumor cell suspensions absorbed out cytotoxic antibody directed against G-cell surface antigens. Therefore, the conclusion was drawn that MSV with envelope characteristics of endogenous G leukemia virus had formed in vivo through a phenotypic mixing phenomenon. The failure of tumors to regress has been interpreted as mainly due to the partial unresponsiveness of host immune reactivity towards G-MuLV specified antigens.

Since MSV-tumors arose in AKR mice after a very long latent period, the possibility was considered that this relative resistance might depend on immunologic mechanisms. In fact, M-MSV-injected AKR mice immunodepressed by goat antimouse lymphocyte serum or rendered partially tolerant by neonatal M-MuLV inoculation developed sarcomas with higher incidence and with a shorter latency. Furthermore, the MSV recovered from these early tumors proved to be the original Moloney pseudotype.

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