NONPRODUCER MALIGNANT TUMOR CELLS WITH RESCUABLE SARCOMA VIRUS GENOME ISOLATED FROM A RECURRENT MOLONEY SARCOMA

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The characteristic features of sarcomas induced in mice by Moloney murine sarcoma virus (MSV-M),[1] namely rapid development and spontaneous regression, have prompted extensive investigations on humoral and cell immune response in this model (1). A cause-and-effect correlation between immune response and tumor regression can be concluded from observations in immunologically incompetent hosts. In X-ray-irradiated (2), thymus-deprived (3, 4), or congenitally athymic mice (5, 6), Moloney sarcomas always show progressive growth. In immunocompetent mice, the majority of Moloney sarcomas completely regress, though few may continue to grow or recur later (7–12). It has been proposed that this recurrence is due to the disappearance of a resistance state acquired during tumor regression (10), although the possibility that the recurrent tumor may differ from the primary one with respect to, for instance, immunosensitivity and/or antigenicity, was not considered. We have therefore studied the virological, oncogenic, and antigenic properties of a transplantable tumor that reappeared at the site of a primary regressed Moloney sarcoma. Also, two oncogenic cell lines derived from this tumor, and for comparative purposes, ascitic MSV-M-producing cells (13) and a Moloney helper virus-producing cell line established from these ascitic cells were included in these studies. The purpose of this investigation was to obtain information on the escape mechanism of tumor cells from immune destruction.

Materials and Methods

Animals, Virus and Cells. STU-inbred mice, MSV-M, ascitic MSV-M-producing cells (asc-MSV-M), and its helper-virus-producing culture derivative (Bc) were the same as those described in preceding studies (13–15). MSV-M was obtained from Flow Laboratories, Inc. (Rockville, Md.; code no. MSV-B-62). asc-MSV-M was derived from a weanling mouse that had developed an ascitic tumor after intramuscular (i.m.) injection of MSV-M; this ascitic tumor has since been

[1] *Abbreviations used in this paper:* AMC, antibody-mediated cytotoxicity; asc-MSV-M, ascitic MSV-M-producing cells; Bc, cell line Bc derived from ascitic MSV-M cells; C′, complement; CMC, cell-mediated cytotoxicity; ffu, focus-forming units; [^{3}H]proline-MA, [^{3}H]proline microcytotoxicity assay; i.m., intramuscular; i.p., intraperitoneal; MLV-F, Friend murine leukemia virus; MSV-M, Moloney murine sarcoma virus; PBS, phosphate-buffered saline; pfu, plaque-forming units; Sac(−), cell line derived from ascitic cells of secondary tumor; Sac(+), Sac(−) cells infected with helper virus (L^+-C-type); S^+L^+, cells producing focus- and XC plaque-forming virus; ST, secondary tumor.

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maintained for more than 4 yr by intraperitoneal (i.p.) inoculations at weekly intervals. The Bc cell line was established from the asc-MSV-M.

Recurrent Secondary Tumor (ST) and Its Culture Derivatives. A 5-wk-old STU mouse was injected i.m. with 10^4 focus-forming units (ffu) in 0.1 ml of a MSV-M-tumor homogenate, and developed a palpable tumor 7 days later. After peak tumor development, regression followed and was complete 3 wk later, as far as could be assessed by palpation. 6 wk after the regression of the primary MSV-M-induced tumor, a second tumor developed at the same site. Single cell suspensions for transplantation were obtained by mincing parts of the peripheral tumor mass followed by filtration through sterile gauze. After washing once in phosphate-buffered saline (PBS) supplemented with antibiotics, a milky suspension of cells was transferred i.m. into four 4- to 5-wk-old unconditioned STU mice. 2 wk after transplantation, tumor development was observed in the recipients. The tumor has since been maintained for more than 2 yr by serial parallel i.m. and i.p. inoculations at intervals of 2–4 wk in the case of i.m. transplantation, and at weekly intervals with the ascitic form. A nonproducer cell line was established from the ascitic form [Sac(−) cells]. Using helper virus released from Bc cells for rescue of a sarcoma virus genome from Sac(−) cells (16), a producer cell line of the sarcoma-helper virus complex was established [Sac(+) cells].

Virus Assays. Focus and XC plaque assays, [^{3}H]uridine incorporation, and electron microscopic studies were performed by applying usual procedures (17–20).

Light Microscopy. Cell monolayers in plastic Petri dishes were fixed with Bouin's solution and stained with hemalum and eosin.

Determination of Tumorigenicity. Tumor induction was determined by i.m. inoculation of carefully graded doses of the various cells in a 0.1-ml volume into STU mice of at least 6 wk of age. In addition, $\cong 10^5$ Bc cells were inoculated into newborn mice. The tumorigenic potency of supernates from Sac(+) cultures was investigated in adult mice using clarified cell-free culture fluid undiluted. Development of tumors was observed for at least 42 days. In the case of Bc cells transplanted into adult mice, the observation period was extended over a period of 12 mo.

Transplantation Protection Assay. The ability of the various cells to induce transplantation resistance to asc-MSV-M, ST, Sac(−), and Sac(+) cells was compared by i.m. inoculation of viable asc-MSV-M (10^5 nuclear cells), ST (10^5 cells), Sac(−) (5×10^3 cells), Sac(+) (5×10^3 cells), or Bc (10^6 cells) cells into the right thigh of STU mice $\cong$ 6 wk of age. Challenge was performed by i.m. inoculation of graded doses of tumor cells into the left thigh $\cong$ 2 wk later; at this time asc-MSV-M cell-induced tumors began to regress, tumors induced by the above-mentioned relatively small number of Sac(+) cells also started to regress or showed deadlock of tumor growth, and at the sites where ST and Sac(−) cells had been inoculated, tumor growth became visible. In addition, mice that had received 10^6 Bc cells were challenged 1 yr later with asc-MSV-M. Age-matched mice that had received no primary inoculation were used as controls. Observation of tumor development after challenge was monitored thrice weekly for at least 40 days; after inoculation of ST or Sac(−) cells the observation period was shorter because of intercurrent death of the tumor bearers. Amputation of limbs bearing ST tumors before challenge in order to increase the survival time was not successful, as fatal metastatic tumors had already developed.

Isolation of Effector Cells and Cytotoxic Antibodies. Inoculation of the above-mentioned cells was done by injecting the desired number of cells in a 0.1-ml volume i.m. into the thigh region of mice about 6 wk of age. Lymphoid spleen cell suspensions were prepared 8 and 12 days after cell inoculation by the method previously described (14, 15). Pooled spleen cells of two or three mice were used in each test. Blood from the inoculated mice was taken at the days indicated in Results by puncture of the retro-orbital plexus, and the sera pooled from at least 3 animals were stored at −20°C until use.

[^{3}H]Proline Microcytotoxicity Assay for the Demonstration of Cell-Mediated Cytotoxicity (CMC) and Antibody-Mediated Cytotoxicity (AMC). A modification of the [^{3}H]proline microcytotoxicity assay ([^{3}H]proline-MA) developed by Bean et al. (21) was used and has been described previously (15). The assay measures target cell detachment as an expression of cytotoxicity. In the meantime, few minor modifications have been applied. We now prefer the model 48 TC Linbro tissue culture plates (Linbro Chemical Co., Inc., New Haven, Conn.). For assay of CMC, 6×10^3 [^{3}H]proline-labeled cells in 0.1 ml were added to each well, and for the assay of AMC, the number of cells per 0.1 ml was 3.5×10^3. Subsequently, the cultures were incubated at 37°C in a 5% CO_2 incubator.

In the CMC assay, the cells were incubated for 4 h, then lymphoid cells in a 0.05-ml volume

were added at ratios indicated in Results. After a 42-h incubation, the plates were dipped twice into PBS supplemented with 2% fetal calf serum at a temperature of 37°C. After trypsinization, the cells were transferred directly into scintillation vials. Calculation of percent reduction of target cells was performed as described previously (15).

For determination of AMC, the target cell cultures were incubated for 16–18 h. Then, the culture supernates were sucked off using a peristaltic pump (Varioperpex, LKB, Stockholm, Sweden) and 0.05 ml of serial threefold dilutions of the sera to be tested were added; after incubation for 1 h at 37°C, 0.05 ml of rabbit complement (C′) was added. Before use, a rabbit serum was diluted 1:3 with PBS and then absorbed with agarose (40 mg/ml) for 1 h at 4°C; after centrifugation at 5,000 rpm for 20 min, and filtration through a membrane with a pore size of 45 μm, the serum was used at a final dilution of 1:9 or 1:12. After addition of rabbit C′, the cultures were incubated for 2–3 h at 37°C and subsequently harvested for scintillation counting as described above. Target cells treated with either rabbit C′ or the serum dilutions to be tested served as controls. Since sera in the absence of rabbit C′ were without cytotoxicity, evaluation was performed by comparing the arithmetic mean of radioactivity of a group comprising three or four replicates treated with a serum dilution and rabbit C′ with that of a corresponding control group to which only rabbit C′ had been added. The degree of cytotoxic antibody activity is expressed for each serum dilution by the percent reduction of target cell radioactivity taking the radioactivity of the control group as basis for the calculation of the percent reduction.

Results

Virological Properties of the Different Cell Lines. The virological properties of asc-MSV-M cells have been described earlier (13). Although they produce both sarcoma and helper virus, supernates of a cell line (Bc cells) originating from asc-MSV-M cells produced no foci but plaques in the XC plaque test; 20 h after the change of the culture medium of confluent cell cultures (5.2×10^6 cells/dish), the supernates (4 ml per dish) contained 10^6 plaque-forming units (pfu)/0.2 ml. For ST cells, no indication of oncornavirus release was obtained. Suspensions of ST cells derived either from ST tumors or from ascitic ST cells were found negative in the XC plaque test and they did not produce foci. Also, electron microscopic examinations revealed no C-type particles. A cell line derived from ST cells in their ascitic form, the Sac(−) cell cultures, were found free of XC plaque-forming and focus-forming virus. In addition, experiments with [^{3}H]uridine, as well as electron microscopic examinations, gave no evidence for release of C-type particles from these cells. Also, no p30 antigen could be detected in these cells by radioimmunoassay using rabbit anti-p30 Friend murine leukemia virus (MLV-F) serum, (kindly performed in the laboratory of Dr. W. Schäfer, Max Planck-Institute for Virus Research, Tübingen, West Germany), and goat anti-gp 69/71 (MLV-F) serum possessed no cytotoxic activity against Sac(−) cells in the [^{3}H]proline-MA, but it was cytotoxic towards Sac(+) cells. The Sac(+) cells were obtained after infection of Sac(−) cells with the supernate of the above-mentioned Bc cell line. The release of oncornavirus from these Sac(+) cells was demonstrated by the detection of XC plaque- and focus-forming virus in the supernate of these cultures (cultures with 6.4×10^6 cells contained 10^4 ffu/0.2 ml and 10^5 pfu/0.2 ml), as well as by [^{3}H]uridine incorporation into material banding at a density of 1.16 g/ml.

Light Microscopy. The cell line producing focus- and plaque-forming virus [Sac(+) cells], as well as the sarcoma genome containing nonproducer cells [Sac(−) cells] appeared predominantly round or fusiform and refractile. Smooth or occasionally branched processes were present in Sac(+) as well as in Sac(−)

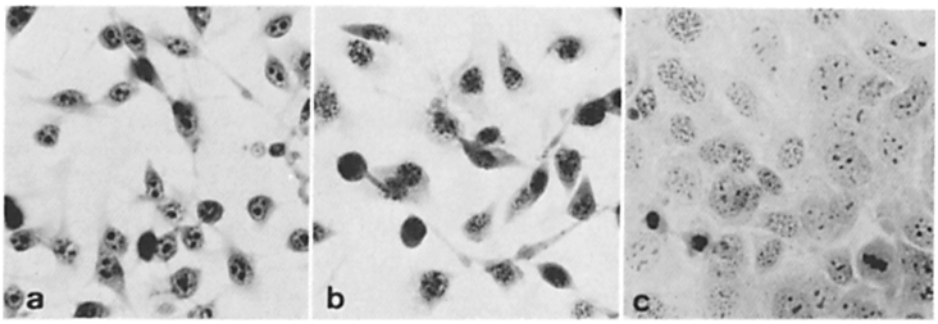

FIG. 1. Morphology of in vitro cell lines. Sac(+) cells (a) and Sac(−) cells (b) appear predominantly fusiform; Bc cells (c) differ by their flattened morphology. Magnification × 340.

cultures. By contrast, Bc cultures predominantly consisted of flattened, poorly refractile cells (Fig. 1).

Growth of Tumors Induced in Mice by the Different Cell Lines. Fig. 2 demonstrates the growth of tumors induced by the cells under study. Tumor induction by graded doses of asc-MSV-M cells has been described previously (13). Briefly, i.m. transplantation of these cells usually induces local tumors which regularly regress in immunocompetent hosts. Fig. 2a shows a representative course of tumor development and regression. Deviating from this typical course, an asc-MSV-M-induced tumor may sporadically grow progressively or may exhibit a biphasic growth pattern (22): after complete or incomplete regression, reestablishment of progressive tumor growth occurs and leads to death of the host. In the present studies, the helper virus producing Bc cells (Fig. 2b) never led to local tumors after i.m. inoculation, or to ascitic tumors after i.p. transplantation in adult mice. During an observation period of ≅ 12 mo, white blood changes could not be observed. However, after i.m. inoculation of Bc cells into newborn mice, leg enlargement became observable 7–8 wk later, and death occurred 8–10 wk after tumor development. Tumor induction after i.m. transplantation of graded nonproducer ST cell concentrations is shown in Fig. 2c. The time between transplantation and tumor establishment lasted from 1 to ≅ 3 wk and depended upon the concentration of the transplants. After tumor establishment, ST tumors exhibited a continuous growth pattern and led to the death of the tumor bearers between 3 and 7 wk after tumor cell transplantation, this interval varying with the transplanted tumor cell concentration. Transplantation of graded doses of nonproducer Sac(−) cells (Fig. 2d and Table I) caused tumor development between 4 and ≅ 25 days later, and these tumors exhibited a continuous growth pattern, death of tumor bearers occurring between 16 and ≅ 38 days after cell inoculation. Such tumors showed no regression, nor did they show a reduction in tumor size. Tumors caused by inoculation of 10^6 Sac(+) cells, a cell line obtained after infection of Sac(−) cells with helper virus, grew progressively. The tumors became palpable 4 days after cell inoculation, and the mice died about 4–7 wk after tumor induction. Inoculation of doses containing 10^2–10^4 Sac(+) cells were also followed by the development of tumors. Some of these tumors grew progressively, but slower

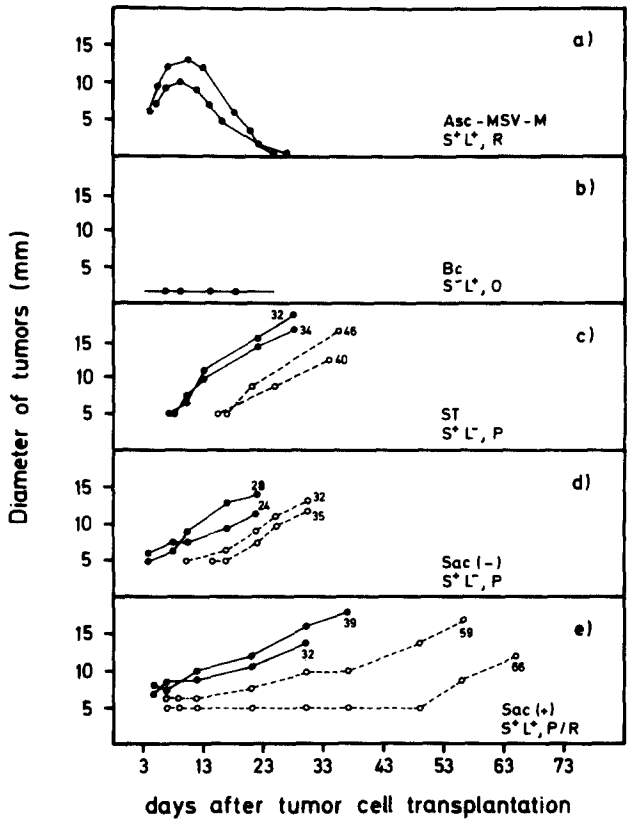

FIG. 2. Course of tumor development after i.m. inoculation of the various cell strains. Two lines for each experiment represent two mice. S^-L^+, cells producing only XC plaque-forming virus (helper virus); S^+L^-, cells carrying the sarcoma genome rescuable by helper virus; P, tumor growth without regression; R, tumor growth followed by regression; P/R, after inoculation of 10^6 cells the tumor growth is progressive without regression, but inoculation of about 10^4 cells results in tumors growing progressively until death of the animals, or tumors stop growing for a variable length of time and either start to grow again or regress; O, no tumor development in adult mice. (●), after inoculation of 10^6 cells/mouse; (○), after inoculation of 10^4 cells/mouse. Number at the end of a curve indicates the day of death after inoculation. Diameters of tumors were determined using a caliper.

than after inoculation of 10^6 cells; others stopped growing for a variable length of time and either started then with a steady continuous growth until the death of the animals, or regressed (Fig. 2e and Table I). These growth patterns of Sac(+) cells may be explained in the case of continuous growth by the selection of nonproducer cells, for example cells with properties of ST cells, or immuno-resistant cells. Results of the following experiments, however, do not support these conceptions: a tumor was induced by i.m. inoculation of 5×10^4 Sac(+) cells in the right thigh of a mouse. 5 days later, this mouse was protected against a challenge with 5×10^4 Sac(+) cells in the left thigh. The tumor on the right thigh reached a diameter of 1.1 cm on the 20th day after inoculation and it was then removed for the preparation of a cell suspension. As early as 72 h after the start of the focus assay, focus development was observed up to cell

TABLE I
*Cross Transplantation Immunity**

Cells used for primary inoculation‡	Groups of mice§	Types and number of cells used for challenge														
		asc-MSV-M			ST			Sac (−)				Sac (+)				
		10^6	10^5	10^4	10^6	10^5	10^4	10^5	10^4	10^3	10^2	10^6	10^5	10^4	10^3	10^2
asc-MSV-M (S^+L^+, R)	Ino.	1/5‖ (13)	0/5	0/5	5/5 (14–20)	2/5 (20–22)	0/5	5/5 (6)	5/6 (8–20)	2/5 (11–15)	0/5	3/5 (<5–9)	2/6 (7–12)	0/6	0/2	
	C	5/5 (5)	5/5 (5)	5/5 (5)	5/5 (8)	4/5 (11–18)	2/5 (18–20)	6/6 (4–6)	6/6 (8–18)	4/6 (11–13)	1/6 (13)	4/4 (<5) 5P	5/5 (<5) 5P	5/5 (<5–7) 5P	5/5 (<5–9) 5P	
ST (S^+L^-, P)	Ino.	5/5 (6)	4/4 (6)	5/5 (6–8)	5/5 (8)	3/4 (14–32)	¶									
	C	5/5 (6)	5/5 (6)	5/5 (6)	5/5 (8)	4/5 (11–18)	2/5 (18–20)									
Sac(−) (S^+L^-, P)	Ino.							12/12 (7)	9/11 (11–18)	¶	¶			5/5 (7–12)	4/5 (7–9)	3/5 (9)
	C							8/8 (7)	11/11 (11–16)	10/11 (11–25)	5/12 (14–21)			5/5 (<5–7) 4P/1R	4/5 (7–9) 2P/2R	3/6 (9–12) 3R
Sac(+) (S^+L^-, P/R)	Ino.		0/5**	0/5**				4/4 (6–8)	4/6 (12–16)	0/6	0/6	4/5 (5–7)	2/6 (9, 14)	0/5	0/5	
	C		5/5 (3–6)	5/5 (6)				4/4 (6)	4/5 (8–14)	6/6 (12–19)	1/6 (12)	4/4 (<5) 4P	5/5 (<5) 5P	5/5 (<5–7) 4P/1R	4/5 (7–9) 2P/2R	
Bc (S^-L^+, O)	Ino.	0/5	0/5	0/5	5/5 (8–11)	3/5 (14)	1/5 (22)	6/6 (4–8)	5/6 (8–15)	5/6 (13–21)	4/6 (13–19)	5/5 (5–7)	0/5	0/5	0/5	
	C	5/5 (6)	5/5 (6)	5/5 (6)	5/5 (8)	4/5 (11–18)	2/5 (18–20)	6/6 (4–8)	5/6 (8–13)	4/6 (13–15)	1/6 (15)	4/4 (<5)	5/5 (<5)	5/5 (<5–7)	4/5 (7–9)	

* Primary inoculation of cells was done i.m. into the right thigh of 6-wk-old mice; ≅ 2 wk later, i.m. challenge inoculation into the left thigh was performed.
‡ See legend of Fig. 2.
§ Ino., groups of mice with primary cell inoculation; C, control groups.
‖ Number of mice with tumors/number of inoculated mice; number in parentheses indicates the day after challenge at which a tumor was palpable.
¶ Intercurrent death due to the primary tumor.
** 5×10^5 and 5×10^4 cells were used for challenge.

concentrations of 10^2 nuclear cells/0.2 ml, thus indicating the presence of ffu-releasing cells, since formation of foci induced by the division of transformed nonproducing cells would require more time. Moreover, the foci reacted positively in the XC plaque assay performed 6 days after the beginning of the test. Simultaneously, the tumor cell suspension was transplanted i.m. into normal mice and into mice that had received Sac(+) culture supernate i.m. 11 days before. Doses containing 10^6 nuclear cells and a 3-, 9-, and 27-fold dilution of it were used. Each group of mice consisted of 5–6 animals. 18 of the 20 mice of the four control groups developed tumors with a progressive (12 cases) or regressive (6 cases) course, whereas no tumor was palpable in the 22 mice pretreated with Sac(+) virus.

After we had obtained the results described above, the virological and growth properties of the different cell strains were designated in the tables and figures as indicated in the legend of Fig. 2.

Growth of Tumors Caused by Virus Released from Sac(+) Cells. The observation that the sarcoma-helper virus complex-producing Sac(+) cells induced in many cases progressively growing tumors without regression and also produce focus- and XC plaque-forming virus, prompted us to study whether this virus causes tumor development with growth characteristics similar to that induced by the cells releasing this virus. Intramuscular inoculation of supernates from Sac(+) cells containing 10^4 ffu/0.1 ml was followed by leg enlargement in 25 of 36 mice which was palpable between 6 and 12 days after virus inoculation. The tumors regressed completely in all cases ≅3 wk after virus inoculation. None of the regressed tumors reappeared during an observation period of ≅2 mo after regression was complete.

Induction of Cross Transplantation Resistance. To study the capacity of the different cell lines to induce homologous and heterologous transplantation immunity, cross tests were performed. Table I summarizes the results. It can be seen that after application of cells releasing either XC plaque-forming virus (i.e., Bc cells) or both focus- and XC plaque-forming virus [i.e., asc-MSV-M, and Sac(+) cells] a transplantation resistance existed against the sarcoma-helper virus-producing cells. After application of the helper virus-producing Bc cells, this resistance was complete against three concentrations of asc-MSV-M cells and strong against Sac(+) cells, i.e. against cells producing the sarcoma-helper virus complex. Even 1 yr after Bc cell inoculation, complete transplantation resistance existed against a challenge with 10^6, 10^5, and 10^4, respectively, asc-MSV-M cells. After inoculation of asc-MSV-M and Sac(+) cells, a strong transplantation immunity was induced against asc-MSV-M cells. Resistance against Sac(+) cells was somewhat less; 10^5 Sac(+) cells were not rejected in all mice challenged with this cell concentration. On the other hand, transplantation of the helper virus-producing cells (Bc) resulted in no resistance against the nonproducer cells [ST and Sac(−) cells] and inoculation with sarcoma-helper virus complex-producing cells [asc-MSV-M and Sac(+) cells] was followed by only very weak resistance against these nonproducer cells. These nonproducer cells also failed to induce transplantation resistance under the conditions tested. The nonproducer ST cells did not induce transplantation resistance against asc-MSV-M cells, and when inoculated into ST cell-pretreated mice they were also not rejected, but the group receiving the lowest challenge concentration could not be observed during the whole required observation period because of intercurrent death of the challenged animals. The results obtained with the in vitro cultured nonproducer Sac(−) cells resembled those observed with the nonproducer ST cells. The nonproducer Sac(−) cells neither induced transplantation immunity against producer cells [10^2 Sac(+) cells], nor against Sac(−) cell inocula containing 10^5 and 10^4 cells. As with the nonproducer ST cells, it could not be decided whether or not the cultured nonproducer Sac(−) cells induced protection against challenge with lower cell concentration because Sac(−) tumor bearers died during the latency period of tumors when using relatively small numbers of Sac(−) cells as challenge.

Comparison of Cultured Cell Lines as Target Cells in CMC and AMC Assays. Experiments were performed to determine whether or not differences exist between the three cultured cell lines with regard to their sensitivity as

TABLE II
Comparison of Three Cell Lines Derived from MSV-M-Induced Tumors as Target Cells in a CMC Assay

Target cells	Lymphoid spleen cells from Sac(+) (S^+L^+, P/R) tumor bearers (12 days after inoculation)		
	Spleen cell:target cell ratio		
	250:1	125:1	62.5:1
		%	
Bc (S^-L^+, O)	79*	78	70
Sac(+) (S^+L^+, P/R)	28	19	0
Sac(−) (S^+L^-, P)	17‡	0	n.t.§

*‡ Percent reduction of target cell radioactivity; values are significant by the Student's t test at $P < 0.001$ with the exception of ‡, where $P < 0.05$.
§ n.t., not tested.

TABLE III
Comparison of Three Cell Lines Derived from MSV-M-Induced Tumors as Target Cells in an AMC Assay

Target cells	Pooled sera from mice inoculated with Sac(+) cells (S^+L^+, P/R)*					Pooled sera from mice inoculated with Bc cells (S^-L^+, O)‡				
	Serum dilutions					Serum dilutions				
	1:27	1:81	1:243	1:729	1:2,100	1:27	1:81	1:243	1:729	1:2,100
					%					
Bc (S^-L^+, O)	74§	75	62	51	14 $P < 0.05$	36	46	41	23	0
Sac(+) (S^+L^+, P/R)	96	96	93	81	53	75	83	69	33	0
Sac(−) (S^+L^-, P)	76	61	45 $P < 0.01$	30 $P < 0.01$	n.t.‖	0	0	0	0	n.t.

* Serum was taken 41 days after cell inoculation.
‡ Serum obtained 147 days after cell inoculation.
§ Percent reduction of target cell radioactivity, $P = <0.001$ with exceptions indicated in the table.
‖ n.t., not tested.

target cells in the [^{3}H]proline-MA applied for the demonstration of CMC and AMC.

Lymphoid spleen cells were prepared from mice that had been i.m. inoculated with 10^4 sarcoma-helper virus-producing Sac(+) cells 12 days before, and that had become tumor bearers. A lymphoid spleen cell preparation from uninocu-

lated age-matched mice served as control. The Sac(+) cells, the helper virus-producing Bc cells, and the nonproducer Sac(−) cells were used simultaneously as target cells in the [^{3}H]proline-MA. Table II summarizes the results. It can be seen that effector cells exhibited the strongest cytotoxic activity towards the helper virus-releasing Bc cells; a spleen cell:target cell ratio of 250:1 resulted in a target cell reduction of 79%. At this ratio, the percent reduction of the sarcoma-helper virus complex-producing Sac(+) cells was only 28%, and that of the nonproducer Sac(−) cells, 17%. At spleen cell:target cell ratios of 125:1 and 62.5:1, the difference in sensitivity to effector cell activity was very pronounced between Bc cells on one side and the Sac(+) cells and Sac(−) cells on the other.

Next, the three cultured cell lines were used as target cells for the demonstration of C′-dependent AMC in the [^{3}H]proline assay. The results of representative experiments are shown in Table III. Sera from three mice bearing tumors induced by Sac(+) cells were obtained and pooled 41 days after inoculation of 1.8×10^4 cells. This serum pool possessed cytotoxic antibodies against the helper virus-releasing Bc cells and the Sac(+) cells, and also against the nonproducer Sac(−) cells. Pooled sera taken from five mice inoculated with nontumorigenic Bc cells 147 days after inoculation were cytotoxic only against the two producer cells, but not toward the nonproducer cell line. In both experiments, the sarcoma-helper virus-producing Sac(+) cells were the most sensitive target cell for detection of AMC.

Induction of CMC and AMC by the Different Cell Lines. Induction of CMC was studied after application of the helper virus-producing Bc cells, the two sarcoma-helper virus complex-producing cells [ascitic MSV-M cells, and Sac(+) cells], and the nonproducer Sac(−) cells, respectively. 12 days after inoculation, spleen cells from the inoculated mice were prepared and tested for cytotoxicity in the [^{3}H]proline-MA. The helper virus-releasing Bc cells served as target cells because they had been shown to be the most sensitive cells for the detection of cytotoxic effector cells as outlined above. As can be seen from Fig. 3, all producer cells induced a relatively strong CMC, the helper virus-producing cells being somewhat less effective than the two cell lines releasing the sarcoma-helper virus complex (S^+L^+ cells). It should be noted that there was no significant difference between the regressor S^+L^+ cells (asc-MSV-M cells) and the S^+L^+ cells, growing frequently progressively [Sac(+) cells], and also that the nonproducer Sac(−) cells induced no detectable CMC. In an additional experiment, mice were inoculated with the nonproducer Sac(−) cells and spleen cells were prepared between 1 and ≅4 wk after inoculation. None of these preparations possessed a cytotoxic activity against the helper virus-producing Bc cells; in this experiment the producer Sac(+) and the nonproducer Sac(−) cells were in addition used as target cells, and no cytotoxic reaction could be detected with either one.

The four cell lines were also tested for their capacity to induce AMC. Mice were inoculated with 10^6 helper virus-producing Bc cells, 10^6 asc-MSV-M cells (S^+L^+), 10^4 Sac(+) cells (S^+L^+), and 10^4 nonproducer Sac(−) cells, respectively, and bled 25 days later. The collected sera of each group were examined for the presence of C′-dependent cytotoxic antibodies in the [^{3}H]proline-MA using Bc cells as target cells. Percent reduction of target cell radioactivity caused by

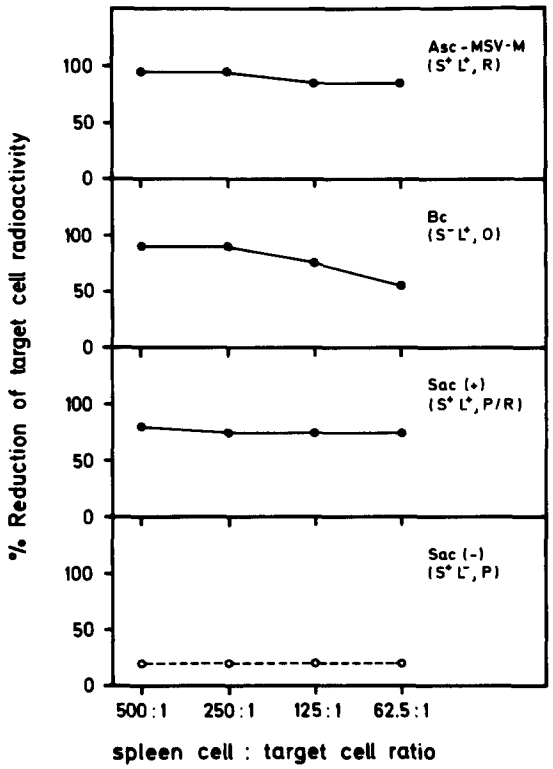

FIG. 3. Comparison of the cytotoxic activity of spleen cells obtained from mice inoculated with cells from one of the four cell lines. Each mouse received 10^6 cells, and spleen cells were prepared 12 days after inoculation. With the exception of Bc cell (S^-L^+, O)-inoculated mice, all spleen cell donors were tumor bearers. CMC was determined in the [^{3}H]proline-MA using Bc cells (S^-L^+, O) as target cells. Percent reduction values of target cell radioactivity are significant by the Student's t test at $P < 0.001$. (O- - -O), no reduction of target cell radioactivity.

serum obtained from mice inoculated with Bc cells and diluted 1:81 was 23%. The corresponding values for sera from mice that had received cells of the two lines producing the sarcoma-helper virus complex were 25% (Sac(+) cells) and 43% (asc-MSV-M cells), whereas 1:9 diluted serum collected from mice inoculated with the nonproducer Sac(−) cells possessed no demonstrable cytotoxic activity. These results indicate that the producer cells induce the development of cytotoxic antibodies regardless of whether they are nontumorigenic, regressor, or prevalently progressor cells. No cytotoxic antibodies against the helper virus-producing Bc cells, the sarcoma-helper virus complex-releasing Sac(+) cells, and the nonproducer Sac(−) cells could be detected in sera from mice inoculated with the nonproducer Sac(−) cells demonstrated above to be a sensitive target cell for cytotoxic antibodies induced by Sac(+) cells (S^+L^+).

Tumor Growth after Simultaneous Inoculation of Producer Cells (S^+L^+) Differing in Their Oncogenic Potency into the Same Mouse. The frequently progressive growth of Sac(+) cell tumors, in spite of the strong capacity of Sac(+) cells to induce transplantation immunity, CMC, and AMC, prompted us

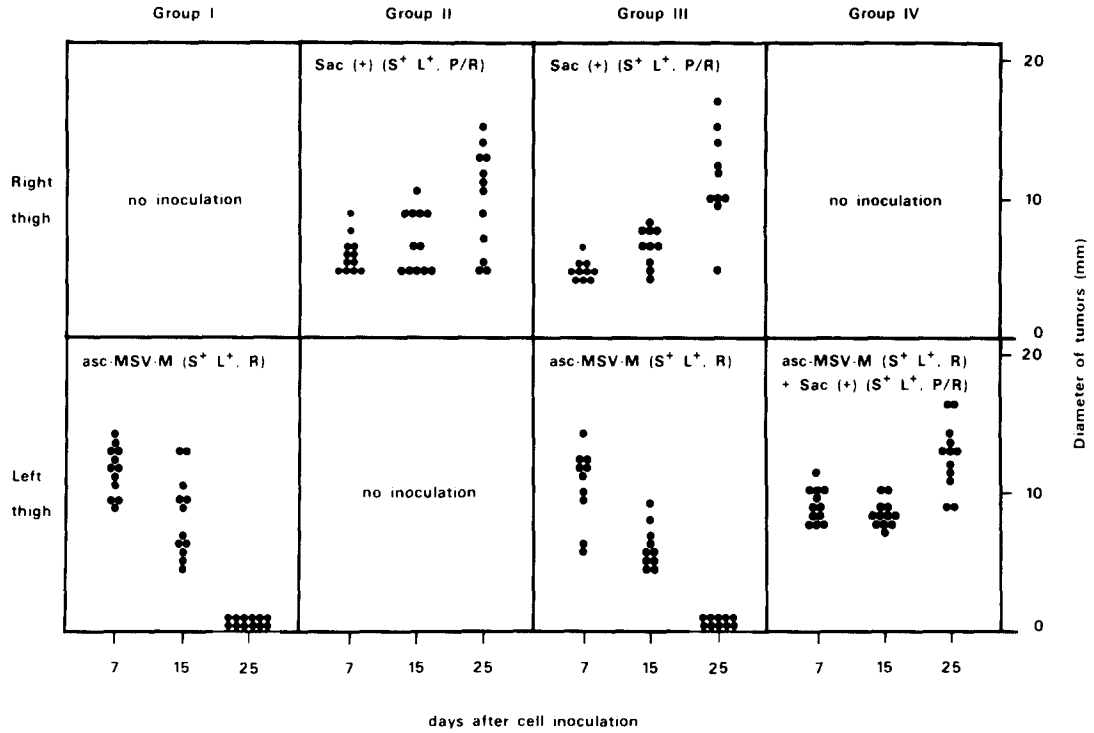

FIG. 4. Simultaneous inoculation of Sac(+) cells (S^+L^+, P/R) and ascitic MSV-M cells (S^+L^+, R) into the same mouse. Each group consists of 10–12 animals of both sexes; the mice were inoculated at the age of about 7 wk. Group I: inoculation of 10^6 nuclear ascitic MSV-M cells (S^+L^+, R)/mouse i.m. into the left thigh. Group II: 5×10^4 Sac(+) cells (S^+L^+, P/R) into the right thigh. Group III: 10^6 ascitic MSV-M cells (S^+L^+, R) into the left thigh and 5×10^4 Sac(+) cells (S^+L^+, P/R) into the right thigh. Group IV: a mixture consisting of 10^6 ascitic MSV-M cells (S^+L^+, R) and 5×10^4 Sac(+) cells (S^+L^+, P/R) in the left thigh. Tumor development was monitored three times weekly by measuring the tumor diameter using a caliper.

to study whether or not there is an indication for the presence of blocking factors which protect the tumor cells in vivo from anti-tumor immune responses. Therefore, we performed an experiment in which Sac(+) cells and asc-MSV-M cells (the latter also producing the sarcoma-helper virus complex but inducing regressing tumors) were inoculated in a group of mice simultaneously at separate sites of a mouse to see whether or not development of Sac(+) cell tumors prevent the regression of tumors induced by asc-MSV-M cells. Fig. 4 (group III) shows that under such circumstances, regression of the tumors induced by asc-MSV-M cells occurred in a course comparable with that of the control group (group I). The tumor appearing at the site where Sac(+) cells had been inoculated also had a growth pattern like that of the corresponding control group (group II). In both groups II and III, the Sac(+) tumors grew rather slowly but progressively; no regression was observed. After inoculation of a mixture of asc-MSV-M cells with Sac(+) cells into the left thigh, tumors appeared with the growth properties of Sac(+) tumors, but with a slightly enhanced development in comparison with the tumors which had been induced by inoculation of Sac(+) cells into the mice of groups II and III.

Discussion

According to Ribacchi (10), the recurrence of MSV-M tumors supports the hypothesis that the resistance state acquired during regression is a transient condition. However, recurrence may also be explained by the conception that transformed cells differing in particular properties from tumor cells eliminated by immune mechanisms can escape the immune response of the host. Such properties are, for example, growth behavior, antigenicity, and/or immunoresistance. To our knowledge there is no report on the properties of a recurrent tumor with regard to its virological and immunological properties. Therefore, in the present studies we attempted to characterize cells of a secondary tumor appearing in a Moloney regressor mouse at the site of a primarily regressed MSV-M induced tumor; cell strains derived from these tumor cells, as well as ascitic MSV-M cells inducing regressing tumors and a helper virus-producing cell line derived from these ascitic cells, were included for comparison. No evidence for the release of C-type particles from the cells of the ST was obtained, but rescuable sarcoma virus genome could be demonstrated in the ascitic form of the ST tumor as well as in its culture derivative, Sac(−) cell line. These nonproducer Sac(−) cells induce tumors which grow without regression in immunocompetent adult mice. As in the nonproducer mouse line derived from in vitro MSV-M-transformed BALB/3T3 cells (16), no evidence for viral antigen was obtained. Neither the presence of MuLV group-specific p30 nor gp69/71 could be detected in Sac(−) cells. However, no comparison between the nonproducer BALB/3T3 and our Sac(−) cells with regard to tumorigenicity can be made, since the nonproducer cells of Aaronson and Rowe (16) had been inoculated into irradiated mice.

The antigenicity of transformed nonproducer murine cells is not regularly demonstrable, and has to be considered to be weak since repeated immunizations are necessary to induce a demonstrable immune response (23). No immunogenicity of MSV-transformed nonproducer cells was detected by Strouk et al. (24), and Stephenson and Aaronson (25), and the failure in the present study of ST and Sac(−) cells to induce an immune response supports these observations. The progressive growth of tumors induced by the nonproducer Sac(−) cells can be explained by their poor antigenicity. A suppression of an immune response in ST tumor-bearers against MSV-M producer tumors could not be detected in a preceding study (26).

The Sac(+) cells obtained after infection of the nonproducer Sac(−) cells with helper virus were shown to release focus- and XC plaque-forming virus, and to possess a strong antigenicity. Despite their capacity to induce transplantation immunity, CMC, and AMC, tumors induced by the Sac(+) cells frequently grew progressively. This frequent progressive growth of tumors induced by Sac(+) cells, despite an ongoing anti-tumor immune response, cannot be explained by antigenic modulation of tumor-associated antigens or immunological enhancement, since we observed rejection of tumor transplants in preimmunized mice. Inhibition of CMC by serum blocking factors and immunosuppression of the host's immune response seems also not to be operative as demonstrated by the regression of asc-MSV-M (S^+L^+)-induced tumors in mice simultaneously inoculated with Sac(+) cells (S^+L^+), which became bearers of progressively growing Sac(+)-tumors.

Failure to induce demonstrable transplantation immunity, CMC, and AMC was not accompanied with failure to react with products of an immune response against cells producing the sarcoma-helper virus complex as demonstrated with the Sac(−) cells. These nonproducer Sac(−) cells reacted as target cells for the demonstration of antibody-mediated cytotoxicity, and to a small extent as target cells for the demonstration of cell-mediated cytotoxicity. The properties of the component of the nonproducer Sac(−) cells reacting with Sac(+) cell-induced cytotoxic antibodies remain to be determined; it may represent a transformation-specific surface antigen.

The observation that the sarcoma-helper virus complex-releasing Sac(+) cells and the helper virus-producing Bc cells, both producing the same helper virus, differ in their reactivity against cytotoxic effector cells induced by sarcoma-helper virus complex-producing cells is of considerable interest. The relative insensitivity of Sac(+) cells is not mediated by components released from these cells since addition of Sac(+) cells to Bc cells did not decrease the sensitivity of the latter (result not shown). The difference in sensitivity between Bc cells and Sac(+) cells in CMC may be due to a smaller amount of helper virus produced per Sac(+) cell in comparison to Bc cells. However, this difference in the amount of helper virus production is without influence on the sensitivity of the two cells in the AMC assay. Furthermore, in view of the difference in the morphological phenotype between Sac(+) and Bc cells, it would be interesting to study whether or not the insensitivity of Sac(+) cells for CMC in contrast to AMC is due to the reduction of cytoskeletal elements that seems to be correlated with the phenotype in oncornavirus-transformed cells (27, 28). Such a decrease in number and distribution of cytoplasmic microfilaments may result in an interference with steps in the lytic process in CMC (29, 30). Besides this, one may consider that H-2 restriction exists in CMC in the MSV-M mouse system (31), and one can speculate that differences in the expression of H-2 between the two cell lines are responsible for their different reactivity against effector cells.

The presented results stress the importance of the cell type used as target cell for the detection of CMC. Contradictory results reported on the demonstration of cytotoxic effector cells in mice with Moloney sarcoma (for review see Levy and Leclerc, 1) can be explained by the use of target cells differing in sensitivity.

The described relative insensitivity of Sac(+) cells (S^+L^+) against cytotoxic effector cells, and possibly a growth behaviour which protects tumor cells within the tumor mass from immune attack may be responsible for the escape of Sac(+) tumors from immune surveillance. The transplantation resistance of mice preimmunized with producer cells against a challenge with Sac(+) cells may be explained with the assumption that mice that have already mounted a strong CMC and AMC response are able to reject a relatively small number of Sac(+) cells. The observation that the sarcoma-helper virus complex released from Sac(+) cells induced tumors which regress, makes it difficult to assume that the escape of Sac(+) cell-induced tumors from immune destruction is the result of at least some fraction of newly infected cells becoming nonproducer, particularly in the presence of antibodies which neutralized helper virus. This observation makes it more reasonable to assume that the progressive growth of tumors induced by the producer cell line derived from ST cells is a property of these transformed cells per se. Such a property, together with a poor antigenic-

ity, may also allow the nonproducer Sac(−) cells to escape from immune surveillance.

Summary

Cells from a secondary tumor developing at the site of a regressed Moloney sarcoma virus-induced tumor could be passaged in adult STU mice by intramuscular and intraperitoneal inoculation. The tumors induced by these cells, as well as by a cell line derived from it, grew progressively and led to death of the animals between 3 and 7 wk after tumor transplantation. No evidence for production of virus from these cells was obtained or for the presence of viral antigens (p30, gp69/71). From both cell variants, sarcoma virus genome could be rescued by infection with helper virus, resulting in the establishment of a cell line producing focus- and XC plaque-forming virus. The rescued producer cells very frequently also produced tumors which finally grew progressively. The nonproducer cells were not immunogenic, as was demonstrated in cross transplantation tests and in studies for cell-mediated cytotoxicity (CMC) and complement-dependent antibody-mediated cytotoxicity (AMC). The producer cells, however, were demonstrated to possess a strong immunogenicity. The nonproducer cells, though nonimmunogenic, revealed a weak immunosensitivity when used for challenge in the transplantation protection assay or as target cell for the demonstration of AMC and CMC, if the immune response was induced by cells producing the sarcoma-helper virus complex, but not by cells producing only helper virus. The nonproducer cells, as well as their rescued producer derivative, showed a stronger reactivity with cytotoxic antibodies than with cytotoxic cells, whereas the helper virus-producing cell line was comparably suitable as target cell for AMC and CMC. The recurrence of a regressed Moloney sarcoma is assumed to be the result of the occurrence of transformed nonproducer cells escaping immune destruction, and not as a consequence of a depleted immune resistance in the host.

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References

1. Levy, J. P., and J. C. Leclerc. 1977. The murine sarcoma virus-induced tumor: exception or general model in tumor immunology? *Adv. Cancer Res.* 24:1.
2. Fefer, A., J. L. McCoy, and J. P. Glynn. 1967. Studies on the growth and regression of a transplantable Moloney sarcoma. *Cancer Res.* 27:2207.
3. Law, L. W., R. C. Ting, and M. F. Stanton. 1968. Some biologic, immunogenic, and morphologic effects in mice after infection with a murine sarcoma virus. I. Biologic and immunogenic studies. *J. Natl. Cancer Inst.* 40:1102.
4. Collavo, D., A. Colombatti, G. Biasi, L. Chieco-Bianchi, and A. J. S. Davies. 1976. Immune reactivity in the Moloney strain of murine sarcoma virus oncogenesis: requirement of thymus-derived lymphocytes for in vivo protection. *J. Natl. Cancer Inst.* 56:603.
5. Stutman, O. 1975. Delayed tumor appearance and absence of regression in nude mice infected with murine sarcoma virus. *Nature (Lond.).* 253:142.

6. Davis, S. 1975. Moloney sarcoma virus-induced tumors in athymic (nude) mice: growth pattern and antibody responses. *J. Natl. Cancer Inst.* **54**:793.
7. Fefer, A., J. L. McCoy, K. Perk, and J. P. Glynn. 1968. Immunologic, virologic, and pathologic studies of regression of autochthonous Moloney sarcoma virus-induced tumors in mice. *Cancer Res.* **28**:1577.
8. Hellström, I., and K. E. Hellström. 1969. Studies on cellular immunity and its serum mediated inhibition in Moloney-virus induced mouse sarcomas. *Int. J. Cancer.* **4**:587.
9. Gazdar, A. F. 1970. The response of NZW × NZB F_1 hybrid mice to a murine sarcoma virus. *Int. Arch. Allergy Appl. Immunol.* **38**:509.
10. Ribacchi, R. 1970. Studies of progressing and regressing MSV-induced tumours in BALB/c mice. *In* Immunity and Tolerance in Oncogenesis. L. Severi, editor. University of Perugia, Perugia, Italy. 437–452.
11. Tamerius, J. D., and I. Hellström. 1974. In vitro demonstration of complement-dependent cytotoxic antibodies to Moloney sarcoma cells. *J. Immunol.* **112**:1987.
12. Hinshaw, V. S., F. L. Schaffer, and M. A. Chatigny. 1976. Evaluation of Moloney murine sarcoma and leukemia virus complex as a model for airborne oncogenic virus biohazards: survival of airborne virus and exposure of mice. *J. Natl. Cancer Inst.* **57**:775.
13. Weiland, E., and F. Weiland. 1974. Transplantierbarer Aszitestumor aus einer Maus mit einem Moloneysarkom: Tumor-, Antigen- und Viruseigenschaften. *Z. Immunitaetsforsch.* **148**:151.
14. Weiland, E., and M. Mussgay. 1975. Tumor inhibiting capacity of spleen and lymph node cells from mice with murine sarcoma virus (MSV-M)-induced tumors. *Z. Immunitaetsforsch.* **150**:414.
15. Weiland, E., and M. Mussgay. 1976. Detection of cytotoxic lymphoid spleen cells from STU-mice with Moloney sarcoma by a ^{3}H-proline microcytotoxicity assay. *Med. Microbiol. Immunol.* **162**:81.
16. Aaronson, S. A., and W. P. Rowe. 1970. Nonproducer clones of murine sarcoma virus transformed Balb/3T3 cells. *Virology.* **42**:9.
17. Ting, R. C., and A. V. Bader. 1969. A quantitative study on transformation of hamster embryo cells in vitro by murine sarcoma viruses (Harvey and Moloney). *Virology.* **39**:194.
18. Rowe, W. P., W. E. Pugh, and J. W. Hartley. 1970. Plaque assay techniques for murine leukemia viruses. *Virology.* **42**:1136.
19. Ball, J. K., J. A. McCarter, and S. M. Sunderland. 1973. Evidence for helper independent murine sarcoma virus I. Segregation of replication-defective and transformation-defective viruses. *Virology.* **56**:268.
20. Weiland, F., S. Ueberschär, O. C. Straub, O. R. Kaaden, and B. Dietzschold. 1974. C-type particles in cultured lymphocytes from highly leukemic cattle. *Intervirology.* **4**:140.
21. Bean, M. A., H. Pees, J. E. Fogh, H. Grabstald, and H. F. Oettgen. 1974. Cytotoxicity of lymphocytes from patients with cancer of the urinary bladder: detection by a ^{3}H-proline microcytotoxicity test. *Int. J. Cancer.* **14**:186.
22. Russel, S. W., and C. G. Cochrane. 1974. The cellular events associated with regression and progression of murine (Moloney) sarcomas. *Int. J. Cancer.* **13**:54.
23. Aoki, T., J. R. Stephenson, and S. A. Aaronson. 1973. Demonstration of a cell-surface antigen associated with murine sarcoma virus by electron-microscopy. *Proc. Natl. Acad. Sci. U. S. A.* **70**:742.
24. Strouk, V., G. Grundner, E. M. Fenyö, E. Lamon, H. Skurzak, and G. Klein. 1972. Lack of distinctive surface antigens on cells transformed by murine sarcoma virus. *J. Exp. Med.* **136**:344.

25. Stephenson, J. R., and S. A. Aaronson. 1972. Antigenic properties of murine sarcoma virus-transformed Balb/3T3 nonproducer cells. *J. Exp. Med.* 135:503.
26. Weiland, E., and M. Mussgay. 1975. Presence of splenic suppressor cells in mice bearing regressively growing Moloney sarcomas and their absence in progressor mice. *Eur. J. Cancer.* 13:705.
27. Altenburg, B. C., K. Somers, and S. Steiner. 1976. Altered microfilament structure in cells transformed with a temperature-sensitive transformation mutant of murine sarcoma virus. *Cancer Res.* 36:251.
28. Wang, E., and A. R. Goldberg. 1976. Changes in microfilament organization and surface topography upon transformtion of chick embryo fibroblasts with Rous sarcoma virus. *Proc. Natl. Acad. Sci. U. S. A.* 73:4065.
29. Cerottini, J.-C., and K. T. Brunner. 1972. Reversible inhibition of lymphocyte-mediated cytotoxicity by cytochalasin B. *Nat. New Biol.* 237:272.
30. Henney, C. S., and J. E. Bubbers. 1973. Antigen-T lymphocyte interactions: inhibition by cytochalasin B. *J. Immunol.* 111:85.
31. Gomard, E., V. Duprez, Y. Henin, and J. P. Levy. 1976. H-2 region product as determinant in immune cytolysis of syngeneic tumour cells by anti-MSV T lymphocytes. *Nature (Lond.).* 260:707.