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Characterization of Tumor Cell Lines from a Spontaneous Rat Sarcoma Expressing an Endogenous Retrovirus*

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Abstract—*We have characterized various biologic, immunologic and growth properties of several cell lines established from a spontaneous rat sarcoma that was discovered more than 60 yr ago. The tumors consisted of mixed cell types with no detectable host cellular immune response. Cultures derived from single-cell clones of the parental cell line were non-invasive but highly tumorigenic even in adult rats. The cultured cells spontaneously released replication-competent endogenous rat type C virus which did not carry a transforming gene in its genome. Since normal cells from the same rat strain did not produce a retrovirus, it is possible that production of the endogenous retrovirus may have triggered specific cellular changes necessary for the oncogene expression and development of this tumor.*

INTRODUCTION

ONE OF the important questions underlying the etiology of animal neoplasia concerns the role of endogenous type C retrovirus genes present in most vertebrate cells and their potential interaction/s with various cellular genes and other environmental factors. Among the cellular genes are DNA sequences called oncogenes (or c-onc), which are highly conserved in evolution, are not related to viral genes and can be transduced by replicating retroviruses to form highly oncogenic viruses [1, 2]. The sarcoma viruses isolated from naturally occurring solid tumors of most vertebrates have been shown to carry cell-derived oncogenic sequences called v-onc [3–5].

Although the transforming genes have not yet been identified from any of the naturally occurring malignancies of rats, two oncogenes from the Harvey and Kirsten strains of murine sarcoma viruses (Ha-v-ras and Ki-v-ras respectively) have been isolated by serial *in vivo* passage of murine leukemia virus (MuLV) in separate rat strains. A third viral oncogene, designated Ra-v-ras, has been identified in the Rasheed strain of rat sarcoma virus (RaSV) which was isolated *in vitro* by cocultivation of a rat leukemia virus (RaLV)-productive culture with chemically induced tumor cells [2]. The Ra-v-ras is related to Ha-v-ras but differs from both Ha-v-ras, Ki-v-ras and the normal cellular gene (c-ras) in that it encodes for a transforming protein of 29,000 daltons (p29) instead of the 21,000-dalton (p21) protein of Ha-v-ras and Ki-v-ras [6, 7]. Immunoprecipitation studies and nucleotide sequence analyses of Ra-v-ras revealed that this transforming gene was formed by recombination of a portion of RaLV gag-gene with the c-ras [6–8].

In the past few years several human tumors have been shown to express c-ras genes related to both Ha-v-ras and Ki-v-ras [9–11]. Thus the viral oncogenes have provided useful tools for understanding mechanisms of oncogenesis in naturally occurring tumors. Since transforming genes have not yet been isolated from spontaneous rat tumors, we studied a naturally occurring sarcoma that was orginally discovered in 1919 in a

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rat, *Rattus norvegicus* var. *albus*, strain Galliera [12, 13]. This tumor, designated Sarcoma Galliera (SG), has been maintained for over 60 yr as a transplant tumor in the Galliera rat strain [12, 13]. This unusual sarcoma, with an exceedingly long *in vivo* passage history, provides a unique resource to identify genes involved in the development of spontaneous tumors (i.e. those of unknown etiology). Our studies on the SG tumor indicate that it consists of a heterogenous population of morphologically distinct cells which are highly tumorigenic but non-invasive *in vivo*. These cells produce replication-competent type C virus particles which do not transform rat cells *in vitro* or cause tumors *in vivo*. In this report we describe the properties of the SG tumor cell line, the virus isolated from these cells and our preliminary efforts to isolate an oncogene from this tumor.

MATERIALS AND METHODS

Characterization of tumor cell lines

A long-term cell line designated SGS was initially established from a spontaneous rat sarcoma that had been serially transplanted in *Rattus norvegicus* for more than 60 yr [12, 13]. Cultures were grown in minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 2 mM glutamine and 50 μg/ml of gentamicine and maintained in a humidified incubator at 37°C in the presence of 5% CO_2 in air. Cell cultures were cloned by plating dilutions containing one cell per 0.1 ml of medium in microtiter plates. Several cell lines were established from single-cell clones of the parental culture and tested for various growth characteristics, including the ability to grow in soft agar [14]. Cell kinetics, measurements of DNA synthesis by [^{3}H]-thymidine uptake and chromosome analysis were carried out according to the previously described techniques [15, 16].

Ultrastructural studies were carried out on cells fixed in 2.5% cacodylate-buffered glutaraldehyde (pH 7.4), postfixed in 1% chrome osmium and embedded in Epon Araldite after dehydration [17]. Ultrathin sections were stained with uranyl acetate and lead citrate and examined by an electron microscope. Cell cultures were also examined by scanning electron microscopy according to previously described techniques [18].

In vivo *transplantation*

The SG tumor and several SGS cell lines were serially transplanted in *Rattus norvegicus* var. *albus* Galliera rat strain in which it was originally observed in 1919. Approximately 200 mg of finely minced tumor or 2×10^5 cultured cells were inoculated subcutaneously in 2- to 4-month-old rats. Since the inoculated preparations produced large numbers of tumors in Galliera rats we also tested the tumorigenic potential of these cells by subcutaneous inoculation in young weanling Wistar rats, BALB/c mice, hamsters and guinea pigs. Animals were not immunosuppressed before or after inoculation of tumor cells.

Virus assays

Presence or absence of the virus was judged by electron microscopy and by infectivity assays. Culture fluids were also tested for reverse transcriptase activity in the presence of exogenous template primer poly rA-oligo dT according to the previously described methods [14]. Host range of the virus was determined on mouse, rat, mink, rabbit, dog, cat and human cells as described earlier [19–21]. The indicator cells were plated at a density of 3×10^5 per 60-mm dish in maintenance medium containing 8–10 μg/ml of polybrene and after 24 hr medium was removed and duplicate cultures were exposed to 0.5 ml each of filtered (0.45 μ Millipore) supernatant from SGS cultures. Virus was allowed to adsorb for about 1 hr prior to the addition of the maintenance medium. All the virus-exposed and unexposed control cells were subcultured when confluent and after 21, 30 and 60 days supernatant fluid from each culture was tested for reverse transcriptase activity.

Viral core antigen was detected in the sonicated tissue culture cell packs by complement fixation (CF) tests with the use of highly specific antisera. The immune sera were made in guinea pigs against isoelectric focus-purified major core proteins of the rat leukemia virus (RaLV), murine leukemia virus (MuLV) and feline leukemia virus (FeLV).

The virus released from SGS cells was tested for its possible syncytia-inducing property in XC-rat cells essentially as previously described [20]. To test the ability of this virus to rescue sarcoma virus genome from non-producer cells the SGS cultures were cocultivated with cells non-productively transformed by Kirsten murine sarcoma virus (KiMSV) and RaSV [21, 22]. After 7–10 days supernatants of the cocultures were separately assayed for focus-forming activity on freshly plated normal rat kidney (NRK) cells. Foci were scored after 9–10 days.

Rabbit immune serum to banded SD-RaLV [20] was also tested *in vitro* for its ability to neutralize focus formation by the KiMSV pseudotypes of virus released from SGS cells. Approximately 100 focus-forming units of virus were mixed with equal volumes of each of the sera dilutions (1:10–1:640), left at room temperature for 30 min and incubated with target cells (NRK) for the next 30 min before addition of medium containing 10%

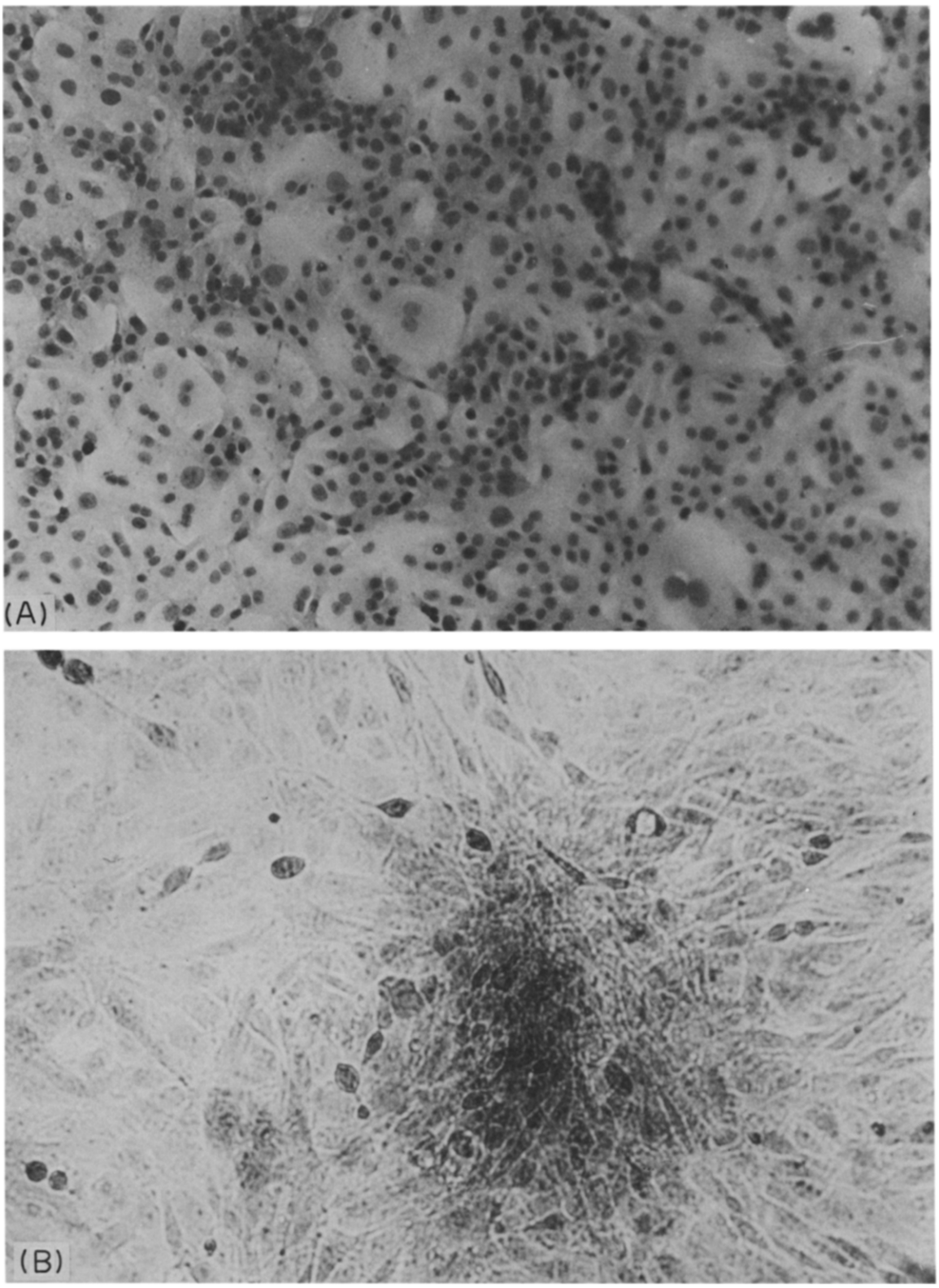

Fig. 1. SGS cell cultures. (A) Note several multinucleated giant cells in SGS-C10 clone (×400); *(B) a focus of crowded SGS cells* (×1000).

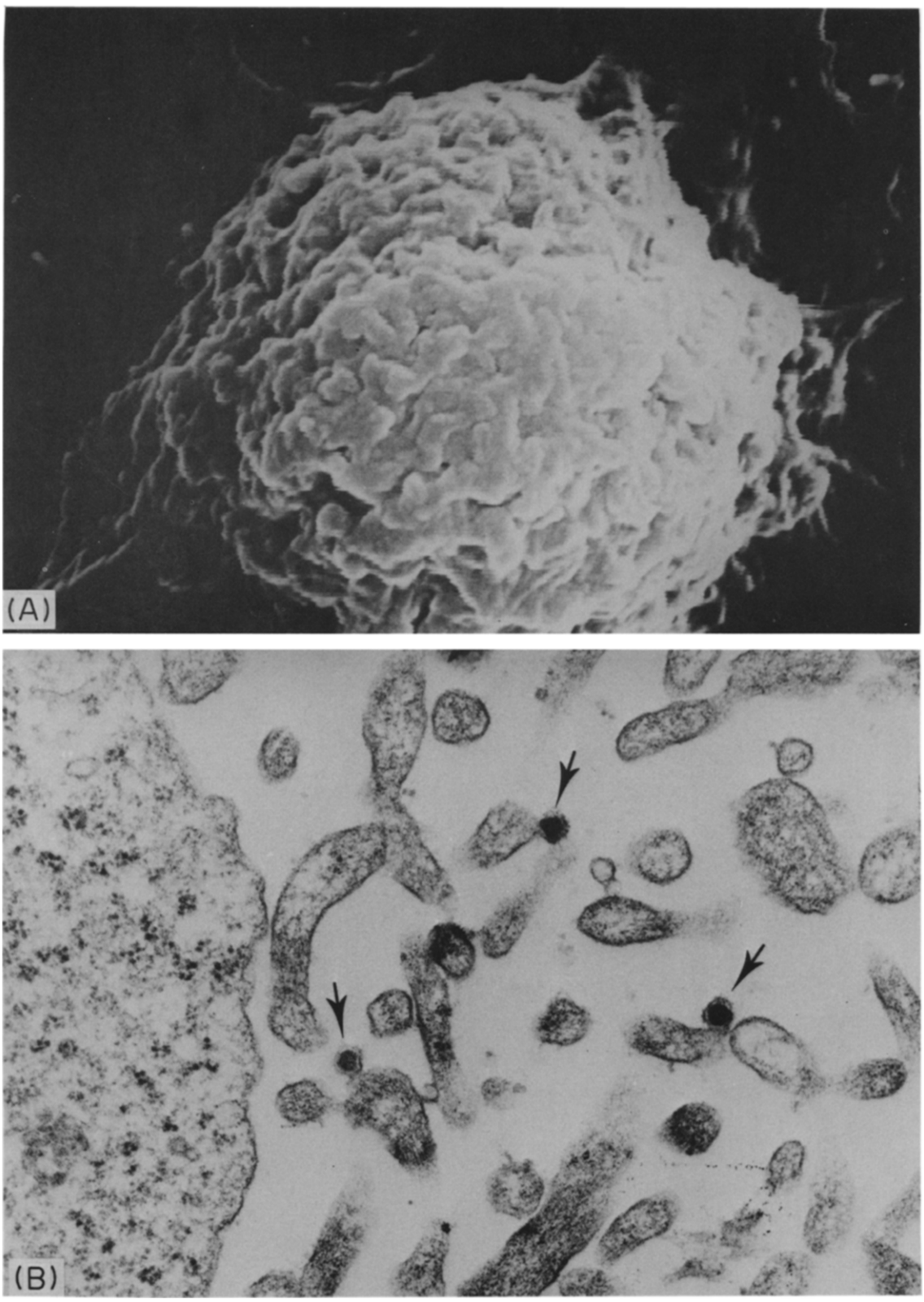

Fig. 2. Scanning (A) and transmission (B) electron microphotographs of SGS cells (×10,000). Note type c particles in B.

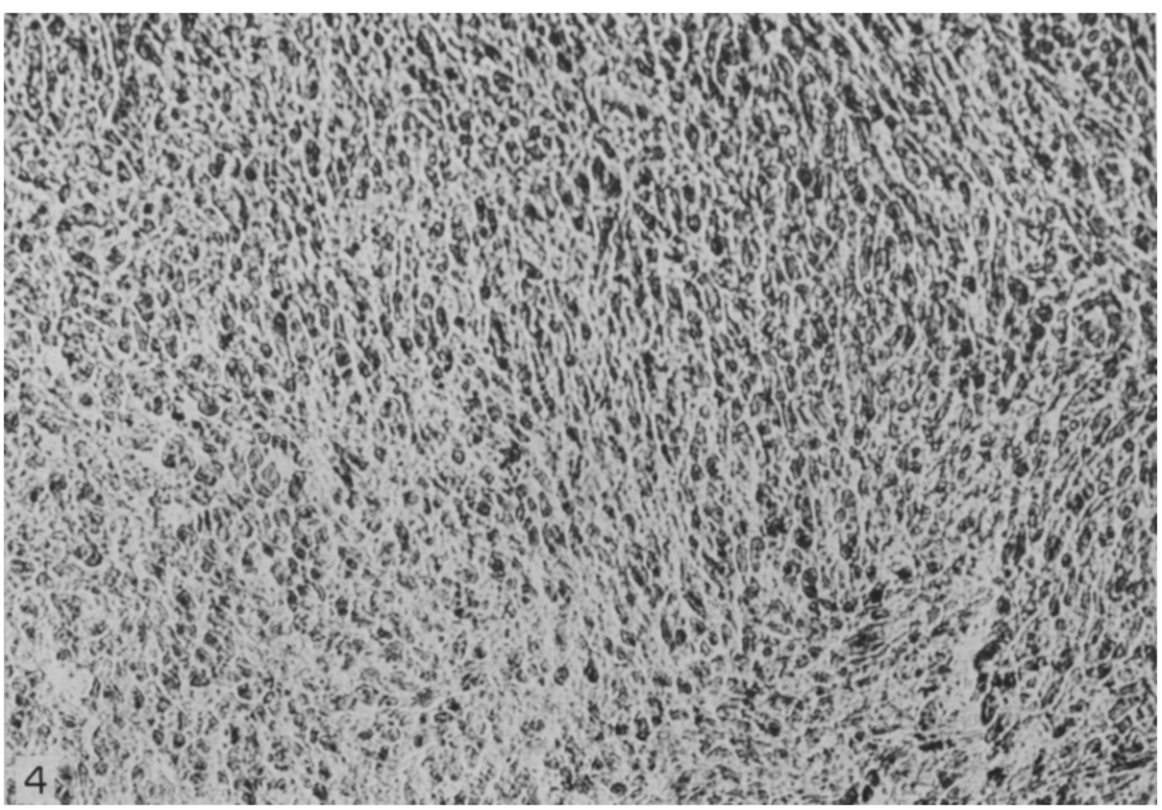

Fig. 4. Paraffin-embedded section of SGS-induced tumor stained with hematoxylin and eosin; note the polymorphocellular aspect of Sarcoma Galliera (×70).

calf serum and 1% dimethyl sulfoxide. Foci were counted after 7–8 days. Neutralization activity was scored positive when 67% or more foci were inhibited.

RESULTS

Characterization of tumor cell lines

The SGS cell line, initially established from the SG tumor, consisted of mixed cell types ranging from fusiform to somewhat rounded and epithelioid morphology with occasional foci of multilayered rounded cells (Fig. 1, A and B). Transformed rounded cells were also observed with the scanning electron microscope but by transmission microscopy the cultured cells appeared very similar in morphology to those present in the *in vivo* induced SG tumors reported earlier [13] (Fig. 2, A and B). The cells grew to relatively high densities with an increased rate of DNA synthesis as compared to the normal control fibroblasts (FG) from the same Galliera rats (Fig. 3). The parental SGS cells produced small colonies in soft agar with a relatively low efficiency compared to the clonal cell lines. The rat origin of the SGS cells was confirmed by chromosome analysis, which indicated aneuploid karyotypes.

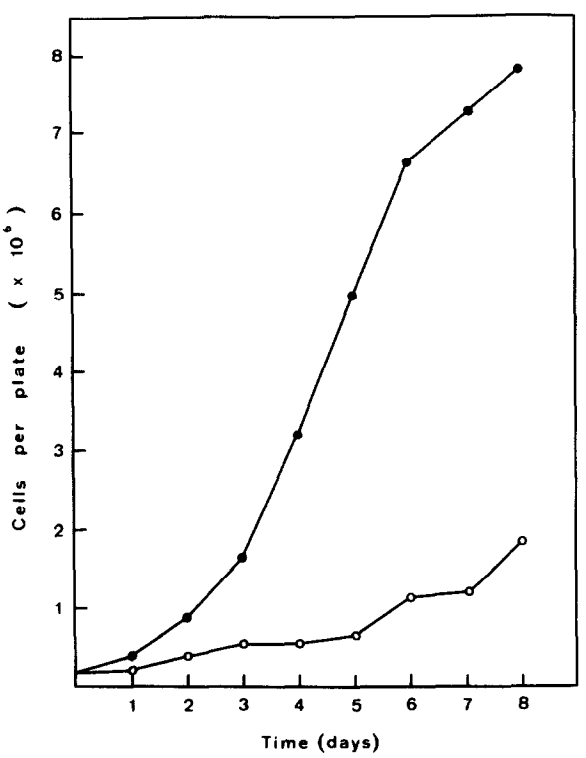

Fig. 3. SGS (●) and FG (○) cells were subcultured and 2 × 10⁵ cells were seeded in 60-mm dishes. Special care was taken to ensure uniform distribution in all culture dishes at the beginning of each experiment. Cell counts were made with a Coulter counter on cell suspensions at the time of seeding (day 0) and daily for 8 days with no medium change. The counts in duplicate dishes differed by less than 5% throughout the experiments [15].

Several cultures were derived from single-cell clones of the parental SGS cells. These clonal cell lines differed in their morphology, growth rate and extent of contact inhibition (Table 1). In general, the contact-inhibited cells (SGS-C1 and SGS-C11) were flat and epithelioid, and showed a relatively slower rate of growth *in vitro* and low tumorigenic potential *in vivo*. Clonal cells that were rounded in morphology grew to high saturation densities and produced tumors in short periods of time. Cultures that were highly tumorigenic also contained several multinucleated giant cells, which were not discernible in cultures that showed low tumorigenic potential (Fig. 1A and Table 1).

Finely minced SG tumor preparations produced non-invasive tumors in 25–30 days in 90% of the inoculated Galliera rats (Table 2). Whereas the parental SGS cells induced tumors in 100% of the inoculated rats with a very short latency period of only 4–6 days, some of the SGS cell clones (SGS-C10 and SGS-C4) showed a slightly higher tumorigenic potential (Table 2). Histologically all *in vivo* induced tumors were polymorphic and appeared to be similar to those described earlier (Fig. 4; [13, 16]).

Properties of an endogenous retrovirus isolated from SGS cells

Although type C particles were detected occasionally by electron microscopy of the original SG rat tumors, a replication-competent virus was not isolated until the SGS cell line was established *in vitro*. The cultures contained extracellular virus particles in abundance and occasionally type C virus particles were seen budding from the plasma membranes of SGS cells (Fig. 2B). The virus particles sedimented at a density of about 1.14 g/ml in an equilibrium sucrose gradient and showed high levels of reverse transcriptase activity. By complement fixation tests the sonicated SGS cell packs reacted positively with a highly specific antiserum made against RaLV core antigen of about 27,000 daltons (p27). This indicated that SGS cells spontaneously released a retrovirus related to RaLV (designated SG-RaLV) [21, 22].

The SG-RaLV was capable of rescuing defective sarcoma virus from both Ki-MSV and RaSV transformed non-producer cells (Table 3). Foci induced by pseudotypes of SG-RaLV (Ki-MSV) were totally neutralized by antiserum made against SD-RaLV. Infectivity assays indicated that SG-RaLV replicated only in the cells derived from various rat strains but not in any of the mouse, human, mink, rabbit, dog or cat cells tested. As indicated in Table 4, for positive controls we used several other strains of rat and

Table 1. Growth properties of the clonal cell lines of Sarcoma Galliera (SGS)

Clone	Morphology	Adhesion	Contact inhibition	Growth rate	Tumorigenicity
SGS-Cl and C11	flat, epithelioid, opaque and granular cells	good	present	moderate	low
SGS-C6 and C8	flat, granular, with numerous multinucleated cells	poor	present	high	high
SGS-C2 and C10	rounded, with numerous multinucleated cells	poor	absent	high	high
SGS-C9	small, round, with clumps with few multinucleated cells	poor	absent	high	NT
SGS-C5 and C7	fusiform fibroblasts	poor	present	low	NT
SGS-C3 and C4	epithelioid, granular, with no multinucleated cells	poor	absent	moderate	high

Cell clones were derived from the parental SGS cell line by limiting dilutions in microtiter plates. Cell morphology, adhesion and contact inhibition were determined by examining live and fixed cell cultures by the light microscope. Growth rate and tumorigenicity were evaluated by cell kinetics and inoculation of cells in Galliera rat, as described in the text. NT = not tested.

Table 2. In vivo tumorigenicity of Sarcoma Galliera

Inoculum*	No. of rats inoculated	% positive	Tumor induction time (days)
SG tumor	500	90	25-30
SGS	50	100	4-6
SGS-C10	20	100	3-5
SGS-C4	20	100	3-5
FG	20	0	-

*200 mg of minced SG tumor preparation or 2×10^5 *in vitro* grown cells (SGS) were inoculated subcutaneously in 2- to 4-month-old male rats (Galliera strain). SGS-C10 and SGS-C4 cultures were derived from single-cell clones and normal fibroblasts (FG) were obtained from a rat embryo of Galliera strain.

mouse retroviruses, which infected these cells. SG-RaLV did not produce syncytia in XC cells nor did it induce cell transformation in exogenously infected rat, mouse or mink cells.

Treatment of early passage cultures with 100 μg/ml of 5′-bromodeoxyuridine did not increase virus production, indicating that it was spontaneously induced in these cells. Furthermore, the lack of cell-transforming ability of SG-RaLV confirmed that it is a helper-independent rat endogenous virus which does not carry an oncogene in its genome. The SG-RaLV also failed to rescue any oncogene from several chemically or polyoma virus-transformed rat cells after prolonged cocultivation [2, 8].

DISCUSSION

We have established and characterized tumor cell lines (SGS) from a spontaneous rat sarcoma that was passaged *in vivo* for over 60 yr [12, 13, 23]. The SGS cells are highly tumorigenic but show a remarkable host specificity. Tumors are induced only in Galliera rats within 3-6 days. Guinea pigs, mouse and other rat strains are not susceptible. Moreover, no immunosuppression is required for tumor induction in Galliera rats and tumors grow rapidly even when SGS cells are inoculated in 4-month-old rats. No infiltration of the surrounding tissues was observed in any of the SGS cell induced tumors. In our experience it has been virtually impossible to induce tumors in adult or even young weanling rats by retroviruses or *in vitro* transformed rat cells [S. Rasheed, unpublished data]. The high tumorigenic potential of these cells in adult Galliera rats therefore indicates a lack of host immune response against the SGS tumor.

The SGS cells spontaneously release an endogenous RaLV (SG-RaLV) which is poorly infectious to the rat cell but does not replicate in other mammalian cells tested. Similar to other

Table 3. Recovery of an endogenous rat retrovirus (SG-RaLV) from SGS cells*

Cell culture	Polymerase activity†	Spontaneously sarcoma virus	Sarcoma virus recovery‡ after			XC plaque formation‖
			Induction	Cocultivation	Rescue§	
SGS	167,269	-	-	-	+	-
FG	35	-	-	-	-	-

*SG-RaLV = Sarcoma Galliera rat leukemia virus.

†Reverse transcriptase values in culture fluids represent [^{3}H]-dTTP-incorporated radioactivity, cpm/ml.

‡Presence of sarcoma virus was tested directly (spontaneous release) and after treatment with 100 μg/ml of 5′-bromodeoxyuridine or after cocultivation with chemically and DNA virus-transformed cells.

§As positive controls we cocultivated SGS and FG cultures with KiMSV- and RaSV-transformed non-producer cell lines as described in Materials and Methods.

‖XC plaque assay was performed as previously described [22].

Table 4. Host range of SG-RaLV

Virus	Mouse	Rat	Human	Mink	Rabbit	Dog	Cat
SG-RaVL	-	+	-	-	-	-	-
ARK-MuLV	+++	+	-	-	-	-	-
X-NZB/K-M1c	-	+	++	+++	++	++	++
KiMSV	+++	++	±	±	±	±	+
SD-RaLV	-	+	-	-	-	-	-

*Indicator cells were exposed to filtered (0.45 μ) supernatants from SGS cells. Subcultures were made when confluent and culture fluids were tested for reverse transcriptase activity up to 60 days. SG-RaLV = Sarcoma Galliera rat leukemia virus; AKR-MuLV = AKR strain of murine leukemia virus; X-NZB/K-M1c = cloned xenotropic MuLV isolated from NZB kidney cells; KiMSV = Kirsten strain of murine sarcoma virus; SD-RaLV = Sprague-Dawley strain of RaLV. + = 3000-50,000 cpm/ml; ++ = 51,000-100,000 cpm/ml; +++ = >100,000 cpm/ml; - = ≤500 cpm/ml. (±) indicates that although foci of transformed cells were induced after infection with KiMSV in various cultures, completely transformed cell lines were not obtained in those marked ±.

endogenous RaLV strains [20, 21], this virus also does not transform rat cells, indicating that it is not a transforming virus. Unlike SD-RaLV, which can occasionally transduce a rat cellular oncogene by cocultivation with a particular chemically transformed non-producer culture [2], the SG-RaLV cannot rescue oncogenes from the same chemically transformed cells *in vitro*. Our preliminary experiments indicate, however, that transfection of SG tumor DNA on mouse NIH 3T3 cells results in transformation of the recipient cells. This indicates that the oncogenic sequences from the SGS tumor have been transferred to the NIH 3T3 cells. Analysis of the DNA from the transformed cells is now underway to characterize genes involved in spontaneous development of Galliera rat tumors.

Although the causes of most human cancers are unknown, retroviruses have been implicated in many naturally occurring cancers. The viruses that carry transforming sequences produce neoplasia by direct insertion of viral oncogenes in the cellular genomes [24, 25]. However, several mechanisms have been proposed for development of tumors by retroviruses that do not carry oncogenes [3, 10, 24-26]. These retroviruses may cause cancer by random insertion of the proviral sequences in the host genome in a manner that would induce mutations in the regulatory sequences or in the cellular genes which allow for expression of a previously repressed gene. This process may also result in gene amplification or translocation of oncogenic sequences to unique active sites. Because the normal fibroblasts from Galliera rat strain do not express detectable levels of the endogenous rat virus and the SGS cells spontaneously release this virus, it is possible that the replicating SG-RaLV in this rat strain may have caused a rat cellular oncogene to be expressed, amplified or mutated in SG tumors. Although the presence of a small number of acute-transforming retroviruses containing an oncogene cannot be completely excluded, no morphological change was observed over a period

of 12 weeks in rat cells exogenously infected with SG-RaLV.

The clonal cell lines that show differences in growth rate and tumorigenic potential will be extremely useful in understanding various stages in tumor development and in correlating these properties to the oncogene expression. Experiments are now in progress to molecularly clone and identify the oncogene(s) involved in production of spontaneous SG tumor.

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