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FBJ Osteosarcoma Virus in Tissue Culture

III. Isolation and Characterization of Non-Virus-Producing FBJ-Transformed Cells

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Hamster and rat cell lines have been established that have been transformed by FBJ murine sarcoma virus (FBJ-MuSV) but that do not produce virus. The hamster cell line originated from an osteosarcoma that appeared in a hamster inoculated at birth with an extract of a CF#1 mouse FBJ-osteosarcoma. The rat cell line was obtained by transferring the FBJ-MuSV genome to normal rat kidney cells in the absence of the FBJ type C virus (FBJ-MuLV), which, usually in high concentration, accompanies the FBJ-MuSV. Both transformed hamster and rat cell lines contain the FBJ-MuSV genome, which can be rescued by ecotropic and xenotropic murine type C viruses. This rescued genome produces characteristic FBJ-MuSV foci in tissue culture and, in appropriate animal hosts, induces osteosarcomas typical of those induced by FBJ-MuSV. FBJ-MuSV was isolated originally from a parosteal osteosarcoma that occurred naturally in a mouse. Since there was no previous history of passage of the agent through any other animal species, these non-virus-producing hamster and rat cells transformed by FBJ-MuSV should be very helpful in molecular studies examining the origin of spontaneous sarcoma genomes in mice.

A naturally occurring murine sarcoma virus (MuSV) was isolated by Finkel et al. from an osteosarcoma that occurred spontaneously in the thoracic spine of a male CF#1 mouse (5). This sarcoma virus, named FBJ-MuSV, induces parosteal osteosarcomas in mice after as short a latent period as 3 weeks (4, 6). Accompanying FBJ-MuSV is another murine type C virus (MuLV), designated FBJ-MuLV. This virus resembles other MuLV by tissue culture studies, including the inability to produce foci of cell alteration in culture (12, 13). FBJ-MuLV has no known pathological role in mice (12, 13). Both FBJ viruses are neutralized by antiserum against AKR-MuLV and are N-tropic, since they preferentially infect N-type and not B-type mouse cells (12). In tissue culture, FBJ-MuSV produces foci of cell alteration that are distinctly different in both morphology and time of appearance (9 to 16 days) from foci induced by other strains of MuSV.

Although we suspect that FBJ-MuSV, like other MuSV, is defective for replication, this question has not yet been adequately answered. The one-hit kinetics of focus formation observed in tissue culture with the virus (12) can be explained by certain properties of the FBJ-MuSV preparations. First, FBJ-MuSV, in contrast to other MuSV, can transform secondary mouse embryo cells into autonomously replicating transformed cells. This fact was demonstrated by the inability of anti-FBJ-MuSV antiserum to suppress focus formation in tissue culture cells after virus inoculation (12). Second, the FBJ-MuLV is present in such great excess (13) that mouse cells can be easily infected by both FBJ viruses, so titration patterns of FBJ-MuSV cannot be readily evaluated.

The large amount of FBJ-MuLV accompanying FBJ-MuSV also makes it difficult to detect and quantitate FBJ-MuSV. Autoinhibition occurs (13): the associated FBJ-MuLV blocks infection of cells by FBJ-MuSV so that extracts and tissue culture fluids must be diluted to demonstrate the presence of FBJ-MuSV. Moreover, the high concentration of FBJ-MuLV has prevented the isolation of non-virus-producing FBJ-MuSV-transformed cell lines.

FBJ-MuSV can transform rat cells in culture but so far has not induced tumors in rats. In contrast, hamster embryo cells in tissue culture resist infection and replication of the virus (12), but some hamsters inoculated with preparations of FBJ-MuSV have developed osteosarcomas (4, 5). In this paper, we evaluate the role of FBJ-MuSV in the induction of these hamster osteo-

sarcomas by characterizing one of them established in tissue culture.

MATERIALS AND METHODS

Cell cultures. NIH Swiss and BALB/c mouse embryo (ME) cells were purchased from Microbiological Associates, Bethesda, Md. Hamster embryo cells were obtained from Syrian hamster/ANL fetuses. Human foreskin (HuF) cells, provided by Miriam Debby, University of California, San Francisco, were used up to passage 10, when their sensitivity to xenotropic virus diminished (11).

The clone 1 cell line (derived from the NZB-Q mouse cell line provided by Marilyn Lander, National Institutes of Health [NIH], Bethesda, Md.) has been described (14). It produces approximately 10^3 infectious xenotropic virus particles per ml. The C57L-D cell line (dog sarcoma cells chronically infected with the C57L xenotropic virus) was provided by P. Arnstein and J. Riggs, California Department of Health, Berkeley (1). It produces about 10^5 infectious xenotropic virus particles per ml. The normal rat kidney cell line, NRK (3), was originally obtained from R. Ting, Biotech Research Laboratories, Rockville, Md. A clone of these cells susceptible to infection by both ecotropic and xenotropic MuLV was used. A line of these NRK cells, nonproductively transformed by the Harvey strain of MuSV (the NRK-Harvey line), was established in our laboratory in San Francisco (9). We also established an NIH-Swiss-ME line infected with FBJ-MuLV by making seven successive clonings by end point dilutions in NIH-Swiss-ME cells. XC cells, a rat cell line transformed by but not producing Rous sarcoma virus, were provided by Wallace P. Rowe, National Institute of Allergy and Infectious Diseases (NIAID), NIH.

Media. All cell lines were grown in Eagle minimal essential medium supplemented with 10% fetal calf serum, 1% glutamine, and antibiotics (250 IU of penicillin per ml; 250 μg of streptomycin per ml). For focus formation assays, the fetal calf serum was replaced with 5% heated (56°C, 30 min) calf serum.

Viruses and virus assays. For virus assays, all cell lines were pretreated with DEAE-dextran to increase their sensitivity to infection (2). Ecotropic (mouse-tropic) MuLV was detected by the XC plaque assay (17); titers are expressed as PFU per milliliter. Xenotropic virus was measured by cocultivation assays as described previously (14). Briefly, fluids were titrated on HuF cells. Inoculated monolayers were passed weekly for 3 weeks, and the cells were then cocultivated with NRK-Harvey cells. The titer of xenotropic virus was estimated on the basis of pseudotype virus formation and represented the end dilution of the virus preparation that gave HuF the ability to produce MuSV pseudotypes. Focus formation assays for MuSV were conducted with mouse, human, and rat cells as described previously (14). Titers are expressed as focus-forming units (FFU) per milliliter. Stock FBJ virus preparations containing FBJ-MuSV and FBJ-MuLV were obtained from chronically infected NIH-Swiss-ME cell lines (12). The FBJ-MuSV preparation had a titer of approximately $10^{3.5}$ FFU/ml and 10^7 PFU/ml. FBJ-MuLV was separated from FBJ-MuSV by end point dilution in NIH-Swiss-ME cells and had a titer of $10^{7.3}$ PFU/ml. Rauscher-MuLV and AKR-L1-MuLV were originally obtained from J. Hartley, NIAID, NIH, and were passed in NIH-Swiss-ME cells. NIH-Swiss-ME cells chronically infected with either of these viruses produced approximately 10^6 PFU of Rauscher-MuLV per ml and $10^{6.3}$ PFU of AKR-MuLV per ml. Assays of mouse tumors were performed with 10% extracts as described previously (12). Osteosarcoma induction was assayed by the injection of virus-containing materials into newborn mice (4–6).

Antiserum and neutralization assays. The preparation of rabbit antiserum to the NZB strain of xenotropic virus has been reported (14). Antisera to AKR and Rauscher strains of ecotropic MuLV were obtained from J. Hartley. Neutralization tests were conducted as described earlier (14). Briefly, approximately 500 FFU of the various MuSV were mixed with 2 to 4 neutralizing units of antiserum for 30 to 45 min at room temperature. The mixtures were then diluted 10-fold and added to tissue culture cells. Neutralization was considered to have occurred when focus formation in the experimental cultures was one-third or less than that in the control cultures, which had received only virus.

RESULTS

Induction and isolation of tumors in hamsters. Nine 1-day-old Syrian hamsters received a 0.2-ml intra-abdominal injection of CF#1/An1 FBJ osteosarcoma extract. This preparation produced multiple osteosarcomas in CF#1/An1 mice within 2 to 3 months (Table 1A). The hamsters were examined for tumor development by frequent palpation and roentgenography. Five of the nine died from natural causes; the last death occurred 1,002 days after injection. The other four developed osteosarcomas by 127, 175, 815, and 854 days. Fragments of tumor tissue from the animal that was sacrificed at 127 days were transplanted under the dorsal skin of nine 15-day-old hamsters. After 28 days the transplant from one of these was used to establish the line of hamster tumor cells described below.

Cultivation of the hamster tumor cell line HT-FBJ. The hamster osteosarcoma described above was minced and plated in tissue culture dishes with Eagle minimal essential medium as described. Small islands of cells developed in 5 days, and within 2 weeks confluent monolayers of tumor cells could be identified. This cell line (HT-FBJ) was passed weekly. Three clones of tumor cells, A-6, D-9, and F-6, were derived from HT-FBJ by plating individual cells in microtest (Terasaki) dishes by techniques already described (14) in spite of the difficulties imposed by a plating efficiency of less than 5%.

Assays of hamster tumor cells for viruses. Supernatant fluids from the parental hamster cell line, HT-FBJ, and the three cell clones were assayed for focus-forming activity

TABLE 1. *Induction of osteosarcomas in mice with FBJ-MuSV preparations*

Virus	No. of recipient mice	Strain	Mean days to death (range)	Mean no. of osteosarcomas per mouse (range)	Mice with osteosarcoma (%)
A. FBJ-MuSV (FBJ-MuLV)[a] prepn no. 73-2	12	CF#1/An1	53.9 (41–91)	3.2 (2–5)	100
B. Pseudotype viruses					
FBJ-MuSV (Rauscher-MuLV)[b]	9	NIH-Swiss	31.1 (20–35)	2.7 (1–5)	100
	15	CF#1/An1	37.1 (33–56)	2.6 (1–4)	100
FBJ-MuSV (Rauscher-MuLV) passage 2[a]	6	NIH-Swiss	29.5 (29–30)	2.2 (1–4)	100
	5	CF#1/An1	29.4 (29–30)	3.6 (2–6)	100
FBJ-MuSV (AKR-MuLV)[b]	14	CF#1/An1	124.7 (37–238)	1.4 (1–3)	92

[a] Viruses were 0.45-μm-filtered, Hanks balanced salt solution extracts of tumor tissue.
[b] Viruses grown in tissue culture.

on mouse and rat cells. Monolayers of these cells were examined for ecotropic MuLV by the XC plaque assay (17). No infectious virus was detected. Culture fluids were tested for the presence of reverse transcriptase by techniques already described (16), and enzyme activity was not observed.

The parental cell line and the three clones were cocultivated with NIH-Swiss-ME and NRK cells, and these mixed cultures were passed weekly for 3 weeks. No infectious virus was detected in the supernatant fluids from these cultures.

Rescue of the MuSV genome from hamster tumor cells. The hamster tumor cell line HT-FBJ and the three clones derived from it were each cocultivated with NIH-Swiss-ME cells chronically infected with either Rauscher-MuLV, AKR-MuLV, or FBJ-MuLV. They were also cocultivated with the NZB-Q clone 1 line and the C57L-D line. Control cultures included the cell lines alone and cell lines cocultivated with non-virus-infected NIH-Swiss-ME cells. Focus-forming virus was present in the supernatant fluid of the cocultivation cultures whenever an MuLV was present in the added monolayer cells (see Table 2 for representative experiments). Supernatant fluids from cultures containing ecotropic virus induced foci in both mouse and rat cells, whereas fluids from cultures with murine xenotropic virus produced foci in rat and HuF cells but not in mouse cells. The foci in NRK cells were typical of those previously described for FBJ-MuSV and different from those induced by standard MuSV (12). The foci in HuF cultures could not be distinguished from those induced in these human cells by xenotropic pseudotypes of other MuSV (10). As reported for conventional FBJ-MuSV (12), foci did not appear until 9 to 16 days after virus inoculation.

TABLE 2. *Rescue of FBJ-MuSV genome from non-virus-producing transformed hamster cells*

Cell line	Cocultivated monolayer cell[a]	Foci/ml induced by coculture supernatant fluid in cells from: Mice (NIH-Swiss-ME)	Rats (NRK)
HT-FBJ	NIH-Swiss-ME (Rauscher-MuLV)	40	120
	C57L-D[b]	—[c]	10
	NIH-Swiss-ME (FBJ-MuLV)	30	15
	NIH-Swiss-ME	—	—
Cell clones of HT-FBJ			
A-6	NIH-Swiss-ME (Rauscher MuLV)	200	>500
	C57L-D	—	10
	NIH-Swiss-ME	—	—
D-9	NIH-Swiss-ME (Rauscher-MuLV)	70	NT[d]
F-6	NIH-Swiss-ME (FBJ-MuLV)	30	10
	NIH-Swiss-ME (Rauscher-MuLV)	30	100
	C57L-D	—	20
	NIH-Swiss-ME	—	—

[a] The virus present in the cell lines used for cocultivation is given in parentheses.
[b] A dog cell line releasing the C57L xenotropic virus.
[c] —, No detectable virus.
[d] NT, Not tested.

The parental cell line (HT-FBJ) after cocultivation yielded up to 3 logs of focus-forming virus per ml, as detected in NRK cells. The clones, however, varied in their ability to express the viral genome. A-6 produced up to 3 logs of

focus-forming virus, D-9 yielded only about 1 log, and F-6 produced very few focus-forming particles. The reason for this difference among the clones is not known. The parental cell line, when cocultivated with NIH-Swiss-ME cells chronically infected with the cloned FBJ-MuLV, yielded a virus preparation that titrated $10^{2.8}$ FFU/ml in NRK cells and $10^{5.3}$ PFU/ml in NIH-Swiss-ME cultures. Cocultivation supernatant fluids containing focus-forming virus did not induce foci in hamster embryo cells.

Cultivation of rescued FBJ-MuSV viruses. Some mouse and rat cell cultures that had been infected by an ecotropic MuLV pseudotype of FBJ-MuSV became completely transformed after three passages. Preparations of viruses were obtained that titrated as high as $10^{3.5}$ FFU/ml and $10^{6.0}$ PFU/ml, as detected in mouse and rat cells. The various helper viruses were present at levels somewhat lower than the vast excess found in standard FBJ preparations.

HuF cells infected with the NZB and C57L xenotropic virus pseudotypes of FBJ-MuSV were similarly passed until they appeared to be completely transformed. Preparations of the xenotropic FBJ-MuSV titrated around $10^{2.0}$ FFU/ml in both HuF and NRK cells.

Neutralization of virus isolates. The focus-forming viruses rescued by Rauscher-MuLV, AKR-MuLV, FBJ-MuLV, and xenotropic virus were examined by neutralization tests in tissue culture to determine their envelope coat properties. As shown in Table 3, each of the viruses was appropriately neutralized by antiserum against the respective helper MuLV.

Induction of osteosarcomas in mice. Different preparations of the ecotropic virus pseudotypes of FBJ-MuSV were injected into newborn NIH Swiss and CF#1/An1 mice. The results of these studies are shown in Table 1B. A total of 48 of the 49 mice inoculated with Rauscher or the AKR MuLV pseudotype of FBJ-MuSV developed at least one osteosarcoma. All tumors were typical of FBJ osteosarcomas, regardless of the pseudotype virus used.

Assay of virus from osteosarcomas induced in mice by the pseudotype FBJ viruses. Extracts of the tumors induced in the mice by the tissue culture-grown ecotropic virus pseudotypes of FBJ-MuSV were assayed for focus-forming activity in tissue culture. In all cases, foci typical of FBJ-MuSV were induced. These foci could be neutralized by appropriate antiserum against the original helper virus that formed the pseudotype MuSV (Table 3).

Isolation of non-virus-producing rat cells. In attempts to transfer the FBJ-MuSV genome to rat cells, a xenotropic virus pseudotype of FBJ-MuSV was titrated on NRK cell monolayers. Those cultures with only one or two foci were maintained, and the foci were isolated with a Pasteur pipette. This technique yielded two NRK-FBJ cell lines of transformed cells that did not release any focus-forming virus into the supernatant fluid. Upon superinfection by ecotropic MuLV or xenotropic MuLV, focus-forming virus with the envelope coat property of the helper MuLV was produced. These NRK cell lines were subsequently cloned in Terasaki dishes, and cell lines from 10 individual cells were established. These 10 lines had properties similar to the parental non-virus-producing NRK cell lines.

TABLE 3. *Neutralization of MuLV pseudotypes of FBJ-MuSV*

FBJ-MuSV pseudotype + antisera	FFU/ml in NRK cells
FBJ (Rauscher-MuLV)[a]	42
+ Anti-Rauscher-MuLV	0
+ Anti-AKR-MuLV	40
FBJ (NZB-MuLV)[a]	21
+ Anti-Rauscher-MuLV	22
+ Anti-AKR-MuLV	20
+ Anti-NZB-MuLV	0
FBJ (Rauscher-MuLV)[b]	80
+ Anti-Rauscher-MuLV	3
+ Anti-AKR-MuLV	100

[a] Virus was grown in tissue culture.
[b] Virus was obtained from osteosarcoma extracts.

Virus induction by IUdR. The parental hamster cell line, HT-FBJ, was cultivated in the presence of 30 μg of iododeoxyuridine (IUdR) per ml by standard techniques (15) in attempts to induce an FBJ-MuSV in a hamster type C virus coat. Supernatant fluids from the IUdR-treated cultures were added to NIH-Swiss-ME, NRK, and hamster embryo cell cultures. No foci of cell alteration were noted. However, similar experiments with the transformed NRK nonproducer cell lines yielded low titers of a virus that induced foci only in NRK cells. Presumably, this virus is a rat type C virus pseudotype of FBJ-MuSV.

DISCUSSION

This paper describes the viral characteristics of an osteosarcoma that developed in a hamster inoculated with FBJ-MuSV. The data indicate that the cell line, HT-FBJ, cultivated from this tumor contains the FBJ-MuSV genome and strongly implicate this virus as the etiological agent in these neoplasms. Tumor induction in hamsters by sarcoma viruses has been a very

successful technique for generating non-virus-producing cells (7, 8). The HT-FBJ cell line established in culture and the clones derived from it have all of the characteristics of other non-virus-producing tumor cell lines, such as the HT-1 (8) and NRK-Harvey (9) lines. They do not release any infectious virus as detected by biological and biochemical techniques. The sarcoma genome, however, is rescuable when the cells are cocultivated with cells producing either an ecotropic or xenotropic MuLV. The demonstration that the FBJ-MuSV genome had been rescued was supported by the consistent production by pseudotype FBJ-MuSV of (i) foci typical of standard FBJ-MuSV in tissue culture and (ii) osteosarcomas in mice. These data indicate the stability of the FBJ-MuSV genome even after integration and subsequent rescue from heterologous host cells.

By cocultivating the HT-FBJ line with cells producing xenotropic MuLV, a xenotropic virus pseudotype of FBJ-MuSV was formed that readily permitted the transfer of the sarcoma genome to NRK cells. From these cells, two non-virus-producing FBJ-MuSV-transformed NRK cell lines were derived. These cell lines, NRK-FBJ, and their clones have all of the biological characteristics of HT-FBJ. Moreover, NRK-FBJ cells can be induced with IUdR to produce an infectious rat-tropic FBJ-MuSV that presumably carries the rat type C virus envelope.

Although the fact that hamster and rat cells fail to produce FBJ-MuSV suggests that the virus is defective for replication, as are other MuSV, this question will not be completely resolved until non-virus-producing FBJ-MuLV-transformed mouse cell lines are obtained. Competent Rous sarcoma viruses, for instance, do not replicate in mammalian cells (8). These non-virus-producing FBJ cell lines will permit an examination of the FBJ-MuSV genome free of the high concentration of FBJ-MuLV, which generally accompanies standard FBJ-MuSV preparations.

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