Neoplastic Transformation and Derivation of a Focus-Forming Sarcoma Virus in Cultures of Rat Embryo Cells Infected with a Murine Osteosarcoma (FBJ) Virus[1] (34373)

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An osteosarcoma-inducing virus—FBJ virus—was recently isolated by Finkel *et al.* (1) from a bone tumor which occurred spontaneously in a CF-1 mouse. Huebner *et al.* (2) subsequently confirmed these findings, and concentrated and purified the virus so that it produced bone sarcoma locally within several weeks after intramuscular injection into newborn CF-1 and NIH Swiss mice. The FBJ virus has also been shown to be serologically identical to "C"-type RNA virus of the Gross serotype, which has been found to be highly prevalent in normal CF-1 mice (2). Unlike other mouse sarcoma viruses (MSV), but like the C-type RNA virus isolated from spleens of normal CF-1 mice, the FBJ virus was found (2) to replicate, but not to produce foci in NIH mouse embryo tissue culture (NIH-METC).

The usefulness of rat embryo (RE) cells for the complement-fixation test for murine leukemia viruses, or COMUL test (3) and for assay of MSV (4) has been described. In this paper, we report studies of the FBJ osteosarcoma virus in rat embryo cell cultures. As reported herein, the FBJ virus produced no transformations in primary rat embryo cultures, nor did it produce cytopathic effects during 25 subcultures. Morphological changes were, however, observed at 4 months, after nine subcultures. Subsequently, whole-cell preparations were inoculated into NIH Swiss mice and induced fibrosarcomas which yielded a fibrosarcoma virus, which in turn induced foci in NIH-METC, as well as fibrosarcomas in mice.

Materials and Methods. Virus. A CF-1 mouse tumor bearing Finkel's osteosarcoma (A82 19, FBJ-6) was obtained from Dr. M. P. Finkel, Argonne National Laboratory, Argonne, Illinois. This tumor was transplanted once in newborn CF-1 mice. A Moloney procedure concentrate of the CF-1 tumor, injected into NIH Swiss mice, induced osteosarcomas which were then transplanted into NIH mice. Moloney procedure concentrate was prepared as seed virus for the experiments described below.

Cell cultures and media. Primary cultures of Fisher rat embryo and of NIH-METC were prepared as previously described (6), or were obtained from Microbiological Associates, Inc., Bethesda, Maryland. Growth medium was 10% fetal bovine serum in Eagle's minimum essential medium (10% FBS-EMEM) with 2 m*M* glutamine and 100 units of penicillin and 100 μg streptomycin per ml.

Establishment of rat embryo cell cultures. Approximately 40 ml of primary rat embryo cell suspension (1×10^6 cells/ml) in growth medium was planted in two "B" flasks (approximately 50 sq cm surface area each) and incubated at 37° under 5% CO_2 in air. The next day they were infected with 1.0 ml of undiluted virus per bottle. By day 5 after planting, a complete monolayer was obtained. Twenty days after inoculation, one of the cultures was subdivided by trypsin treatment. This was repeated every 1 to 2 weeks

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thereafter, except for two intervals of several weeks. The medium was renewed at 3- to 4-day intervals. Uninoculated cultures were established and grown the same way.

Tests for infectious virus. Rat cell lines were tested for infectious murine leukemia virus by the COMUL (6) test, and for MSV by a focus-forming (7) test. To insure that supernatant fluids from infected rat embryo cells were cell-free, they were filtered through HA (0.45 μ) Millipore filters.

Cell pack preparation of complement-fixing (CF) antigen from infected and normal rat embryo cells was done as previously described (8).

Complement fixation. CF tests were carried out in the microtiter technic described for tumor antigen studies (9). Titers were recorded as reciprocals of the highest dilution giving 3+ to 4+ fixation of 1.8 units of complement.

Antiviral sera. Rat antisera used in the CF test were obtained from Fisher rats carrying transplanted sarcomas induced by the Moloney strain of murine sarcoma virus (M-MSV) (10).

Staining. Acridine orange staining techniques used in this study have been described in detail (11).

Animals. Newborn NIH mice and Fisher rats were used to determine the oncogenicity of the infected cells. Newborn mice were inoculated intrasacrally and intramuscularly or subcutaneously. Rats received the same amounts subcutaneously or intrasacrally.

Portions of tumor were homogenized with a Waring Blendor and processed according to a modification of the Moloney procedure (5). Organs other than tumors were triturated in a chilled mortar, sonicated, and made up to a 10–20% suspension (based on packed-cell volume) in cold culture medium.

Neutralization tests. These were performed in mice with homologous mouse and MSV and AKR rat serum. Equal volumes of undiluted virus and diluted inactivated serum were mixed. Serum–virus mixtures and control virus preparations were held at room temperature for 30 min before inoculation of 0.05 amounts into newborn NIH Swiss mice.

Results and Discussion. Morphological alteration. During the first 4 months after inoculation, no differences were noted between the inoculated and the uninoculated rat embryo cultures. Both contained regularly growing, smooth monolayers of fibroblastic cells with occasional multilayered areas caused by local retractions of the cell sheet. An abnormal pattern of growth was first noted in the ninth subculture of the infected cultures. A few altered cell foci were found (Fig. 1). These foci were principally composed of polygonal and spindle-shaped cells which stained vividly with acridine orange. There were far fewer round cells than are found in foci induced by MSV (4, 7). After a few additional subcultures, the transformed cells replaced the original fibroblastic monolayers. The uninfected control cell cultures remained entirely fibroblastic in appearance. Subsquently, cell lines were obtained of both

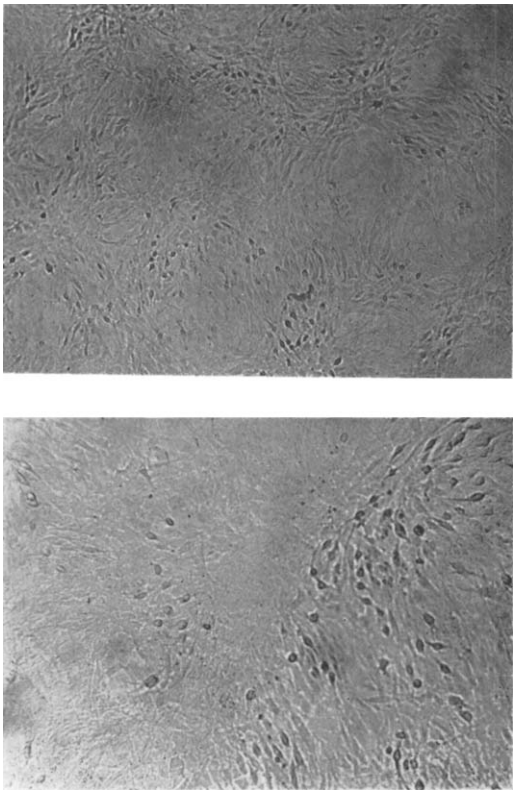

FIG. 1. Foci of transformed rat embryo cells at the ninth subculture (×40 and 70).

TABLE I. Presence of CF Antigens and Virus in FBJ-Infected Rat Embryo Cell Line.

Subculture	Cumulative no. of days	CF titers vs MSV rat serum	Virus recovery (COMuL test)[a]		
			NIH-METC	RETC	HETC
1	20	1:4	—	—	—
2	56	1:8	—	—	—
3	84	1:8	—	—	—
4	95	>8	+	+	—
8	118	1:8	+	+	0
10	132	>8	+	0	0
11	143	>16	+	0	—
12	159	>16	+	+	0
14	182	>16	+	+	—
15	191	>16	—	—	—
17	211	>16	—	—	—
20	237	>4	—	—	—
25	273	>4[b]	+	—	—

[a] — = not done; + = CF titer 1:2 or more; 0 = CF titer <2; NIH-METC = NIH mouse embryo tissue culture; RETC = rat embryo tissue culture; HETC = hamster embryo tissue culture.

[b] Moloney concentrate.

FBJ-infected rat embryo cells, and of the control. At the present time, they have been carried through more than 25 cell transfer passages, during a period of 9 months.

Presence of complement-fixing antigens of murine leukemia–sarcoma virus in the FBJ-infected rat cell line. The group-specific murine leukemia–sarcoma virus CF antigen was demonstrated in cell pack preparations of all the subcultures tested. As shown in Table I, a CF antigen titer of 1:4 was obtained in the primary culture after 20 days' incubation. The CF titers appeared to increase in proportion to increasing subculture levels. CF antigen was also demonstrated in the Moloney procedure extract of the 25th subculture of infected cells. No positive reactions were found in similar preparations of the normal, uninfected cells.

Presence of infectious virus in the infected rat embryo cell line. The COMUL test and focus-forming tests for the presence of infectious virus were performed in mouse, rat, and hamster embryo cells. The results indicate that CF antigen was induced in all the NIH-METC within 3 weeks after exposure to the supernatant fluids of the infected cultures. Some of the RETC also demonstrated the presence of CF antigen, but none of the HETC tested (Table I). Foci were not noted in any of these cultures during the 3-week observation period.

Oncogenicity of infected cells. The results are shown in Table II. Newborn mice inoculated intrasacrally and subcutaneously developed tumors at the site of inoculation very quickly, after an average latent period of 18–33 days; although tumors were not observed in all the mice inoculated. The mouse tumors were transmissible by cell-free extracts in newborn mice, where they have now reached the fourth passage.

Tumors also developed, after 40–49 days, in the newborn rats inoculated subcutaneously. In rats inoculated by the intrasacral route with cells from the 10th subculture level, however, tumors were not observed. An uninfected rat embryo cell line prepared at the 10th and 11th subculture levels produced no tumors in animals inoculated with it, nor did a Moloney-procedure extract of the 25th subculture of FBJ-infected rat embryo culture.

Characteristics of tumors. The characteristics of mouse and rat tumors induced by rat embryo cells infected with FBJ osteosarcoma virus are shown in Table III.

Pathology. The mouse tumors showed considerable histologic variation from region to

TABLE II. Production and Passages of Tumors from an FBJ-Infected Rat Cell Line in Newborn Animals.

Expt. no.	Tumor passage no.	Animal	Inoculum (per animal)	Route of inoculation[a]	No. with tumor/no. inoculated[b]	Day tumor first noted
I[c]	Primary	NIH mice	3.3×10^7 cells	IS	15/17	18
	1	NIH mice	0.05 ml Moloney extr.	IS	11/16	29
	2	NIH mice	0.05 ml Moloney extr.	IM and IS	9/13	20
	3	NIH mice	0.05 ml Moloney extr.	IM and IS	10/18	19
	4	NIH mice	0.05 ml Moloney extr.	IM and IS	25/34	20
	Primary	Fisher rat	3.3×10^7 cells	IS	0/15(102)	
II[d]	Primary	NIH mice	3.3×10^7 cells	SQ	0/4 (88)	
	Primary	Fisher rat	3.3×10^7 cells	SQ	2/4	49
III[e]	Primary	NIH mice	3.9×10^6 cells	SQ	4/9	27
	Primary	Fisher rat	3.9×10^6 cells	SQ	1/8	40
IV[f]		NIH mice	0.1 ml Moloney extr.	SQ	0/9 (101)	
		Fisher rat	0.2 ml Moloney extr.	SQ	0/5 (101)	

[a] IS, intrasacral; IM, intramuscular; SQ, subcutaneous.
[b] Numbers in parentheses represent days under observation.
[c] Inoculum from 10th subculture level.
[d] Inoculum from 11th subculture level.
[e] Inoculum from 18th subculture level.
[f] Inoculum from 25th subculture level.

region, and with tumor passage levels. Histologic types included osteoblastic sarcoma, osteogenic sarcoma, and fibrosarcoma with osteoblasts and chondrocytes.

The first cell-free passage tumor from primary mouse tumor induced by the infected

TABLE III. Presence of CF Antigen and Virus in Tumors and Organs of Mice and Rats Inoculated with FBJ Osteosarcoma Virus-Infected Rat Embryo Cells.

Tumors	Passage	Pathology finding	T-Number	Specimen	CF titers	Foci	Virus recovery (COMuL test)[a]	
						METC	METC	RETC
Mouse	Primary	N.A.[b]	T 19723	Mol. tumor[c]	1:16	No	+	+
			T 19723A	20% tumor[d]	1:8	No	+	0
			T 19724	20% spleen	1:16	No	+	0
			T 19725	20% thymus	1:8	No	+	0
	1	Osteosarcoma	T 20713	Mol. tumor	1:16	No	+	0
			T 20713A	20% tumor	1:16	No	+	0
			T 20714	20% spleen	1:16	No	+	0
			T 20715	20% thymus	1:8	No	+	0
	2	Fibrosarcoma	T 21525	Mol. tumor	1:8	Yes	+	0
	3	Fibrosarcoma	T 21980	Mol. tumor	>4	Yes	+	0
Rat	Primary	Undifferentiated sarcoma	T 21230	10% tumor	1:16	No	+	0
			T 21231	10% spleen	1:8	No	0	0
			T 21232	10% thymus	1:4	No	0	0

[a] —, not done; +, CF titer >4; 0, CF titer <4.
[b] N.A. = not available; specimens not satisfactory.
[c] Moloney concentrates.
[d] Clarified extracts.

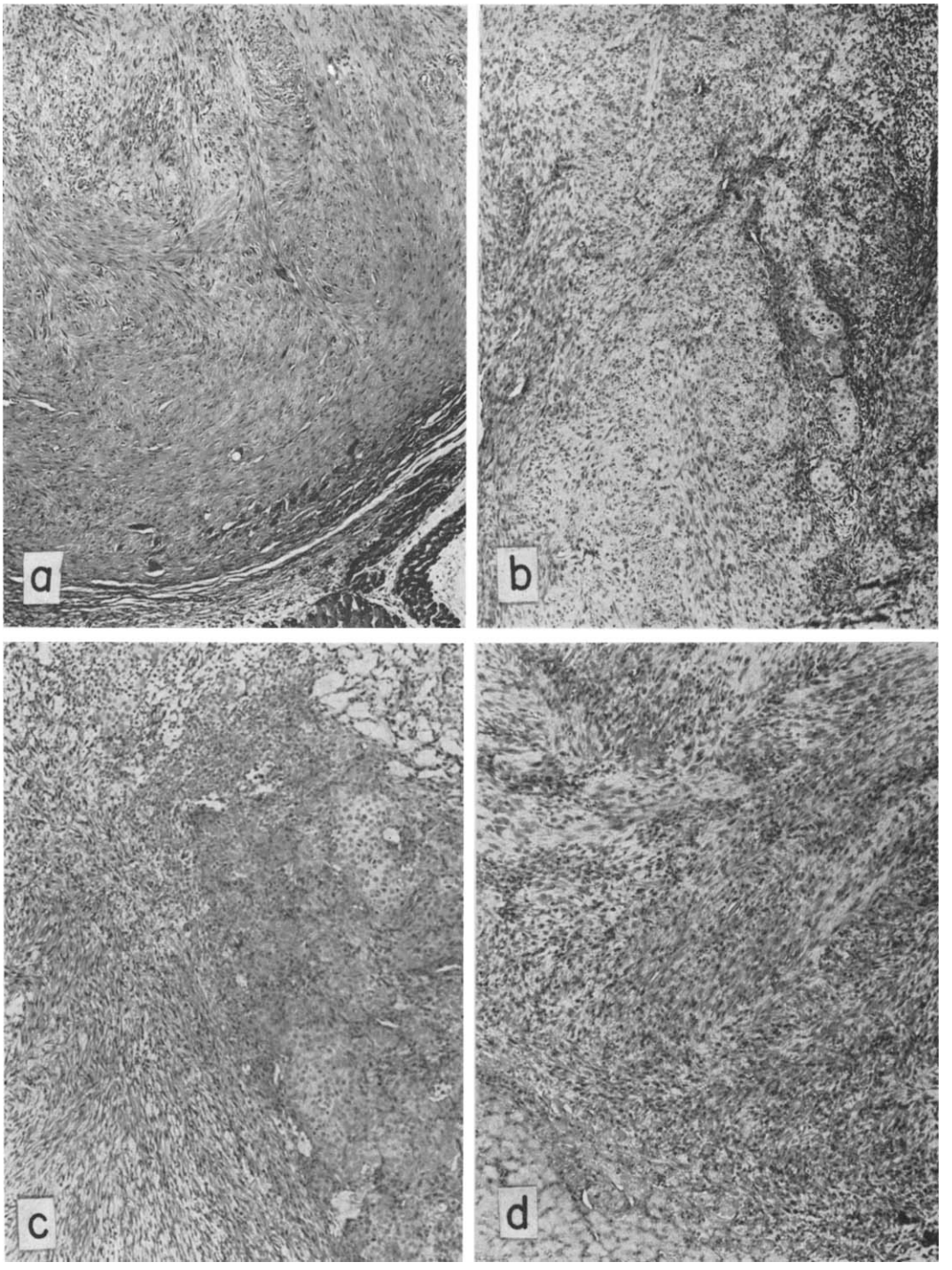

FIG. 2. Mouse tumors induced by FBJ-transformed rat embryo cells. (a) Sarcoma with elongated, spindle-shaped, fibroblastic cells and cells that resemble osteoblasts (×40). Growth induced by the first cell-free passage tumor from primary mouse tumor. (b) Fibrosarcoma with nests of osteoblast-like cells (×40), induced by the second cell-free passage tumor. (c) Fibrosarcoma with numerous nests of chondrocytes (×40), induced by the third cell free passage tumor. (d) Osteogenic sarcoma with fibroblastic and collagenous tissue and one area with a number of bone spicules (×40), also induced by the third passage.

rat embryo culture was a sarcoma consisting of interlacing bands of elongated, spindle-shaped fibroblastic cells and numerous nests of cells that resembled osteoblasts. No bone was present (Fig. 2a). The second-passage tumor was a fibrosarcoma with nests of cells suggestive of osteoblasts. The tumor consisted of parallel bands and whorls of elongated, spindle-shaped cells with elongated ovoid nuclei, and usually small central nucleoli, in an abundant fibrillar matrix. In one portion of the growth, there was a heavy deposit of collagen, throughout which were small cells that resembled osteoblasts; and in one portion there was primitive cartilage (Fig. 2b). The third-passage tumors were fibrosarcomas with numerous nests of chondrocytes and osteoblasts (Fig. 2c). Here there was no frank bone or cartilage. There was a severe hyperplasia of the sacral bones, indicating a healing process after damage to the bone. There was also an osteogenic sarcoma in the third cell-free passage of mouse tumors, consisting of bands and whorls of alternating fibroblastic and collagenous tissues, and one area with a number of bone spicules (Fig. 2d).

A rat tumor induced by the infected rat embryo cultures was an undifferentiated sarcoma. There were pleomorphic cells in whorl patterns forming a thick periphery around a central core of degenerated cells. Many of the cells were ovoid to elongated, with eccentrically located nuclei, and vesicular cytoplasm suggestive of cells typically seen in tumors induced by M-MSV in rats; and there were also some multinucleated giant cells. No bone or cartilage was observed.

Presence of CF antigens and virus in tumors. Complement-fixing antigens were demonstrated in all the Moloney and 20% extracts of mouse and rat tumors; antigen was also present in the spleens and thymuses of tumor-bearing mice and rats. In the case of the tumor-bearing mice, the C-type RNA leukemia-like virus was regularly demonstrated in tumors, spleens, and thymuses in the COMUL test, using NIH-METC. In one at tempt to isolate virus from the tumor-bearing rats, the virus was isolated from the tumor, but not from the spleen or thymus (Table III).

Altered foci (Fig. 3) similar to those originally observed in the infected rat embryo

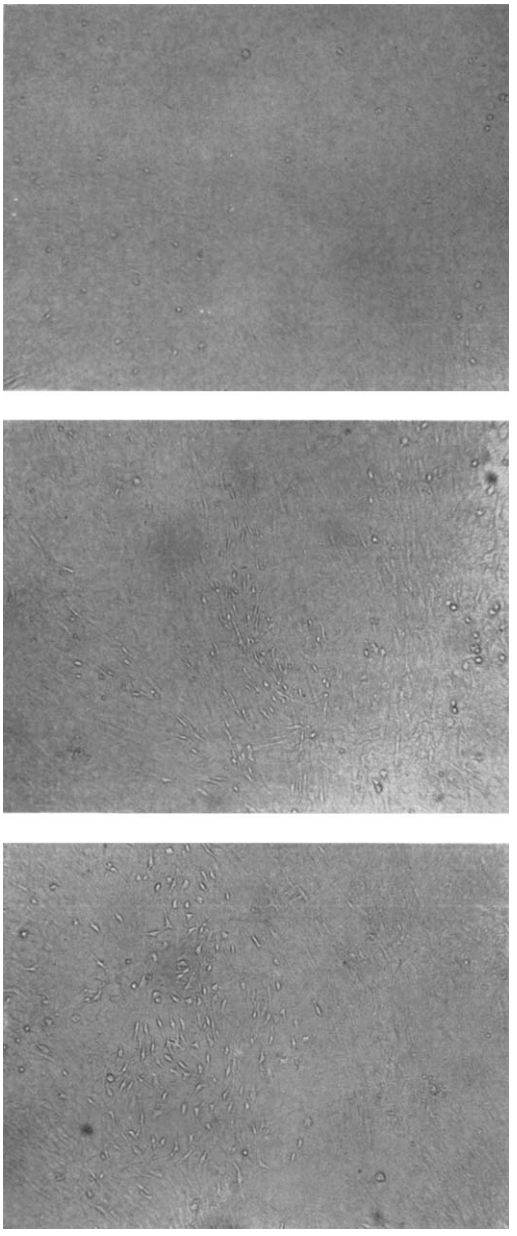

FIG. 3. Altered foci in NIH–Swiss mouse embryo tissue cultures inoculated with cell-free extracts of mouse tumors (passage 2) induced by FBJ-transformed rat embryo cells (×40) (middle and bottom). Uninoculatd NIH–Swiss mouse embryo tissue cultures (upper).

TABLE IV. *In Vivo* Neutralization of FBJ Virus (T-21980)[a] by Various Antisera.

Antiserum used	Dilution	No. with tumor /no. inoculated	Serum titer[b]
Medium only	1:1	6/14	
Normal NIH mouse serum (77841)	1:10	11/16	<10
Anti-FBJ mouse serum (93630)	1:10	0/16	>10
Normal Fisher rat serum (79315)	1:40	8/16	<40
MSV Fisher rat serum (18996)	1:40	10/16	<40
	1:80	6/16	
AKR Fisher rat serum (Pool 5)	1:40	0/16	
	1:80	1/16	>80

[a] An FBJ mouse sarcoma virus (3rd cell-free Moloney mouse tumor extract, T-21980) derived from FBJ-transformed rat embryo cell-induced mouse tumor.

[b] Titer shown as reciprocal of serum titer resulting in 70% reduction of tumors.

cultures, have been found in NIH-METC inoculated with a Moloney extract of mouse tumors from the second and third cell-free Moloney tumor passages (Fig. 3). These foci, however, differed in morphology, and appeared significantly later (7–14 days) than those induced in NIH-METC by other MSV (7). FBJ foci were also too diffuse for precise counting.

Neutralization tests. Table IV gives the results of the *in vivo* neutralization test in the case of one FBJ mouse sarcoma virus (third cell-free Moloney mouse tumor extract, T-21980) derived from FBJ-transformed rat embryo cell-induced mouse tumor. The mouse tumor was inhibited by homologous serum from FBJ-tumor-bearing mice, and by AKR rat serum, but not by MSV rat serum. However, as is true with other strains of MSV, the sera from the tumor-bearing mice were negative for homologous and MSV CF antibodies. These data confirm the original observation by Huebner *et al.* (2) that FBJ belongs to the Gross-AKR subgroup.

Further characterization and identification of *in vitro* FBJ-focus-forming virus is under way. We have also recently established FBJ-transformed NIH-METC cells in continuous cultures, and have now induced tumors in mice using cell-free supernatant fluid of this transformed culture. Detailed results will be published later.

The findings of this report suggest that rat embryo cells may favor the selection of sarcoma-inducing virus particles, a possibility also implied by the emergence of sarcoma-inducing viruses in the rat-passage experiments of Harvey (12) and Kirsten (13). The naturally occurring FBJ osteosarcoma virus, like the avian osteosarcoma and fibrosarcoma virus described by Rous (14) suggests that osteosarcoma and related muscle and fibrous tissue sarcomas can occur independently as competent viruses. This is important, because it indicates that direct attempts should be made to isolate such viruses in *in vitro* tissue culture systems, including rat cells as well as a variety of mouse cells.

Summary. Neoplastic transformation of rat embryo cells *in vitro* by an osteosarcoma (FBJ) virus is reported. Foci of transformed cells consisted largely of spindle-shaped cells which stained vividly with acridine orange. The transformed cells produced virus and complement-fixing (CF) antigen characteristic of the murine leukemia–sarcoma virus complex. Tumors were produced when transformed cells were injected into newborn mice and rats. The mouse tumors had the histologic pattern of osteosarcoma, which was transmissible in cell-free passages in newborn mice. Cell-free extracts of the mouse tumors also produced transformed foci *in vitro*. The rat rumors were undifferentiated sarcomas, also yielding virus and CF antigens.

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