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In vitro Properties of FBR Murine Osteosarcoma Virus (40650)

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We have previously reported the isolation of two murine osteosarcoma viruses, both having Type C morphology. FBJ virus was derived from a spontaneous osteosarcoma occurring in a CF No. 1 mouse (1) and FBR virus from a ^{90}Sr-induced osteosarcoma in an X/Gf mouse (2, 3). Since their isolation, FBJ-MuSV[2] and FBR-MuSV have been maintained by continuous cell-free passage in their respective host strains. Currently at their 102nd and 67th passages, respectively, both viruses are potent inducers of parosteal osteosarcomas. Intraperitoneal injection of either virus into newborns of its respective host strain results in tumors in as early as 3 weeks with a final incidence of 100%. These two viruses are biologically distinct: Whereas each is highly tumorigenic in its own strain, FBJ-MuSV is poorly tumorigenic in X/Gf mice and FBR-MuSV is poorly tumorigenic in CF No. 1 mice (2, 3).

FBJ and FBR, together with the Gazdar (4) and BALB-MuSV (5) comprise the few natural MuSV isolates. Other MuSVs were recovered from sarcomas resulting from the injection of leukemia virus into mice (6, 7) and rats (8, 9).

The specific osteosarcomagenic property of FBJ-MuSV and FBR-MuSV is unique among murine sarcoma viruses as none of the other isolates induces bone tumors in mice (10). However, the Moloney isolate can induce osteosarcomas if injected directly into the bone marrow of rats (11–13).

In this report we describe the properties and propagation of the FBR isolate in tissue culture. Some of the biological properties of the FBJ isolate in tissue culture have been reported earlier (14–16).

Materials and methods. Viruses. A pool of FBR virus (Pool No. 77-1) was prepared from 10 g of osteosarcomas that had been induced by tumor-derived FBR virus injected into newborn X/Gf mice (Animal Passage No. 54). The tumors were homogenized (MicroMill, Chemical Rubber Company, Cleveland, Ohio) and extracted with 100 ml of Hank's balanced salt solution (GIBCO). The extract was clarified (International High-Speed refrigerated centrifuge, rotor No. 856, 9000 rpm, 10 min), filtered (Millipore, Millex 0.45 μm), and stored in liquid nitrogen in 1-ml aliquots in sealed glass ampules. The tumorigenicity of Virus Pool No. 77-1 was tested by injecting 0.1 ml of the preparation into the abdominal cavity of X/Gf newborns. The undiluted pool produced multiple osteosarcomas in all 12 recipients in 25–28 days. A 1:5 dilution produced multiple osteosarcomas in all 14 animals in 32–57 days.

Virus from tissue culture sources was obtained from 1-day-old spent media collected from confluent FBR-MuSV(MuAV)-infected cultures. The media were clarified (clinical table centrifuge) and filtered (Millipore, Millex, 0.45 μm).

Cells. Established cell lines were obtained from the following sources: Dr. R. H. Bassin (National Cancer Institute), D152, D55, Sc-1, NIH/3T3; Dr. P. Fischinger (National Cancer Institute), XC, BALB/c-3T3; Dr. G. Todaro (National Cancer Institute), E36, 64, 8155, 88; Dr. J. Stephenson (National Cancer Institute), NRK, sheep (SL). Cell lines BC352, A5700, BE849, AP737, AM237, and A0134 were supplied by the Naval Biomedical Research Laboratory, Oakland, California, under a contract from the Division of Cancer Cause and Prevention, National Cancer Institute.

Secondary whole mouse embryo cultures were derived from mouse embryo primary

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[2] Abbreviations used: MuSV, murine sarcoma virus; MuAV, murine-associated virus; ffu, focus-forming unit; pfu, plaque-forming unit.

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cultures of NIH Swiss, C57BL/6, and BALB/c mice from the colonies maintained at Argonne National Laboratory.

All cultures were grown in plastic dishes or flasks (Falcon Plastics) and kept in 37° humidified CO_2 incubators in McCoy's 5A medium (GIBCO) supplemented with 100 μg/ml streptomycin and 100 units/ml penicillin, 1% glutamine, and 10% heat-inactivated fetal calf serum (56°, 30 min). Media were changed every 2–3 days and cultures were subcultured with trypsin every 5–7 days. Other supplements used in some experiments were: tryptose phosphate broth (GIBCO), polybrene (Aldrich), and dexamethasone (Sigma).

Focus assay. Transformation assays for sarcoma viruses (17) were routinely carried out on D55 indicator cells; exceptions in some experiments are specified. Falcon plastic gridded petri dishes (60 mm) were seeded with 10^5 cells per dish. Twenty to twenty-four hours later, the cultures were treated with 2 ml of medium containing 25 μg/ml DEAE-dextran (Pharmacia) without serum. After a 20-min incubation, the dishes were drained of medium and the cells were washed with medium containing no serum. Duplicate dishes were then infected with 0.2 ml serial 10-fold dilutions of virus inocula (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}). After 1 hr incubation, 4 ml of complete medium was added. All cultures were fed fresh media every 2–3 days. Foci of transformed cells were counted under an inverted light microscope on Days 10–14 postinfection. Titers were given in focus-forming units/ml (ffu/ml) of undiluted virus inoculum.

Plaque assay. Standard XC plaque assays (18) for associated nontransforming viruses were routinely carried out on duplicate D55 cultures which were infected as described above except with a wider range of virus dilutions. On Day 6 postinfection, all cultures were irradiated with ultraviolet light (G. E. Germicidal Lamp, 1500 ergs/mm^2) and overlaid with 10^6 XC cells per dish. When the XC cells reached confluency in 3–4 days, the mixed cultures were fixed and stained with a solution of 1% methylene blue in methanol, 100% methanol, and 1% carbol fuchsin in methanol in a ratio of 3:2:1, respectively. Infectious centers were counted under a dissecting microscope and expressed as pfu/ml of undiluted virus inoculum.

Polymerase assay. Various cell cultures were tested for virus by carrying out reverse transcriptase assays on high-speed pellets (1 hr at 100,000 *g*) obtained from preclarified media (10 min at 10,000 *g*). After draining, each pellet was resuspended in 120 μl of preincubation buffer (0.01 *M* Tris, pH 8.2, 0.01 m*M* dithiothreitol, and 0.33% nonionic detergent NP40), and incubated for 10 min on ice. A 50-μl aliquot of the suspension was assayed with 50 μl of reaction mixture containing a final 100-μl reaction volume: 0.05 *M* Tris, pH 8.2, 0.01 *M* NaCl, 2 m*M* dithiothreitol, 50 μM [^{3}H]dGTP (ICN, diluted to 4 Ci/mmole), 25 μg/ml oligo(dG)$_{8-12}$:poly(rC) (P. L. Biochemicals). For control, a matched 50-μl aliquot of the resuspended pellet was incubated with an equal volume of the same reaction mixture but without oligo(dG)$_{8-12}$:poly(rC). After incubation at 37° for 1 hr, both test and control reactions were terminated with 20% cold trichloroacetic acid. Acid-precipitable products were collected on glass fiber circles (Whatman GFC), dried under an infrared lamp, and assayed in a liquid scintillation counter. A sample was considered positive when the tritium incorporation of the test assay was more than twice that of the control assay.

Cell cloning. Sc-1 cells, chronically infected with FBR virus from Pool No. 77-1, were cloned by an endpoint dilution technique in microwell plates (Tissue Culture Cluster 96, Costar). Of 30 wells that were seeded, 11 had no cells, 14 had single cells, and 5 had more than one cell. Clones 1–5 were derived from the single-cell wells.

Results. Host range studies. Sixteen normal cell lines derived from a wide range of animal species were inoculated with serial 10-fold dilutions of FBR virus, Pool No. 77-1. Infectivity was monitored by examination for morphologic transformation of the treated cultures and by focus, plaque, and reverse transcriptase assays on culture media. FBR virus proved to be infectious for all murine cells including those of rat (Table I). However, cells from 10 different nonmurine species, including human, were totally resistant to infection by this virus. After two passages 4–5 weeks postinfection, all infected cultures

TABLE I. HOST RANGE OF FBR-MuSV(MuAV)

	Initial infected cultures				Infected cultures at passage two		
		Virus detection[b]			Virus detection[d]		
Species (cell line)	Focus assay[a] (ffu/ml)	Focus assay (ffu/ml)	Plaque assay (pfu/ml)	Polymerase assay[c]	Focus assay (ffu/ml)	Plaque assay (pfu/ml)	Polymerase assay
Mouse (NIH 3T3)	5.0×10^2	1.0×10^2	2.5×10^2	+	5.5×10^2	1.5×10^4	+
Mouse (D152)	6.4×10^5	1.0×10^5	2.1×10^7	+	4.0×10^5	4.7×10^7	+
Mouse (Swiss D55)	1.2×10^5	1.5×10^4	3.6×10^7	+	1.5×10^4	1.2×10^6	+
Mouse (Feral Sc-1)	***	2.0×10^4	1.5×10^6	+	1.0×10^4	2.9×10^7	+
Rat (OM—NRK)	1.5×10^2	0	1.5×10^5	+	0	2.0×10^5	+
Chinese hamster (E36)	0	0	0	–	0	0	–
Mink (64)	0	0	0	–	0	0	–
Sheep (SL)	***	0	0	–	0	0	–
Rabbit (BC352)	0	0	0	–	0	0	–
Rabbit (A5700)	0	0	0	–	0	0	–
Cat (BE849)	0	0	0	–	0	0	–
Dog (8155)	***	0	0	–	0	0	–
Bat (88)	0	0	0	–	0	0	–
Cow (AP737)	0	0	0	–	0	0	–
African green monkey (AM237)	0	0	0	–	0	0	–
Human (A0134)	0	0	0	–	0	0	–

[a] In the cases indicated by *** transformation could not be assessed because of the detachment of the cells, whether infected or uninfected, prior to the time required for the development of visible foci in permissive host cells.

[b] Culture media were tested for presence of virus by the indicated assays 8–12 days postinfection.

[c] For the polymerase assay (+) = a positive reaction (–) = a negative reaction.

[d] Media were tested 3–6 days after second passages.

were retested. Results comparable to the first test were obtained.

Transforming and nontransforming activities. In appropriate murine cells, such as BALB/c (A31, D152) and Swiss (D55), FBR virus readily induce morphologic transformation. With diluted virus inoculum, discrete foci of disoriented, refractile, rounded, and spindle-shaped cells were visible (Fig. 1). Whereas similar foci induced by other MuSVs usually appeared on Days 5–6 (8), those induced by FBR virus appeared later. In infected BALB/c monolayer cultures, recognizable FBR foci first appeared on Days 7–8 postinfection and were best quantitated on Days 10–14. On standing, these foci continued to enlarge and overlap one another. Totally transformed cultures often resulted. Attempts to speed up focus development with a polybrene medium supplement and high serum concentrations were unsuccessful.

For focus assay, D55 and D152 are the best indicator cells. They yield the highest transforming titers (Table I) and the most stable foci, and their monolayers remain smooth and attached for more than the required 2-week period. Focus induction assay using NIH/3T3 cells is not satisfactory as these monolayer cultures tend to lift early. Even with low initial cell densities and low serum concentrations, NIH/3T3 cells reach confluency rapidly and usually detach before Day 7 and prior to the usual time required for the development of visible foci in susceptible indicator cells. FBR virus induces foci in rat NRK cells that are morphologically different from those induced in mouse cells. Rat foci appear on Days 6–7 postinfection and are small and transient, consisting of clusters of small, rounded-up cells that detach easily.

As with all other mammalian sarcoma virus isolates, FBR-MuSV has an associated nontransforming virus detectable by a standard infectious center plaque assay with XC cells (Fig. 2). This associated virus, FBR-MuAV, is present in two to three logs higher concentration than the transforming component (Table I).

Focus titration assays of FBR-MuSV-(MuAV) consistently yielded single-hit patterns whether foci were counted early (Day 9) or late (Day 15). Single-hit kinetics was also observed whether the infecting virus

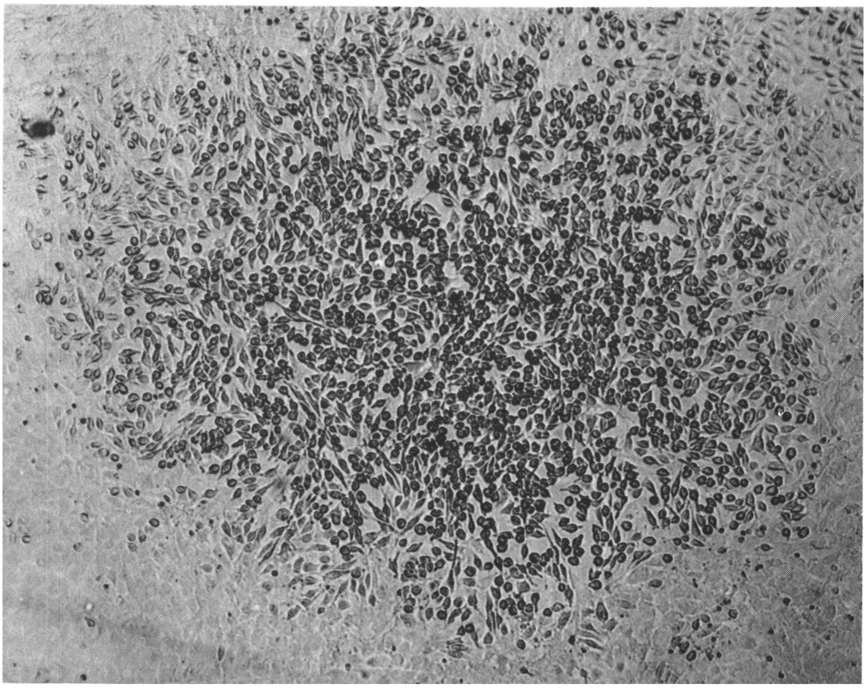

FIG. 1. Focus of transformed cells induced by FBR-MuSV(MuAV) on D55 cells. Photograph shows a single representative focus of morphologically altered cells 14 days postinfected in a plate infected with virus inoculum at a 1:100 dilution, × 170.24.

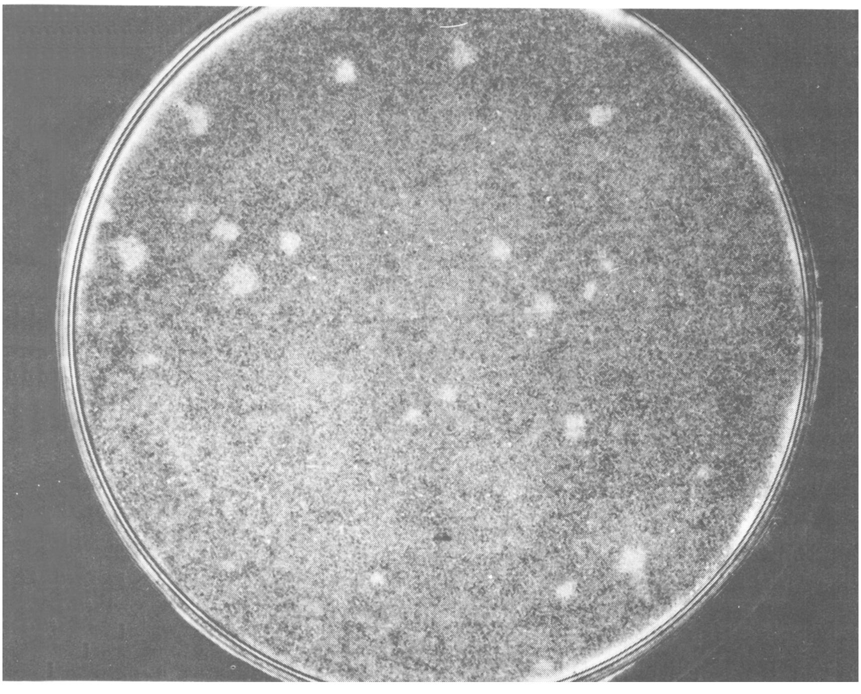

FIG. 2. Syncytial plaques formed between XC cells and irradiated D55 cells infected with FBR-MuSV(MuAV). The D55 cells were infected with a 10^{-4} dilution of virus inoculum (Pool No. 77-1), × 2.66.

stock had a one, two, or three log excess of the nontransforming component (Fig. 3).

Tropism of FBR-MuSV(MuAV) was determined by comparing the susceptibilities of embryonic cells from mice having different alleles in the Fv-1 gene locus (19–21). FBR-MuSV(MuAV) proved to be b-tropic, showing higher infectivity for b than n cells (Table II). The differences in titers are of the order of two logs for the transforming virus and four logs for the nontransforming virus.

Virus propagation. To determine the best virus–cell system for the large-scale production of FBR-MuSV(MuAV), the transforming and nontransforming titers of viruses produced by various infected murine cell lines were compared. Results of representative experiments are shown in Table III. In three separate trials, infected NIH/3T3 cells yielded low titers of transforming virus, and the transforming titer of chronically infected cells tended to decrease with passage. On the other hand, the nontransforming titer produced by NIH/3T3 cells was fairly high and increased with cell passage to a stable high titer of 10^6–10^7 pfu/ml. Thus, NIH/3T3 cells preferentially replicate the nontransforming component and are suitable for the propagation of FBR-MuAV. Chronically infected BALB/c and Sc-1 cells yielded much higher titers of the transforming component. However, each also produced a two log excess of the nontransforming virus. Attempts to increase virus titers of infected Sc-1 cells by supplementing the media with tryptose phosphate broth, DEAE-dextran, polybrene, or high serum resulted in only minimal improvement. Dexamethasone and 10% conditioned medium did affect a significant but small enhancement (approximately one log). The high virus-producer, FBR-infected Sc-1 cells, were cloned by endpoint dilution in microplates, and the virus titers produced by the single-cell derivatives were compared (Table IV). Of the five clones tested, clone 1 yielded the best ratio of transforming to nontransforming virus. This clone was selected for large scale virus production in roller bottles. Periodic testing showed that virus titers of the clone 1 roller cultures remained essentially unchanged for more than 10 months. In soft agar, these cells have a cloning efficiency of almost 100%.

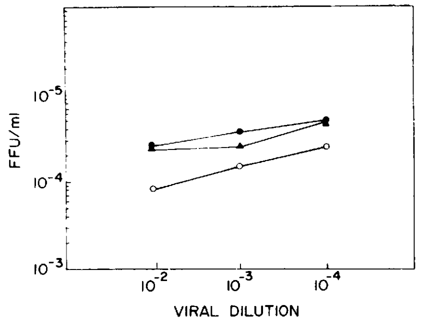

FIG. 3. Focus titrations of FBR-MuSV(MuAV) stocks having varied ratios of transforming to nontransforming viruses. Standard assays were carried out on virus preparations derived from different clones of chronically infected Sc-1 cells. The corrected titers in ffu/ml are plotted against virus dilutions. (○) clone 1, ffu:pfu—1:10; (▲) uncloned, 1:200; (●) clone 5, 1:7,500.

TABLE II. TROPISM OF FBR-MuSV(MuAV)[a]

Strain	Fv-1 genotype	Focus titer (ffu/ml)	Plaque titer (pfu/ml)
NIH Swiss	n	9.2×10^1	5.0×10^2
C57BL/6	b	1.6×10^4	4.2×10^6
BALB/c	b	1.5×10^4	2.8×10^6

[a] FBR virus Pool No. 77-1 was titrated on various secondary embryo cultures derived from mice having different Fv-1 genotypes. Culture dishes were seeded with 3×10^5 cells and inoculated in quadruplicate with serial 10-fold dilutions of the virus. On Day 7, two plates of each dilution were irradiated for XC plaque assay. The matched plates were held for focus counting on Days 10–14.

TABLE III. TRANSFORMING AND NONTRANSFORMING VIRUS TITERS OF CHRONICALLY INFECTED MURINE CELL LINES

Cell line	Focus titer (ffu/ml)	Plaque titer (pfu/ml)
NIH Swiss—3T3	5.0×10^1	2.0×10^5
BALB/c—D152	1.0×10^5	2.0×10^7
Swiss—D55	1.4×10^4	3.0×10^7
Feral—Sc-1	2.5×10^4	1.5×10^6

Discussion. This report describes the properties and propagation of FBR-MuSV-(MuAV) in tissue culture and contains basic information useful for further characterization of this interesting virus. FBR-MuSV(MuAV) is apparently one of the few available authentic murine endogenous sarcoma viruses. Its origin and unique osteosarcomagenic property may provide a model for the study of the role of this virus in ^{90}Sr-induced osteosarcomas.

FBR-MuSV(MuAV) derived from virus-induced bone tumors in X/Gf mice appears to be strictly ecotropic. Although only a limited number of tissue cells from a variety of mammalian species were tested, the data clearly showed that the virus is infectious for murine cells only. All infected cultures were monitored for morphologic transformation and their spent media tested for virus by reverse transcriptase, focus induction, and XC-plaque assays. There was no evidence of infection of the nonmurine cells even on retesting after two cell passages 4–5 weeks postinfection. These negative data for nonmurine cells also indicate the absence of readily detectable xenotropic (22) or polytropic viruses (23).

Tumor-derived virus preparations consist of a transforming MuSV and a nontransforming MuAV. When tested in various mouse embryo secondary cultures, both the transforming and nontransforming activities showed distinct b-tropism. This fact is consistent with the finding that the X/Gf mouse has a Fv-1^b genotype (J. W. Hartley, personal communication), which is restrictive for n-tropic viruses.

The focus-inducing behavior of FBR-MuSV(MuAV) is consistently single-hit. This single-hit pattern may be due to: (i) the presence of excess helper virus, (ii) competence of the sarcoma virus, or (iii) late quantitation of foci. The first explanation is unlikely since the same pattern was observed whether the excess of MuAV over MuSV was one, two, or three logs. The second would be most interesting but it has no precedent. The third is a likely possibility. In contrast with other strains of MuSV that induce foci within 5–6 days, recognizable FBR-induced foci first appear 7–8 days after infection and are not sufficiently well formed for quantitation until 10–14 days. Perhaps FBR foci develop primarily by cell division and not by cell recruitment involving infection of adjacent cells by progeny virus (10, 24–26). Consequently, in 2 weeks all transformed clones, whether originally infected with or without helper virus, will have grown to a sufficient size to be scored. These focus-inducing properties of FBR virus are similar to those of FBJ-MuSV(MuAV) in terms of single-hit kinetics and late appearance of foci (14).

TABLE IV. TRANSFORMING AND NONTRANSFORMING VIRUS TITERS PRODUCED BY VARIOUS CLONES OF FBR VIRUS-INFECTED SC-1 CELLS

	Focus titer (ffu/ml)	Plaque titer (pfu/ml)	Ratio (ffu:pfu)
Clone 1	2×10^4	2×10^5	1:10
Clone 2	5×10^4	4×10^6	1:80
Clone 3	2×10^3	6×10^5	1:300
Clone 4	2×10^4	1×10^6	1:50
Clone 5	8×10^4	6×10^8	1:7, 500
Uncloned	1×10^4	2×10^6	1:200

Further immunologic and biochemical characterizations, especially of sarcoma-specific sequences, require large quantities of sarcoma-rich virus. For this purpose an appropriate virus–cell system was selected for mass culture. A clone of infected Sc-1 cells produces high titers of the transforming virus (10^5 ffu/ml) without a vast excess of the nontransforming component (10^6 pfu/ml). This clonal cell line grows well in roller bottles and remains a good producer of sarcoma-rich virus. These cells are themselves completely transformed as indicated by their high cloning efficiency in soft agar. The viruses they produce are highly transforming *in vitro* and are potent inducers of osteosarcomas *in vivo*. Thus, tissue-culture-derived FBR-MuSV(MuAV) has biological properties similar to its parent virus except for an enrichment in the transforming component.

Summary. This report describes the *in vitro* biological properties of an osteosarcoma virus, FBR, which was originally isolated from a ^{90}Sr-induced osteosarcoma in an X/Gf mouse. The virus complex consists of a transforming component, MuSV, and a nontransforming component, MuAV. Foci of cells transformed by FBR-MuSV(MuAV) are late appearing and consistently yield single-hit focus titration patterns. The isolate is infectious for only murine cells and shows distinct b-tropism. A clone of transformed cells yielding high-titered sarcoma-rich virus has been propagated for the large-scale production of this virus. Tissue-culture-derived FBR-MuSV(MuAV) possesses osteosarcomagenic properties comparable to those of the parent virus.

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