Rat C-type Virus (BV-1): Effect of Amniotic Fluid, Fetal Serum, and Hormones in Cell Culture[1,2] (36403)

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A virus was isolated in this laboratory in 1967 from rat mammary carcinomas induced by 7,12-dimethylbenz(a)anthracene (DMBA) (1). It was provisionally termed the rat mammary tumor-derived virus (RMTDV), and subsequently identified as a C-type virus whose general characteristics (2) and buoyant density (unpublished data) were similar to those of oncogenic RNA viruses. Investigators in other laboratories have also reported the presence of C-type virus particles in various rat mammary tumors (3, 4). Our isolate (RMTDV), henceforth referred to as BV-1 (Breast Virus), appears unique because of its ability to produce typical cell alterations and cytopathogenicity in a rat embryo cell line designated REL (2). The latter is conveniently used for assaying this virus *in vitro*.

In view of the rat DMBA mammary tumor origin of BV-1 and the well documented hormone sensitivity of this type of tumor (5–9), an investigation was carried out in regard to the possible effects of amniotic fluid, fetal serum, estrogen, and progesterone on the growth and cytopathic effect of BV-1 in rat embryo cell cultures. The results are reported in this communication.

Materials and Methods. Production and assay of virus. The methods for production and infectivity titrations of BV-1 have been previously described (2). Briefly, the virus was grown in cultures of an established cell line (REL) derived from Sprague–Dawley rat embryo cells. It was harvested on day 4 postinfection which is the time when infectious virus progeny reaches its peak. After partial purification, the virus was sedimented at 78,000*g* for 1 hr, resuspended in 0.05 *M* citrate, titrated, and stored at —70° in sealed glass ampules.

All infectivity titrations of BV-1 were done in quadruplicate tubes of REL monolayer cell cultures. Serial tenfold dilutions of the virus were made in Hanks' balanced salt solution (HBSS) supplemented with 1% newborn calf serum, and 0.1 ml of a given dilution was inoculated into each culture. Control cultures were inoculated with 0.1 ml of diluent. After adsorption at 37° for 1 hr, 1 ml of medium was added to each tube and the cultures were incubated at 37° for 7 days. At this time the cultures were examined for the characteristic BV-1 lesions (2), which consisted of foci of altered rounded cells, and the infectivity titer ($TCID_{50}$) of the virus was calculated according to the method of Reed and Muench (10).

Cell cultures and media. Wistar/Furth (W/FU) rat embryo cell cultures were used in all of the experiments reported below and the REL cell cultures were utilized for virus infectivity titrations as described. All cultures were maintained at 37° on minimal essential medium (MEM) supplemented with 10% newborn calf serum (MEM–NCS) and 100 units/ml of penicillin and 100 μg/ml of streptomycin.

In experiments where the effect of amniotic fluid on virus–cell interactions was tested, 30% of bovine amniotic fluid (BAF), obtained from North American Biologicals, Inc.

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[2] Presented in part at the Institute for Medical Research Symposium on Mammary Carcinoma, Camden, New Jersey, November, 1971.

TABLE I. Influence of Bovine Amniotic Fluid and Fetal Calf Serum on the Growth of BV-1 in Wistar/Furth Rat Embryo Cell Cultures.

Experiment	Composition of culture medium	Virus titer ($log_{10}TCID_{50}$) on day: 0	2	4	7	10
A	MEM–NCS[a]	2.0	5.0	6.5	6.0	4.0
	MEM–NCS-BAF[b]	2.0	6.0	7.5	7.0	6.0
B	MEM–NCS	0.5	5.0	7.0	6.0	ND[d]
	MEM–FCS[c]	0.5	6.5	7.5	7.0	ND

[a] Minimal essential medium (MEM) supplemented with 10% newborn calf serum (NCS).
[b] MEM–NCS supplemented with 30% bovine amniotic fluid (BAF).
[c] MEM supplemented with 10% fetal calf serum (FCS).
[d] Not done.

(lot No. 3108N004), was added to the MEM–NCS medium. In experiments with fetal serum, the newborn calf serum in the MEM–NCS medium was substituted with 10% fetal calf serum (FCS) from the same supplier. All sera were heat inactivated at 56° for 30 min.

Hormones. Estrogenic substance (95% natural) was obtained from Nutritional Biochemicals Corp. and progesterone ($C_{21}H_{30}O_2$) from Gallard Schlesinger Mfg. Corp. One milligram of each hormone was dissolved in 1 ml of 95% ethyl alcohol and then diluted with MEM–NCS medium to give a stock solution containing 100 μg/ml of hormone. The stock solution was further diluted with the same medium to give the desired concentration of hormone for use in experiments conducted as described below.

Additional details of experimental design will be described under "Results."

Results. In a series of experiments the possible influence of BAF and FCS on virus–cell relationships in W/FU rat embryo cells infected with BV-1 was examined. In the first (A) set of experiments cultures were seeded with 10^5 cells in MEM–NCS medium each. After incubation at 37° for 2 days, the number of cells in a few representative cultures was determined and an appropriate number of these cultures were infected with BV-1 at a multiplicity of infection of about 0.1 $TCID_{50}$ per cell. After adsorption of the inoculated virus at 37° for 1 hr, the cultures were washed three times with HBSS to remove unadsorbed virus. Half of the infected and uninfected control cultures were fed with MEM–NCS and the other half with MEM–NCS containing 30% BAF. In the second (B) set of experiments, conducted under similar conditions, the effect of MEM–FCS was tested by comparison to that of MEM–NCS. All cultures were incubated at 37° and examined microscopically at the time when samples were removed for virus assay. The cells and supernatant of a given sample, consisting of three cultures, were pooled and the cells disrupted by three cycles of freezing and thawing to free intracellular virus. Cellular debris was removed by centrifugation at 1,600 rpm in an International PR-6 centrifuge at 4° and the clear supernatant was assayed in 2-day-old REL cell cultures to determine the titer of BV-1 in the material.

The average results of several experiments of this type are shown in Table I. Minimal to moderate cell alterations (rounded, fusiform, and granular cells) were first observed in cultures on MEM–NCS at day 4 which progressed to abundant lesions, involving the entire cell sheet, by day 7–10. On the other hand, cultures maintained on MEM–NCS–BAF or MEM–FCS were partially protected as evidenced by lack of significant lesions at day 4. Although some areas of altered cells became conspicuous by day 7–10, at least 50% of the cell sheet appeared unaltered. Nevertheless, the titers of BV-1 at all times tested were higher in cultures on MEM–NCS–BAF or MEM–FCS than in cultures on MEM–NCS. We obtained essentially

TABLE II. Dose Related Effect of Estrogen and Progesterone on the Growth of BV-1 in Wistar/Furth Rat Embryo Cell Cultures.

Type of hormone	Dose of hormone (μg/ml of medium[a])	Virus titer[b] ($\log_{10}$TCID$_{50}$)
Estrogen	5.0	6.0
	2.5	6.5
	1.0	6.0
	0.1	5.5
None	—	5.0
Progesterone	5.0	$<$ 3.0
	1.0	3.5
	0.1	4.0
None	—	4.0

[a] Minimal essential medium (MEM) supplemented with 10% newborn calf serum.

[b] Seven days after addition of hormone.

similar results when cultures infected with BV-1 and maintained on MEM–NCS–BAF or MEM–FCS were divided and subcultured in their respective media and MEM–FCS, respectively. These findings have enabled us to establish actively proliferating monolayer rat embryo cell cultures which can be subcultured serially and are persistently infected with BV-1, synthesize infectious BV-1 of high titer, and yet are relatively free of lesions.

Based on the results described above, experiments were carried out in a similar fashion with estrogen and progesterone. Wistar/Furth rat embryo cell tube cultures were infected with BV-1, washed with HBSS, and divided into several groups. Each of these groups was refed with MEM–NCS containing different amounts of estrogen and progesterone, respectively. A control group was fed with MEM–NCS without the hormone. All cultures were incubated at 37° for 7 days at which time the cells and supernatant of 4–6 cultures in each group were pooled, extracted for virus, and titrated in REL cell cultures as described above. Representative data, obtained in several experiments using a dose gradient of estrogen and progesterone, are presented in Table II. The data indicated a stimulating effect of estrogen upon synthesis of BV-1 in W/FU rat embryo cells which was similar to that of BAF and FCS and was dose dependent. Progesterone, at dose levels of 1.0 μg/ml and higher, appeared to be antagonistic to the replication of BV-1 in these cells, whereas lower doses had no effect.

Discussion. The results of this study suggested that BAF and FCS exerted a protective effect on rat embryo cells infected with BV-1 whereby the cell altering and partially lethal effect of this virus was reduced considerably. At the same time there was a virus growth stimulating effect as evidenced by increased titers of viral progeny in cultures maintained in the presence of BAF or FCS. This difference in titer might be attributable to the protective effect of MEM–NCS–BAF or MEM–FCS on the cells. This possibility, however, is ruled out by the fact that even at times when there were no cell lesions detectable in cultures of either group (day 2), the titer of BV-1 was 1–2 $\log_{10}$ TCID$_{50}$ lower in cultures on NCS. The factor and the mechanism responsible for the protective effect of BAF and FCS on cells infected with BV-1 is unknown at this time. Presently, these findings have provided a means whereby rat embryo cell cultures liberating high-titered BV-1 can be maintained in a chronically infected state. On the other hand, the microscopical detection of lesions produced by BV-1 is obscured if cultures are maintained in the presence of FCS or BAF.

The observation that estrogen exerted a growth stimulating effect on BV-1 suggested that the similar effect of BAF or FCS may also be hormone mediated. These findings thus seem to provide interesting possibilities for studying C-type virus–hormone interactions *in vitro* and *in vivo*. In this regard it is important to note that a recent communication (11) described hormone-activated expression of the mouse C-type virus genome and another recent report (12) noted an enhancing effect of FCS on the replication of a C-type virus in cell cultures of lymphocytes from bovine leukemia.

Summary. Bovine amniotic fluid or fetal calf serum markedly reduced the cell alterations which were produced in Wistar/Furth rat embryo cell cultures by a rat C-type virus (BV-1) isolated from chemically-induced mammary tumors. At the same time, higher

titers of infectious BV-1 were produced as early as 2 days post-infection in cultures maintained on medium supplemented with bovine amniotic fluid or fetal calf serum as compared to cultures on medium supplemented with newborn calf serum. A similar stimulatory effect on the growth of BV-1 in these cultures was exerted by estrogen (95% natural) which was dose dependent, 1.0–2.5 μg/ml being the most effective dose under the conditions employed. Progesterone had no stimulatory effect on the replication of BV-1 and appeared to be suppressive at higher dose levels.

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