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Enhancement of Encephalomyocarditis Virus Replication in L Cells Treated with Insulin (41065)

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Abstract. Data are presented which show that treatment of L cells with low levels of insulin results in a marked increase in the yield of encephalomyocarditis virus, strain MM. Maximum yields were obtained when the cell cultures were pretreated with the hormone for 24 hr followed by the presence of insulin after virus infection. The increased virus yields cannot be attributed either to increased cell proliferation or to interference with the interferon system.

A limited number of studies suggest that insulin might influence viral replication. For example, it was reported that the addition of insulin and hydrocortisone (10 μg/ml of each hormone) to culture medium augmented the production of mouse mammary tumor virus (MMTV) in cultures of mammary adenocarcinoma cells of Balb/cfC3H mice (1). Insulin treatment alone resulted in the increased release of MMTV while a combination of insulin and hydrocortisone augmented the production of the virus. Treatment of uninfected and herpesvirus simplex (HSV)-infected lymphoblastoid cells with high levels of insulin (4 IU/ml) inhibited both cellular and viral DNA synthesis (2). Very high levels of insulin (20 IU/ml), added 3 hr after Sindbis virus infection also has been reported to interfere with virus polypeptide cleavage in chicken cells (3). In the present study, we report that the replication of encephalomyocarditis (EMC) virus, strain MM, is markedly stimulated in mouse-cell cultures treated with low (near physiological) levels of insulin.

Materials and Methods. *Cells.* L929 cells (L cells) were grown in Dulbecco's modified MEM supplemented with 10% calf serum (DMEM). Monolayer cultures for experiments were prepared by adding approximately 1.5×10^6 cells in 5 ml DMEM to 60-mm plastic dishes 18–24 hr prior to use.

Virus. A stock suspension of encephalomyocarditis virus, strain MM, was prepared and titrated by methods previously described (4). For infection of experimental cell cultures, the stock virus was diluted in Hanks balanced salt solution supplemented with 2% calf serum (HBSS) to give a multiplicity of infection (MOI) of either 10 or 0.1 plaque-forming units (pfu) per cell as indicated. The virus inoculum was adsorbed for 1 hr at room temperature after which each cell culture was washed twice with HBSS and fed with 5 ml of the appropriate medium. After the indicated time periods at 37° in a humidified atmosphere containing 5% CO_2, the production of free virus was determined by assaying the culture fluids for pfu content. Total virus was determined by scraping the cells with a rubber policeman into the culture fluid. The resulting cell suspension was sonicated (Heat Systems Model W-220F; power setting 3, 10 sec) to release the cell-associated virus. After removing the cell debris by low speed centrifugation (1500 *g*, 5 min), the supernatant fluid was assayed for pfu content.

Production and assay of interferon. Concentrated, partially purified L-cell interferon was prepared by methods previously described (5). To inactivate the virus, samples to be assayed for interferon content were adjusted to pH 2.0 with 1 *N* HCl for a period of 4 days at 5°. Interferon activity was determined by the 50% plaque reduc-

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tion (PR_{50}) technique using MM virus as the challenge agent (5). We have determined that each PR_{50} unit by this method is equivalent to 0.5 NIH (G002-904-511) reference units.

Insulin. Bovine pancreas crystalline insulin (26.8 IU/mg, Sigma Chemical Company, lot No. 28C-0136) was dissolved in sterile distilled water at a concentration of 10 mg/ml by adjustment to pH 2.0. This stock suspension was stored at −30° and for experiments, was thawed and diluted to the specified concentration in DMEM. Unless stated otherwise, cell cultures were washed twice with HBSS following insulin treatment.

Results. Alteration of MM virus infection by insulin was first suggested by the data shown in Table I. Pretreatment of L cells for 24 hr with insulin resulted in a marked dose-dependent increase in plaque formation when the cultures were subsequently challenged with about 100 pfu of MM virus. Increased numbers of pfu were observed in cell cultures treated with insulin at levels as low as 0.0025 μg/ml and with increasing dosages there was a progressive increase in pfu. Subsequent experiments revealed that pretreatment of L cells with 1 μg insulin/ml for 18–24 hr markedly increased the 24-hr yield of free virus (data not shown). A dose response experiment was done to determine the lowest concentration of insulin which stimulated virus replication. Cell cultures were treated with several concentrations of insulin for 24 hr. The cultures were then infected with MM virus at a MOI of 0.1 pfu/cell. The culture fluids were collected 24 hr later and assayed for pfu content. The results (Table II) show that treatment with as little as 0.1 μg insulin/ml markedly increased (472% of control) the yield of free virus. In contrast to the increase in plaque formation (Table I), lower levels of hormone had no effect. Based on these data, insulin was used at a concentration of 0.1 μg/ml for subsequent experiments.

In the previous experiment, only free virus was measured. Therefore, the increase in virus titer following insulin treatment could have resulted from a more efficient release of the virus rather than from increased virus synthesis. To resolve this question, cultures were treated with insulin for either 4 or 24 hr prior to virus infection. The 24-hr free and total virus yields were then determined. The results (Table III) show that both free and total virus yields were elevated by hormone treatment. They also indicate that insulin treatment for 24 hr was more effective than a 4 hr exposure. Since MM virus has been shown to be exceptionally sensitive to the antiviral action of interferon (6), the increase in virus production also could have resulted from an impaired interferon system. To determine the effect of insulin on interferon synthesis,

TABLE I. INCREASED PLAQUE FORMATION IN CELLS PRETREATED WITH INSULIN

Insulin[a] (μg/ml)	Plaque formation[b] (pfu/plate)	pfu (% control)
0.05	340	378
0.02	266	296
0.01	203	226
0.005	150	167
0.0025	136	151
0	90	N/A

[a] Monolayer cultures of L cells in 60-mm plates were treated for 24 hr with the concentrations of insulin indicated.

[b] After insulin treatment the cell cultures were challenged with about 100 pfu of MM virus. The average (four plates at each concentration) number of plaque forming units (pfu) per plate was determined 24 hr later.

TABLE II. EFFECT OF VARIOUS CONCENTRATIONS OF INSULIN ON THE YIELD OF FREE VIRUS

Insulin (μg/ml)[a]	24-hr Virus yield[b] (pfu/ml)	pfu (% Control)
4.0	5.0×10^6	694
2.0	4.3×10^6	579
1.0	4.1×10^6	569
0.5	4.2×10^6	583
0.1	3.4×10^6	472
0.05	6.2×10^5	80
0.01	6.0×10^5	83
0	7.2×10^5	N/A

[a] Monolayer cultures of L cells were treated for 24 hr with insulin at the indicated concentrations.

[b] After the 24-hr treatment period, cell cultures were challenged with MM virus at a multiplicity of infection of 0.1 pfu/cell. Culture fluids were collected 24 hr later and assayed for pfu content.

TABLE III. EFFECT OF INSULIN ON VIRUS REPLICATION AND INTERFERON PRODUCTION

Treatment at[a]		24-hr Virus yield[b] (pfu/ml)		Interferon production[c] (PR_{50} units/ml)	
0 hr	4 hr	Free virus	Total virus[d]	Before sonication	After sonication
Insulin	DMEM	2.4×10^6	4.0×10^6	5,700	2520
DMEM	DMEM	7.5×10^5	1.6×10^6	6,550	1390
Insulin	None[e]	4.8×10^6	1.0×10^7	11,400	3830
DMEM	None[e]	1.1×10^6	1.6×10^6	4,900	1800

[a] At the times indicated monolayer cultures of L cells were treated with either insulin (0.1 μg/ml) or Dulbecco's medium (DMEM). Cultures were washed twice with HBSS between treatments.

[b] Cell cultures were challenged with MM virus (0.1 pfu/cell) 24 hr after the initial treatment. Virus yields were determined 24 hr after infection.

[c] Samples used to determine virus yields were adjusted to pH 2.0 for 4 days and then assayed for interferon activity.

[d] Cell-associated virus released by sonication.

[e] No further treatment, i.e., insulin or DMEM was left on for the entire 24-hr preinfection period.

the above samples were assayed for interferon activity. The data (Table III) show that the amount of interferon produced was markedly increased in cultures treated with the hormone for 24 hr. No effect on interferon production was noticed following the 4-hr treatment. The data also show that interferon was inactivated by sonication. Additionally, the possible interference by insulin with the antiviral activity of interferon was investigated by exposing cell cultures to the hormone either before or after interferon treatment. The results (Table IV) show that insulin had no effect on the protective action of interferon.

TABLE IV. EFFECT OF INSULIN TREATMENT ON THE PROTECTIVE ACTION OF INTERFERON

Treatment at[a]		48-hr virus yield (pfu/ml)[b]
0 hr	4 hr	
IF	DMEM	4.8×10^3
IF	Insulin	7.5×10^3
DMEM	IF	<100
Insulin	IF	<100
Insulin	DMEM	2.1×10^7
DMEM	DMEM	8.2×10^6

[a] Monolayer cultures of L cells were treated with interferon (IF) 2700 PR_{50} units; insulin, 0.1 μg/ml; or Dulbecco's medium (DMEM) at the times indicated.

[b] Twenty-four hours after the start (0 hr) of the experiment the cultures were challenged with MM virus at a multiplicity of infection of 10 pfu/cell. Culture fluids were collected 48 hr later and assayed for pfu content.

In each of the previous experiments insulin was removed by washing the cell cultures and was not present in the medium after virus infection. An experiment was done to determine virus production in the presence of the hormone. The data depicted in Table V show that, in cultures previously treated with insulin, the presence of the hormone after virus infection resulted in higher yields of virus (compare treatment 1 vs 2; 3 vs 4; 5 vs 6). The largest increase in virus yield (540%) was observed with a 24-hr pretreatment followed by the presence of insulin after virus infection (Treatment 5). When the hormone was not present after virus infection, pretreatment for a 48-hr period did not alter the virus yield (Treatment 2). The presence of insulin only after virus infection appeared to have a suppressive effect (Treatment 7).

Discussion. The data in this study show that replication of the MM strain of Encephalomyocarditis virus is enhanced in L-cells treated with insulin. Although other reports suggest that this hormone might influence viral replication (1–3), this is the first study, that we are aware of, in which relatively low levels of the hormone were used. In one experiment (see Table I), the data show that the plaque-forming ability of the virus was increased by an insulin concentration which approached physiological levels (7). Since each plaque represents a

TABLE V. EFFECT OF INSULIN TREATMENT BEFORE AND AFTER VIRUS INFECTION ON VIRUS REPLICATION

Culture	Treatment at[a]			24-hr Virus yield (pfu/ml)	
	−48 hr	−24 hr	0 hr[b]	Total virus[c]	% Control
1	Insulin	Insulin	Insulin	9.0×10^5	142
2	Insulin	Insulin	DMEM	6.0×10^5	95
3	Insulin	DMEM	Insulin	1.5×10^6	238
4	Insulin	DMEM	DMEM	1.2×10^6	190
5	DMEM	Insulin	Insulin	3.4×10^6	540
6	DMEM	Insulin	DMEM	1.3×10^6	206
7	DMEM	DMEM	Insulin	3.5×10^5	56
8	DMEM	DMEM	DMEM	6.3×10^5	N/A

[a] Cultures were treated at the times indicated with either insulin (0.1 μg/ml) or Dulbecco's minimal essential medium (DMEM).

[b] Cultures were infected with MM virus at a multiplicity of infection of 0.1 pfu/cell. Immediately after virus absorption (0 hr) the cultures received either insulin (0.1 μg/ml) or DMEM.

[c] Cell-associated virus was released by sonication.

virus infected cell, an increase in plaque formation indicates that more cells were initially infected and should result in increased virus yields. However, a comparison of the data in Tables I and II shows that much less insulin was required to enhance plaque-formation (2.5 ng) than was required to increase virus yields (100 ng). This apparent discrepancy can be explained by noting that the data in Table II show only the yields of free virus. The total-virus yield in response to insulin (100 ng/ml) treatment was substantially higher than that of free virus (see Table III). This observation suggests that lower concentrations of the hormone should result in increased yields of total virus. We have, in fact, recently determined that pretreatment of L cells with 50 ng insulin/ml results in a two- to threefold increase in the yield of total virus. This finding closely parallels the increase in plaque production seen with a similar concentration of the hormone in Table I.

Stimulation of virus production was seen only in cell cultures which were exposed to insulin before virus infection. It was also observed that pretreatment with the hormone for a period of 4 hr was not as effective as pretreatment for 24 hr while a 48-hr exposure had no effect. The presence of insulin after virus challenge appeared to increase further the final virus yields from cultures pretreated with the hormone.

The mechanism(s) responsible for stimulating virus production is not known. It has been reported that growth of cells in culture is significantly improved by hormones such as insulin (8). Thus, the observed increase in virus replication could have resulted from an increase in the number of cells in the hormone-treated cultures. We have determined, however, that under our experimental conditions, there was no increase in either cell numbers or cell viability in the insulin-treated cultures when compared to untreated controls (data not shown).

Since MM virus is exceptionally sensitive to the antiviral action of interferon (6), another possible mechanism for increased virus production would be interference with the interferon system. This mechanism was ruled out, however, by the data showing that insulin treatment resulted in about a twofold increase in the yield of interferon and had no effect on its antiviral activity. The observation that hormone treatment increased interferon production is in agreement with results reported by Azuma et al. (9) which showed that treatment of human embryonic lung (WI-38) cells with insulin increased interferon production in response to New Castle Disease Virus (NDV) infection.

Other possible mechanisms of action include increased adsorption and/or penetration of the virus. Insulin is known to inter-

act with membrane receptors of mammalian cells (10, 11) and could alter the permeability of the cell membrane allowing easier penetration by the virus. That the cell membrane is more permeable following hormone treatment is suggested by the increase in plaque-forming ability of the virus preparation (Table I) and by preliminary data in our laboratory which shows that Mengovirus (also a member of the EMC group of viruses) is released more efficiently from insulin-treated cultures than from untreated controls. In view of the fact that physiological levels of this hormone have been shown to accelerate DNA synthesis in cell cultures (12, 13), the stimulation of cellular metabolism such as DNA, RNA and/or protein synthesis could also result in more virus being produced. In addition, viral RNA and/or protein synthesis could be altered. Studies currently in progress will investigate each of the above as possible mechanisms of action.

Although the relationship, if any, between the results reported in this study and diabetes is not clear, it is of interest to note that the M variant of encephalomyocarditis (EMC) virus, has been shown to induce diabetes in experimental animals (14, 15).

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