Interferon Synthesis in Human Diploid Cells Pretreated with Insulin

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Interferon production by Newcastle disease virus in human diploid (WI-38) cells was greatly reduced in the absence of serum. Pretreatment of the serum-free cells with insulin in excess of 1.2 IU/ml for 2 h before induction considerably increased the amount of interferon produced. No enhancement of interferon production was seen when the cells were exposed to insulin 2 h after induction or later. The insulin's effect persisted for at least 6 h after its removal, but not for 24 h. A possible mechanism of insulin action is discussed.

Human leucocyte and human diploid cell cultures require the presence of serum in media to yield substantial quantities of interferon (2-4, 8, 9). However, to obtain interferon preparations with high initial specific activities, it would be advantageous to omit serum from the culture medium during the period of interferon production. There have been many reports dealing with the cultivation of mammalian cells in serum-free media of defined composition (5-7, 10). Higuchi and Robinson (5) showed that hormones, such as insulin, cortisol, and thyroxine, significantly improved cell culture growth in defined media.

The purpose of the present work has been to search for substances that improve interferon production in human diploid cells maintained in serum-free defined medium. In this paper we show that insulin increases interferon production in such diploid cells.

MATERIALS AND METHODS

Cell cultures. Human embryonic lung cells (WI-38) were cultivated in Eagle minimum essential medium supplemented with 10% fetal bovine serum. To remove serum components, confluent monolayer cultures in plastic Petri dishes were washed three times with 0.01 M phosphate-buffered saline solution and refed with Ham F10 medium without serum (F10). After cultivation at 37°C for 24 h, the monolayer cultures were washed twice again and used for experiments. Hereafter, these unwashed and thoroughly washed cultures will be referred to as serum-containing and serum-free cultures, respectively.

Viruses. The Miyadera strain of Newcastle disease virus (NDV), used as an interferon inducer, was propagated by allantoic inoculation of 11-day-old chicken embryos. The infectivity titer was $10^{9.2}$ plaque-forming units per ml, as determined on chicken embryo cell primary cultures. For interferon assay, the Indiana strain of vesicular stomatitis virus, propagated in chicken embryo cells, was used as a challenge virus.

The titer was $10^{9.0}$ 50% tissue culture infective dose per ml in WI-38 cells.

Interferon production and titration. The cultures were induced with NDV at a multiplicity of 100 plaque-forming units per cell. After 2 h of incubation, cultures were washed twice with phosphate-buffered saline and refed with maintenance medium. Interferon activity in culture fluids was measured by reduction of the cytopathic effect in vesicular stomatitis virus-infected WI-38 cells, as described previously (1). The human reference standard interferon (reagent number G-023-901-527), with an assigned potency of 20,000 U, had a titer of 12,800 U by our method.

Materials. Insulin (24.3 IU/mg, from bovine pancreas) was purchased from Sigma Chemical Co., St. Louis, Mo., and dissolved in F10 at a concentration of 1 mg/ml by adjustment to pH 2. This stock solution was kept at -20°C, and for experiments the stock solution was diluted to the specified concentrations with F10. Actinomycin D was obtained from Schwarz/Mann, Orangeburg, N. Y. Actinomycin D at a concentration of 0.5 µg/ml inhibited cellular ribonucleic acid (RNA) synthesis by 92% and also completely inhibited NDV-induced interferon production in WI-38 cells. Cycloheximide was obtained from Nakarai Chemical Ltd., Kyoto, Japan. Cycloheximide at 50 µg/ml inhibited cellular protein synthesis by 95%. Eagle minimum essential medium and F10 medium were purchased from Nissui Seiyaku Co. Ltd., Tokyo, Japan. Plastic Petri dishes (50 mm in diameter) were supplied from Wako Pure Chemical Industries, Ltd., Osaka, Japan, and plastic plates (16-mm well diameter) were from Linbro Scientific Co. Inc., Hamden, Conn.

RESULTS

Interferon production in serum-free WI-38 cell cultures. Serum-free and serum-containing cultures were induced with NDV as described in Materials and Methods. After 12 h, interferon production in the absence of serum (160 U/ml) was much less than that in the

presence of serum (1,280 U/ml), in agreement with previous reports (2-4).

Interferon production in serum-free WI-38 cell cultures treated with insulin. Serum-free cultures were refed with F10 supplemented with graded levels of insulin. After 2 h of incubation, cultures were induced with NDV in the presence of insulin for 2 h, washed with phosphate-buffered saline, and then replenished with the same medium containing insulin. Interferon was sampled 12 h after induction and assayed. In preliminary experiments, the presence of 2.4 IU of insulin per ml in interferon samples did not affect per se the interferon assay. Insulin at a concentration higher than 1.2 IU/ml showed about a 10-fold increase in the yield of interferon (Table 1). Since no significant increment of cell numbers was noted during 12 h of insulin treatment (data not shown), the enhancement of interferon production by insulin may be due to a net increase in the interferon yield per cell, rather than to larger cell numbers. In the following experiments, insulin at a concentration of 2.4 IU/ml was used.

Effect of insulin on adsorption and growth of NDV. To establish whether the presence of insulin actually increased adsorption or growth of NDV in WI-38 cells so that the virus could function as a more effective inducer, the propagation of NDV in cells exposed to insulin at a concentration of 2.4 IU/ml was compared with that in untreated cells. There was no difference in the rate of adsorption or in the yield of infective particles and hemagglutinins in these cultures.

Effect of varying time of insulin treatment. Serum-free cultures treated with insulin for different periods of time were induced with NDV. The treatment of cells with insulin before or, at least, simultaneously with induction markedly increased the interferon yield, whereas no increase of interferon resulted from the addition

of insulin 2 h after induction or later (Table 2).

Reversibility of insulin action. Experiments were then performed to determine whether the action of insulin was reversible or not. Serum-free cultures were treated with insulin at 37°C for 2 h and then induced with NDV either immediately or after incubation with F10 for 6 or 24 h at 37°C. The interferon yield was assayed 12 h after induction. The insulin's effect persisted for at least 6 h after its removal, but not for 24 h (Table 3).

Interferon production in serum-free WI-38 cell cultures treated with insulin and inhibitors of macromolecule synthesis. The results of Table 2 suggest that insulin acts on transcription of the interferon gene, but not on translation of interferon messenger RNA or on the release of interferon from the cells. To support this concept, the following experiment was carried out (Fig. 1).

Two (A and D) of five sets of duplicate cultures were treated with 2.4 IU of insulin per ml for 2 h at 37°C. The other three sets (B, C, and E) were left untreated. One of the insulin-treated cultures (A) and two of the untreated cultures

TABLE 2. *Interferon production in serum-free WI-38 cell cultures treated with insulin for different periods*

Time of insulin treatment (h) ^a	Interferon (U/ml) at 12 h after induction
-6 to +12	3,240
-2 to +12	3,240
0 to +12	1,080
+2 to +12	640
+6 to +12	360
No treatment	360

^a Serum-free cultures were treated with insulin at 2.4 IU/ml and induced with NDV at a multiplicity of 100 plaque-forming units per cell at 0 h.

TABLE 3. *Reversibility of insulin action on interferon production*

Cells ^a	Incubation time before induction (h) ^b	Interferon (U/ml) at 12 h after induction
Untreated	None	120
Treated	None	3,240
Untreated	6	360
Treated	6	3,240
Untreated	24	360
Treated	24	360

^a Serum-free WI-38 cell cultures were exposed to F10 (untreated) or F10 containing 2.4 IU of insulin per ml (treated) for 2 h at 37°C.

^b Treated and untreated cell cultures were washed and incubated with F10 at 37°C before induction with NDV at 100 plaque-forming units per cell.

TABLE 1. *Effect of insulin treatment on interferon production in serum-free WI-38 cell cultures*

Insulin treatment of cells ^a (IU/ml)	Inducer ^b	Interferon (U/ml) at 12 h after induction
None	NDV	160
0.12	NDV	320
1.2	NDV	1,280
2.4	NDV	2,560
12.0	NDV	2,560
12.0	None	<10

^a Serum-free cultures were treated with F10 or F10 containing insulin from 2 h before induction until the interferon sampling time.

^b NDV was inoculated at a multiplicity of 100 plaque-forming units per cell.

FIG. 1. Interferon production in serum-free WI-38 cell cultures treated with insulin and inhibitors of macromolecule synthesis. The effects on interferon production are presented on the right (from 7 to 23 h) above each line. Symbols: ■, Insulin (2.4 IU/ml in F10); ▨, NDV (100 plaque-forming units per cell); ○----○, cycloheximide (50 µg/ml); ■—■, actinomycin D (0.5 µg/ml); □ hr, F10.

(B and C) were then induced with NDV for 2 h at 37°C, whereas the other cultures were left as controls. After removal of the virus and washing of the cultures, cycloheximide at a concentration of 50 µg/ml was added for 5 h to permit accumulation of interferon messenger RNA. Actinomycin D at a concentration of 0.5 µg/ml was then added for 30 min to inhibit further transcription of the interferon gene. The cultures were then washed three times with phosphate-buffered saline, and two pairs (B and E) that had remained untreated with insulin were refed with F10 containing 2.4 IU of insulin per ml, whereas the other pairs received F10 alone. Interferon titers were measured 23 h after induction. The interferon yield was enhanced only in those cultures that had been exposed to insulin before induction of interferon was allowed to take place (Fig. 1). This observation suggests that insulin treatment enhances transcription of the interferon gene, thereby causing the observed increment in interferon production.

DISCUSSION

NDV-induced human diploid cells yielded small amounts of interferon in serum-free culture medium, in agreement with a previous report (4). The yield of interferon was increased about 10 times when the cells maintained in serum-free medium were pretreated with insulin. Since the presence of serum was required neither during exposure to insulin nor during interferon synthesis, this method may facilitate efforts to purify interferon from human diploid cells. Whether or not residual insulin in purified

interferon samples may prove detrimental in other respects is now under investigation.

As to the mechanism of insulin action in enhancing interferon production, one may assume for the present that it promotes increased transcription of the interferon gene. However, these studies do not shed light on the manner in which this control is exercised by insulin.

Our recent studies (I. Yoshida, M. Azuma, T. Suenaga, and F. Mizumo, *J. Gen. Virol.*, in press) demonstrated that the guanosine 2':3'-cyclic monophosphate level increased in the femtomole range when serum-free WI-38 cells were treated with insulin at a concentration of 2.4 IU/ml and that the serum-free WI-38 cells treated with dibutyryl guanosine 2':3'-cyclic monophosphate showed an enhancement in the yield of interferon in response to NDV. These results suggest that the enhancing activity of insulin may be mediated by guanosine 2':3'-cyclic monophosphate.

Hadházy et al. (3) also reported that human leukocyte suspensions required the presence of serum in the culture medium to ensure the production of large amounts of interferon. Translation of interferon messenger RNA was reduced when the leukocytes were incubated in serum-free medium, whereas synthesis of interferon messenger RNA proceeded normally under these conditions.

Further investigations should determine whether enhanced transcription by insulin restores the level of interferon messenger RNA translation, which is otherwise reduced in the absence of serum, and by what means this effect is achieved.

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