Effect of Dibutyryl Cyclic AMP on Interferon Production by Cells Treated with Viral or Nonviral Inducers[1] (36677)

F. DIANZANI, P. NERI,[2] AND M. ZUCCA
Institute of Microbiology of the University of Turin, Turin, Italy

Introduction. Previous studies (1) have shown that cells treated with an inducer of interferon develop antiviral resistance through a two-step process in which the production of interferon must precede that of a second protein, the so-called antiviral protein. Cells rendered highly resistant to viral replication by a viral inducer, become temporarily refractory to new induction of interferon. In addition, the interferon mechanism is activated during viral infection and it ceases functioning shortly after the infection is terminated. This sequential induction and subsequent inhibition suggests that a regulatory system is activated in the induced cells. Some recent findings indicate that in some cell systems the production of interferon is potentiated by inhibitors of protein and RNA synthesis, added at critical times after the induction (2, 3). These data have been interpreted to indicate production of proteins which might play a repressive role during interferon production. Since cyclic adenosine 3′,5′-monophosphate (cyclic AMP) plays an important role in the regulatory system of bacteria, as well as in many secretory phenomena of eucharistic cells, and in lymphocyte transformation and activity (4–6), it seemed interesting to determine whether cyclic AMP levels would influence the mechanisms controlling interferon production *in vitro*.

Materials and Methods. Dibutyryl cyclic AMP [DBCAMP, P-L Biochemicals (8)], adrenaline, and theophylline at a concentration of 10 mg/ml, were dissolved in 0.15 *M* phosphate buffered saline. Immediately before each experiment further dilutions were made in Eagle's medium. Unless otherwise specified these compounds were used at a final concentration of 1 mg/ml. Cycloheximide (Sigma) and actinomycin D (Merck, Sharp, and Dohme) were both dissolved in Eagle's medium and used at a final concentration of 20 and 5 μg/ml, respectively. Interferon production was studied as previously described (9) in mouse L cells and in primary or secondary rat embryo cells induced with Newcastle disease virus (NDV, strain F) and with Chikungunya virus, respectively, or with the synthetic polynucleotide complex formed by polyinosinic and polycytidylic acids (In.Cn), added to the cell cultures in the presence of DEAE-dextran. Interferon titrations were performed in homologous cells as previously described using GD-7 virus for L cells or Sindbis virus for rat embryo cells as challenge viruses (H. Oie, C. Buckler, C. Uhlendorf and S. Baron, personal communication).

Results. Effect of cyclic AMP on interferon production by L cells treated with In.Cn. Triplicate tubes of L cells were induced with In.Cn (10 μg/ml) plus DEAE-dextran (200 μg/ml), in the presence or absence of DBCAMP or of adrenaline plus theophylline. Adrenaline and theophylline are compounds which, respectively, induce and stabilize intracellular levels of cyclic AMP in a wide range of cell systems (8). Interferon production was determined after 7 hr of incubation at 37°. The results of a representative experiment are presented in Table I.

As shown the cells induced with In.Cn alone produced a large amount of interferon while its production was undetectable in the presence of DBCAMP or adrenaline plus theophylline. Lower doses of DBCAMP (from

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[2] Istituto Sieroterapico Sclavo, Research Department, Siena, Italy.

TABLE I. Effect of Dibutyryl Cyclic AMP[a] or Adrenaline[a] (A) plus Theophylline[a] (T) on Interferon Production by Mouse L Cells Treated with In • Cn.

Inducer	Treatment	Interferon production (units/ml)
None	DBCAMP	<10
None	A + T	<10
In • Cn	None	100
In • Cn	DBCAMP	<10
In • Cn	A + T	<10

[a] 1 mg/ml.

100 to 0.1 μg/ml) did not exert any detectable effect on interferon production.

In addition adenine, adenosine, 5'-adenosine monophosphate, cytosine monophosphate, guanosine monophosphate and deoxyadenosine monophosphate, used at equimolar amounts were not effective in inhibiting interferon production. These substances although chemically related to cyclic AMP do not influence cyclic AMP levels. Control experiments showed that all the compounds used did not influence viral growth nor exert a visible toxic effect on the cells within the time of the experiments.

Effect of cyclic AMP on interferon production by L cells treated with Newcastle disease virus and by rat embryo cells treated with Chikungunya virus. The inhibitory activity of DBCAMP or adrenaline plus theophylline in L cells treated with In.Cn could be due (a) to an effect on the cellular responsiveness to the inducer (either specifically or nonspecifically related to interferon production); (b) to a direct activity on the inducer itself; or (c) to an effect on the DEAE-dextran used as the enhancer, since In.Cn does not induce detectable amounts of interferon in L cells without the addition of DEAE-dextran (10). The possibilities that the compounds acted directly on In.Cn (b), or on DEAE-dextran (c), were tested by determining the effect of DBCAMP or adrenaline plus theophylline on interferon induced by virus instead of In.Cn plus DEAE-dextran. The results of representative experiments are shown in Table II.

It can be seen that either in L cells induced with NDV or in rat embryo cells induced with Chikungunya virus DBCAMP or adrenaline plus theophylline strongly inhibited interferon production. These data show that the inhibitory activity occurs independently of the kind of inducer used, and also independently of the cell type. Since these results make possibilities (b) and (c) unlikely, it is reasonable to think that these compounds exert their effect by somehow affecting cellular mechanisms underlying the production of interferon.

Further experiments showed that rat embryo cells induced with Chikungunya virus and treated simultaneously with DBCAMP produced normal amounts of interferon if the compound was removed after 2 or 6 hr. Interferon production also took place normally in cells pretreated overnight with DBCAMP and then washed before induction with Chikungunya virus. These findings indicate that the action of DBCAMP is completely reversible.

Experiments were undertaken next to help determine whether protein synthesis was re-

TABLE II. Effect of Dibutyryl Cyclic AMP[a] or Adrenaline[a] plus Theophylline[a] on Interferon Production by Rat Embryo and L Cells Induced with Viral Inducers.

Cell cultures	Treatment with DBCAMP or with adrenaline + theophylline	Inducer	Interferon production (units/ml)
Rat embryo	No	Chikungunya	300
	Yes	Chikungunya	10
L	No	NDV	1000
	Yes	NDV	30

[a] 1 mg/ml.

quired for DBCAMP to inhibit induction of interferon. The experimental design was to determine whether the mRNA for interferon would be produced in cells induced by Chikungunya virus in the presence of DBCAMP plus cycloheximide. It had previously been shown that cycloheximide did not inhibit the induction of mRNA for interferon although it did inhibit translation into the interferon protein. On removal of cycloheximide and addition of actinomycin D to prevent formation of additional mRNA, the mRNA for interferon was translated into interferon protein (11). Using a similar experimental design, rat embryo cells were treated with Chikungunya virus alone or in combination with cycloheximide, DBCAMP, or DBCAMP plus cycloheximide. Five hours later actinomycin D was added to stop any further production of mRNA and 1 hr later the cultures were washed, fed with fresh medium and incubated overnight at 37°. At that time interferon samples were harvested for titration. Control experiments based on measurements of ^{3}H-uridine and ^{14}C-proline incorporation showed that under the experimental conditions cycloheximide inhibited more than 97% of protein synthesis without affecting RNA synthesis. After the removal of cycloheximide and the addition of actinomycin D, protein synthesis resumed fully but RNA synthesis was almost completely and irreversably suppressed. The results are shown in Table III.

TABLE III. Effect of Cycloheximide on the Inhibitory Activity of Dibutyryl Cyclic AMP on Interferon Production by Rat Embryo Cells Treated with Chikungunya Virus.[a]

Treatment	Expt.:	Interferon production (units/ml) 1	2	3
None		1000	300	300
DBCAMP		30	<10	<10
Cycloheximide		300	100	300
DBCAMP + cycloheximide		300	100	300

[a] Cells were treated for 5 hr with Chikungunya virus in the presence of the indicated compounds. At the fifth hour actinomycin D (5 μg/ml) was added. One hour later cells were washed 3 times and fresh medium was added. Interferon samples were harvested after overnight incubation.

As shown, interferon production occurred in cells treated with Chikungunya virus alone or in combination with cycloheximide, but it did not occur in cells treated with Chikungunya virus and DBCAMP. Normal production of interferon was, however, observed in cells treated with Chikungunya virus in combination with cycloheximide and DBCAMP, indicating that the inhibitory activity of the latter was prevented by cycloheximide. This finding is consistent with the interpretation that DBCAMP requires cellular protein synthesis for its action (12, 13).

Another interesting aspect of this experiment was that cells induced with Chikungunya virus in the presence of DBCAMP failed to produce normal amounts of interferon when the DBCAMP was removed and actinomycin D was added (Table III, line 2). When actinomycin D was omitted after removal of DBCAMP, normal amounts of interferon were produced. This finding suggests that DBCAMP may prevent induction or function of the mRNA for interferon.

Discussion. It has been reported that cyclic AMP increases the antiviral activity of interferon in chick embryo cells (14). The present results, indicating an inhibitory effect of cyclic AMP on interferon production by rat embryo and mouse L cells, supports the view that cyclic AMP can play an important role on the regulatory mechanisms of the interferon system. A related and recent observation is the depressive effect of cyclic AMP on the protective activity of In.Cn has been observed in mice infected with Friend leukemia virus (15).

The mechanism by which cyclic AMP decreased interferon production is not yet clear. However, the reversibility of its action, the absence of inhibitory activity in the presence of cycloheximide, and the lack of activity of several related but inactive compounds strongly suggest that the effect is real and not due to some nonspecific effect on the cell system.

It has been established that in cell cultures the rate and duration of interferon produc-

tion vary with the cell type and the kind of inducer. However, after the maximum level has been reached, production ceases and the cells become refractory to a second induction (16, 17). It has been also reported that the addition of inhibitors of protein and RNA synthesis after the induction enhance the production of interferon (2). These findings suggested that the metabolic inhibitors prevent the synthesis of a protein which suppresses interferon production (2, 3). The present finding that the inhibition of interferon production by DBCAMP does not occur in the presence of cycloheximide may suggest the hypothesis that DBCAMP could induce the synthesis of that inhibitory protein. If this hypothesis is correct this protein should be relatively unstable since the action of DBCAMP is completely reversible after 2–6 hr. This point is currently under study.

The present data suggest that DBCAMP inhibits interferon production at the transcriptional level. Cells induced with Chikungunya virus in the presence of cycloheximide or DBCAMP did not produce interferon while either compound was present. However after their removal and the addition of actinomycin D interferon was produced only by the cells induced in the presence of cycloheximide, but it was not produced by cells induced in the presence of DBCAMP. Since only preformed mRNA could be translated into the interferon protein in the presence of actinomycin D, it can be inferred that functional mRNA was produced in the presence of cycloheximide and it was either not produced or it was unstable in the presence of DBCAMP. This hypothesis is now under study.

Summary. Treatment of mouse L or rat embryo cells with dibutyryl cyclic AMP prevents, or strongly reduces, the production of interferon induced either by poly I.poly C or by Newcastle disease and Chikungunya viruses. The same effect was also observed in cells treated with adrenaline plus theophylline (enhancers of cyclic AMP). In addition the inhibitory activity of dibutyryl cyclic AMP was prevented by suppression of protein synthesis with cycloheximide. Preliminary evidence obtained using metabolic inhibitors suggests that the depression of interferon production may be due to an effect at the transcriptional level. The results are interpreted to indicate a possible role of cyclic AMP on the intracellular regulatory mechanisms of the interferon system.

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