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Enhancement of Interferon Antiviral Action in L-Cells by Cyclic Nucleotides (38151)

L. B. ALLEN, N. C. EAGLE, J. H. HUFFMAN, D. A. SHUMAN, R. B. MEYER, JR., AND R. W. SIDWELL

ICN Pharmaceuticals, Inc., Nucleic Acid Research Institute, 2727 Campus Drive Irvine, California 92664

Exogenous interferon (IF) has been successfully utilized in prevention of human viral diseases (1). However, the massive quantity of IF required for such successful applications provides a significant hindrance to general usage of this natural broad-spectrum antiviral defense mechanism. Reduction of the quantity of IF required for effective antiviral applications would increase its practicality as a chemotherapy tool.

The use of other compounds concurrently with IF to stimulate its antiviral activity would be one approach to the reduction of the large quantities of IF required for human treatment. A promising compound in this regard is adenosine 3′, 5′-cyclic phosphate (cAMP).

In addition to the original demonstration of the stimulation of IF antiviral effect by cAMP (2) other work has demonstrated a direct correlation between factors which cause high intracellular levels of cAMP (3–9) and conditions which favor maximal sensitivity to and production of IF (10–14). Various investigators (9, 15, 16, 17, 18, 19) have demonstrated that *in vitro* cell morphology and growth rates can be altered by addition of cAMP or its derivatives to the cell culture medium.

The knowledge of the importance of cAMP in cell growth control, coupled with its possible enhancement of IF action, and our desire to develop practical means of using IF therapeutically, stimulated the present work. In this report we describe studies on the effects of cAMP and certain of its synthetic derivatives on the action of IF in mouse L-929 cells.

Materials and Methods. Cells and Culture Media. L-929 cells (Flow Laboratories, Rockville, MD) were grown in Eagle's minimum essential medium (MEM) containing 10% heat-inactivated fetal bovine serum (FBS), 0.25% $NaHCO_3$, penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml) (20). In order to minimize variation in cell response to IF from test to test, cells were synchronized by the double thymidine block technique (21) prior to seeding in 0.2 ml volumes in cells of micro tissue culture plates (Falcon Plastics, Oxnard, CA) (20) at a concentration of 1.4×10^5 cells/ml. The amount of FBS was reduced to 2% in MEM used in IF assays.

Viruses. Vesicular stomatitis virus (VSV), strain Indiana, obtained from the American Type Culture Collection (Rockville, MD) was used in this study as the sixth L-929 cell passage.

Interferon. The IF used in most experiments was a polyriboinosinic–polyribocytidylic acid (poly I:C) stimulated mouse serum IF with a titer of 320 units/0.2 ml. Mouse serum IF's stimulated by tilorone, statolon, or Newcatle disease virus (NDV) were also used on a limited basis.

Chemicals. Cyclic nucleotides used in these studies were: adenosine 3′, 5′-cyclic phosphate (ICN Nutritional Biochemicals Corp., Cleveland, OH); N^6, 2′-*O*-dibutyryladenosine 3′, 5′-cyclic phosphate (DBcAMP) (Sigma Chemical Co., St. Louis, MO); 8-bromoadenosine 3′, 5′-cyclic phosphate (8-BrcAMP); 8-benzylthioadenosine 3′, 5′-cyclic phosphate (8-BTcAMP); 8-methylthioadenosine 3′, 5′-cyclic phosphate (8-MTcAMP); 8-*P*-chlorophenylthioadenosine 3′, 5′-cyclic phosphate (8-CPTcAMP); 8-benzylaminoadenosine 3′, 5′-cyclic phosphate (8-BAcAMP), and 6-methylthio-9-β-D-ribofuranosylpurine 3′, 5′-

cyclic phosphate (6-MT-PRcP). The latter compounds were synthesized at this Institute as previously described (22, 23).

Interferon Assays. Cell monolayers (18 hr) were treated with test medium (cell and virus controls) or varying two-fold dilutions of IF in test medium, incubated 24 hr, washed with Hanks' balanced salt solution (HBSS), and exposed to medium alone or medium containing 1000 cell Culture 50% Infectious Doses ($CCID_{50}$) of VSV. After incubation for 3 days at 37°, the cells were observed microscopically for virus cytopathic effect (CPE). In each experiment the IF solutions were the same on all plates.

To determine if the cyclic nucleotides alter the response of cells to IF, cells at each IF dilution were exposed to a concentration of compound varying between 10^{-5} and 10^{-2} *M*. Control infected cells were exposed only to compounds to determine any specific antiviral activity exerted by the compound. The IF titer (the reciprocal of the highest IF dilution causing 50% inhibition of viral CPE) was determined for cells exposed to IF alone and for those exposed to both IF and the cyclic nucleotides. The results are expressed as the ratio $\frac{\text{Treated IF titer}}{\text{Control IF titer}}$. Thus, values of 1 indicate no effect, those > 1 indicate enhancement and < 1 indicates inhibition.

In one experiment the average percent inhibition of virus CPE was determined for cells exposed to IF alone and those exposed to 6-MT-PRcP and IF. In addition, total virus concentration was determined for the same cells after freezing and thawing.

TABLE I. IF Action[a] in Presence of Cyclic Nucleotides[b].

Compound	Concentration of compound (*M*)			
	10^{-2}	10^{-3}	10^{-4}	10^{-5}
cAMP	1	1	1	1
DBcAMP	1	1	1	1
8-BrcAMP	1	1	1	1
8-BTcAMP	1	1	1	1
8-MTcAMP	1	1	1	1
8-CPTcAMP	1	1	1	1
8-BAcAMP	1	1	1	1
6-MT-PRcP	1	8	8	4

[a] Data expressed as the radio, $\frac{\text{Treated IF titer}}{\text{Control IF titer}}$.

[b] Cells were exposed to a mixture of IF and cyclic nucleotide for 24 hr, washed, and virus added.

TABLE II. Effect of Varying Treatment Times[a] on Enhancement of IF Action[b] by 6-MT-PRcP.

Compound treatment time (Prior to IF)	Concentration of compound (M)		
	10^{-2}	10^{-3}	10^{-4}
24 hr	1	1	1
12 hr	4	4	1
6 hr	8	4	2
4 hr	32	16	2
2 hr	8	4	1

[a] Cells were exposed to 6-MT-PRcP for the indicated times, compound removed, and IF added. The IF was removed after 24 hr, cells washed and virus added.

[b] Data expressed as the ratio, $\frac{\text{Treated IF titer}}{\text{Control IF titer}}$

Results. The effect of cyclic nucleotides on IF action was determined in an experiment in which cells were exposed to varying concentrations of the cyclic nucleotides and IF for 24 hr prior to virus challenge (Table I). Only 6-MT-PRcP effectively altered the IF action producing a 4–8-fold enhancement, thus 6-MT-PRcP was subsequently used in determining the treatment schedule which would result in optimal enhancement of IF action.

Exposure of cells to 6-MT-PRcP for varying intervals prior to compound removal and addition of IF revealed that the greatest enhancement (16–32-fold) was obtained when the pretreatment time was 4 hr (Table II). Based on this experiment, the various cyclic nucleotides were applied to cells 4 hr prior to IF addition (Table III). At the 10^{-2} *M* concentration of compounds, the degree of enhancement was 16-fold for 6-MT-PRcP, 4-fold for 8-BrcAMP and 8-MTcAMP, and 2-fold for compounds other than 8-BAcAMP, which did not enhance IF action. The narrowest range of enhancing activity (10^{-2} *M*) was seen with cAMP, while DBcAMP, 8-BTcAMP, 8-MTcAMP, and 8-CPTcAMP were active at 10^{-2} and 10^{-3} *M*. The widest range of activity was exhibited by 6-MT-PRcP and 8-BrcAMP. The IF-enhancing ef-

TABLE III. Effect of Cyclic Nucleotides on IF Antiviral Action[a] when Added to Cells 4 hr Prior to IF[b]

Compound	Concentration of Compound (M)		
	10^{-2}	10^{-3}	10^{-4}
cAMP	2	1	1
DBcAMP	2	2	1
8-BrcAMP	4	2	2
8-BTcAMP	2	2	1
8-MTcAMP	4	2	1
8-CPTcAMP	2	2	1
8-BAcAMP	1	1	1
6-MT-PRcP	16	8	2

[a] Cells were treated with compounds for 4 hr, compounds removed, and IF added. After 24 hr, IF was removed, cells washed, and virus added.

[b] Data expressed as the ratio, $\frac{\text{Treated IF titer}}{\text{Control IF titer.}}$

fect of 6-MT-PRcP is illustrated by inhibition of viral CPE (Fig. 1) and virus titer reduction (Fig. 2). The IF titer enhancement as determined by titer increase was similar to that found in the previous experiment (Table II) where 6-MT-PRcP was added to the cells 4 hr prior to IF.

In the above described experiments a poly I:C induced serum IF was used. IF inducers stimulate the production of heterogenous populations of IF (24), although in serum the predominant size of the IF molecule seems to vary with the inducer. The IF enhancing effect of 6-MT-PRcP was therefore studied further using IF stimulated by statolon, tilorone, and NDV, with similar enhancement again demonstrated (Table IV). Normal mouse serum, tested in parallel, had no antiviral effect alone or on cells pretreated with 6-MT-PRcP.

Discussion. These experiments demonstrate that the antiviral activity of mouse IF in L-929 cells can be increased by treatment of the cells with certain cyclic nucleotides. The relative potency, as enhancers of IF activity, of the cyclic nucleotides used in this study is as follows: 6-MT-PRcP > 8-BrcAMP > 8-MTcAMP > 8-CPTcAMP = 8-BTcAMP = DBcAMP > cAMP > 8-BAcAMP.

The IF-enhancing action of 6-MT-PRcP may be related to cell control mechanisms. It has been shown that 6-MT-PRcP is readily hydrolyzed by phosphodiesterases (23) but DBcAMP (25) and the 8-substituted derivatives (22) of cAMP are not. It has also been shown that 6-methylthio-9-β-D-ribofuranosylpurine has anticancer activity by blocking purine synthesis and stimulating pyrimidine synthesis (26). Thus, hydrolysis of 6-MT-PRcP could yield a nucleoside which inhibits cell division by blocking purine synthesis and possibly enhance IF activity in a manner different from the other cyclic nucleotides utilized in this study. Cell membrane transport is another cell control mechanism which may be altered by these compounds and thus cause enhancement of IF activity. Recently, 5′-AMP and 2′, 3′-cAMP were found to inhibit membrane transport of uridine, and adenosine was shown to decrease the uptake of uridine, leucine, and 2-deoxyglucose (19). Ininhibition of membrane transport by cyclic nucleotides or their breakdown products could result in control of cellular metabolic activities similar to that of serum starvation, which results in higher levels of intracellular cAMP (19).

On the other hand, the relatively high concentrations (10^{-5} to 10^{-2} *M*) of cyclic nucleotides, which appear necessary for IF enhancing activity may indicate a requirement for intact molecules to enter the cells to elicit an enhanced response to IF.

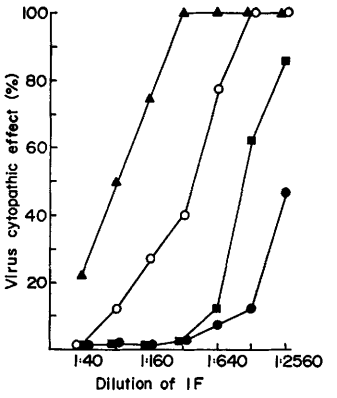

FIG. 1. Enhancement of IF action by 6-MT-PRcP as evidenced by inhibition of virus cytopathic effect. Virus control and virus with 6-MT-PrcP only have 100% CPE or complete cell destruction. ▲ IF alone, ● 10^{-2} *M* 6-MT-PRcP prior to IF, ■ 10^{-3} *M* 6-MT-PRcP prior to IF, ○ 10^{-4} *M* 6-MT-PRcP prior to IF. All 6-MT-PRcP exposures were for a 4 hr period prior to IF exposure.

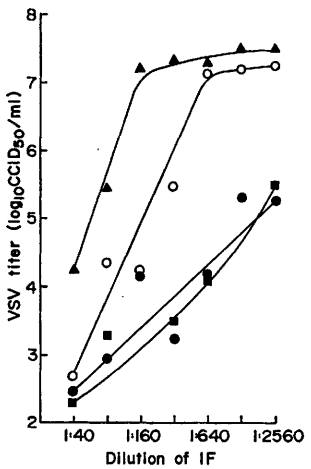

FIG. 2. Enhancement of IF action by 6-MT-PRcP as evidenced by inhibition of virus titer. VSV titers ($CCID_{50}$/ml) for controls are: virus control, 2.3×10^7; 10^{-4} *M* 6-MT-PRcP alone, 3.3×10^7; 10^{-3} *M* 6-MT-PRcP alone, 5×10^6; and 10^{-2} *M* 6-MT-PRcP alone, 2.3×10^6. ▲ IF alone, ● 10^{-2} *M* 6-MT-PRcP prior to IF, ■ 10^{-3} *M* 6-MT-PRcP prior to IF, ○ 10^{-4} *M* 6-MT-PRcP prior to IF. All 6-MT-PRcP exposures were for a 4 hr period prior to IF exposure.

Expression of biological effects of cAMP is often by activation of protein kinases (27). However, comparison of the relative IF enhancement achieved in our studies to the relative activation of bovine brain cAMP-dependent protein kinase by some of the same compounds in studies by Muneyama *et al.* (22) and Miller *et al.* (28) would indicate that this correlation does not exist in the case of IF enhancement by cAMP derivatives. Of course, such comparison of activities of cyclic nucleotides in cell free and cell culture systems are of limited value, since information concerning cell penetration is not available for many of the compounds.

In the present studies using mouse L-929 cells, cAMP was one of the least effective compounds tested for enhancement of IF action. The greater enhancement seen by DBcAMP and other derivatives in the present study over that seen by Friedman and Pastan (2) may have resulted from the change in cell species or the differences in experimental methods. In our experiments, cells were exposed to IF overnight, rather than for one hr, since the longer incubation reduces the amount of IF required to produce an antiviral state. Further, enhancement of IF activity by cAMP, DBcAMP, and the 8-substituted deriveratives required exposure of the cells to cyclic nucleotides prior to addition of IF. Pretreatment of cells with cyclic nucleotides prior to IF addition may permit cells to mimic those static cells previously reported to be very sensitive to IF (10–14). The pretreatment time might represent the period required for the cyclic nucleotides to penetrate cells and initiate activities associated with the higher levels of cAMP. Although work by Kram *et al.* (23) suggests that DBcAMP acts by altering membrane transport, it is not clear whether cyclic nucleotides added to medium of cultured cells act at intra- or extracellular levels. The lack of requirement for pretreatment by 6-MT-PRcP may reflect its structural difference from the other derivatives and suggests that it acts by a different mechanism than cAMP, DBcAMP and the 8-substituted derivatives.

Our studies indicate that the most potent of the IF enhancers studied, 6-MT-PRcP, has some antiviral activity when used alone. However, the maximum antiviral effect seen was a 1 log_{10} reduction in virus titer by the 10^{-2} *M* dose of compound (Fig. 2). This antiviral effect is not sufficient to account for the 2–4 log_{10} reduction of virus titer seen

TABLE IV. Comparison of 6-MT-PRcP Enhancing Effect[a] on IF Stimulated by Various Inducers[b]

Inducer of interferon	Concentration of 6-MT-PRcP			
	10^{-2}	10^{-3}	10^{-4}	10^{-5}
Normal Serum	—[c]	—	—	—
Poly I:C	8	4	2	1
Statolon	4	4	2	1
Tilorone	16	16	2	2
NDV (Newcastle Disease Virus)	16	8	8	4

[a] Cells were exposed to 6-MT-PRcP for 4 hr, compound removed, and a single IF added per plate. After 24 hr, IF's were removed, cells washed, and virus added.

[b] Data expressed as the ratio, $\frac{\text{Treated IF titer}}{\text{Control IF titer}}$.

[c] No protection in presence or absence of 6-MT-PRcP.

in cells exposed to 6-MT-PRcP and later to IF in comparison to cells exposed to IF alone. In addition, this antiviral effect was not sufficient to alter viral CPE development (Fig. 1). Exposure of L-929 cells to 6-MT-PRcP prior to IF treatment seems to have rendered the cells more sensitive to the antiviral activity of IF.

This study supports the results of Friedman and Pastan (2) and extends such work to the cells of another species as well as to a variety of derivatives of cAMP. Development of practical applications of IF-activity enhancement must await further basic work.

Summary. Experiments were described in which mouse L-929 cells were exposed to cAMP and various related cyclic nucleotides. One cyclic nucleotide, 6-MT-PRcP, enhanced IF activity as much as 16 to 32-fold when cells were exposed to a 10^{-2} *M* concentration for 4 hr prior to compound removal and addition of IF. Treatment of cells with 8-BrcAMP and 8-MTcAMP resulted in a 4-fold increase, whereas cAMP, DBcAMP, 8-BTcAMP, and 8-CPTcAMP produced a 2-fold increase. The exact nature of the IF enhancement by these cyclic nucleotides is unknown and may not be the same for all the cyclic nucleotides tested. It is possible that exposure to cyclic nucleotides may alter cells such that they behave as cells under limited growth conditions which respond well to IF.

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