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EFFECT OF CHOLERA TOXIN, DIBUTYRYL cAMP AND ADENOSINE ON THE *IN VITRO* REACTIVATION OF LATENT HERPES SIMPLEX VIRUS.

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ABSTRACT: *Cholera toxin and dibutyryl cyclic adenosine 3', 5'-monophosphate(db-cAMP) increased the rate and number of infections units produced in the in vitro reactivation of latent herpes simplex virus, whereas adenosine diminished them. cAMP concentration in latently infected trigeminal ganglia of mice was greatly increased by cholera toxin but was not affected by adenosine.*

Keywords: *herpes, latent virus, reactivation, cAMP.*

INTRODUCTION

Herpes simplex virus (HSV) establishes latent infections in the sensory or autonomic ganglia and the central nervous system both in humans(Bastian *et al.*, 1972; Brainger and Swoveland, 1973; Fraser *et al.*, 1981) and in experimental animals(Stevens and Cook, 1971; Price *et al.*, 1975; Cabrera *et al.*, 1980). Latent infection is characterized by the detection of infectious virus following culture of an explanted ganglion but not by direct homogenization assay(Stevens and Cook, 1971). The nature of latent HSV is not yet known. Although the whole HSV genome is present in the latent form, it might be different from that found in infectious virus particles(Rock and Fraser, 1983). However, it is well known that reactivation of latent HSV in neurons leads to recurrent HSV diseases in the surface tissues of the body(Stevens, 1975). Although many studies have been done in attempts to understand the mechanisms of the reactivation of latent HSV, the molecular events are not known(Stevens and Cook, 1974; Green *et al.*, 1981; Galloway *et al.*, 1982; Youssoufian *et al.*, 1982).

Recently, some studies suggested the involvement of prostaglandins(PGs) in the exacerbation of recurrent HSV diseases (Trofatter and Daniels, 1979, 1980; Hill and Blyth *et al.*, 1976; Kurane *et al.*, 1984): PGs were found to inhibit antibody-dependent complement cytolysis(ADCC)(Trofatter and Daniels, 1980), but they did not influence the HSV replication in monolayer cells. Hill and Blyth(1976) proposed that PGs enhance the local growth of HSV. They showed that PG E_2 injected into mice at

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previous inoculation sites induced exacerbations of the latent infection as much as ultraviolet light(Blyth *et al.*, 1976) or mild trauma(Hill and Blyth, 1976) did. More recently, Kurane *et al.*(1984) have reported that indomethacin inhibited the reactivation of HSV in latently infected ganglia explanted *in vitro*. Although other cellular changes caused by indomethacin could explain the suppression of the reactivation, they strongly implied that the inhibitory effect of indomethacin may be due to the inhibition of prostaglandin synthesis. However, no study has examined the direct effect of PGs on the reactivation of latent HSV *in vitro* or *in vivo*. Since PGs are known to alter intracellular concentrations of cyclic adenosine 3′,5′-monophosphate(cAMP), and in doing so, PGs could conceivably cause an exacerbation(Hill and Blyth, 1976), the effect of cAMP modulators on the reactivation of latent HSV may provide a crucial information in the reactivation process of latent virus.

In the present study, we have observed the effects of several cAMP modulators i.e., cholera toxin(CTX), dibutyryl cyclic adenosine 3′,5′-monophosphate(db-cAMP) and adenosine, on the *in vitro* reactivation of latent HSV and determined cAMP levels in latently infected trigeminal ganglia of mice. CTX, as a specific and irreversible activator of adenyl cyclase(Moss and Vaughan, 1979) causes a marked increase in the intracellular cAMP concentrations of almost all cells. Db-cAMP exerts an effect on the physiology of cells by acting as an analog of cAMP(Daniels *et al.*, 1973). In some cells, adenosine has an effect on the cAMP pathway, stimulating the accumulation of cAMP in certain cell types but inhibiting or producing a biphasic effect on the accumulation of cAMP in other cells (Londos and Wolff, 1977).

MATERIALS & METHODS

In these experiments, inbred albino male mice(BALB/c strain, 5 weeks old; Charles River Breeding Lab, Wilmington, MA) were inoculated with HSV-1(F-strain, American Type Culture Collection, Rockville, MD). Under pentobarbital anesthesia (60 mg/kg, intraperitoneal injection), the corneas of both eyes were scarficed in a cross-hatched pattern with a 30 gauge needle. Ten microliters of HSV-1(10^5 plaque-forming units) was applied into lower cul-de-sac of each eye with gentle massage. Our preliminary study indicates that the ocular infection results in the development of latent HSV-1 infection in 100% of the trigeminal ganglia of inoculated mice. Four to eight weeks after the viral inoculation, the mice were killed and the trigeminal ganglia were removed for the study using sterile technique.

To determine the effects of CTX, db-cAMP, and adenosine on the reactivation of latent HSV-1 *in vitro*, the excised ganglia were organ cultured in Eagle's minimum essential medium containing CTX(1 ug/ml or 3 ug/ml), db-cAMP(2mM) or adenosine(500uM) for one or two days. At the end of the culture period, the ganglia were homogenized by sonication using a Sonifier(Branson Sonic Power Co., Plainview, N.Y.). The titers of the reactivated viruses in the homogenates were determined by a plaque assay in green monkey kidney cell(CV-1) monolayers as described earlier (Park *et al.*, 1982).

The effects of CTX and adenosine on the concentration of cAMP in the ganglia with latent virus were determined as follows. The ganglia, which had been cultured in medium containing CTX(1 ug or 3 ug/ml) or adenosine(500uM) for 1 or 2 days, were

placed in assay buffer(20mM NaH_2PO_4, 100mM NaCl, 4.5mM KCl, 0.8mM $MgCl_2$, 6.9mM dextrose, and 4mM $CaCl_2$, pH 7.6) for 20 min. at 37°C in the absence and/or presence of 0.5mM isobutylmethylxanthine(IBMX). Then the ganglia were transferred to hot 0.1N KOH, homogenized, and neutralized with 0.1N HCl. After centrifugation, the supernatants were used for radioimmunoassay of cAMP(NEX-132; New England Nuclear Corp., Boston, MA) based on the method of Steiner *et al.*(1972), and protein content was determined from the pellet by the method of Lowry *et al.*(1951).

The time-course effects of CTX, db-cAMP and adenosine on the replication of HSV-1 were determined using an yield reduction assay. Confluent CV-1 monolayers were infected with HSV-1 at a multiplicity of infection(m.o.i.) of three for 1 hour with intermittent rocking at 15min. intervals, then washed twice with phosphate buffered saline(PBS). Medium with or without CTX(1 ug/ml or 3 ug/ml), db-cAMP(2mM) or adenosine (500uM), was added in triplicate to the appropriate petridishes. All cultures were incubated at 37°C for 12, 24 or 36 hours in a CO_2 incubator. They were then gently washed twice with PBS therapy preventing a carry-over of chemicals to the pla-

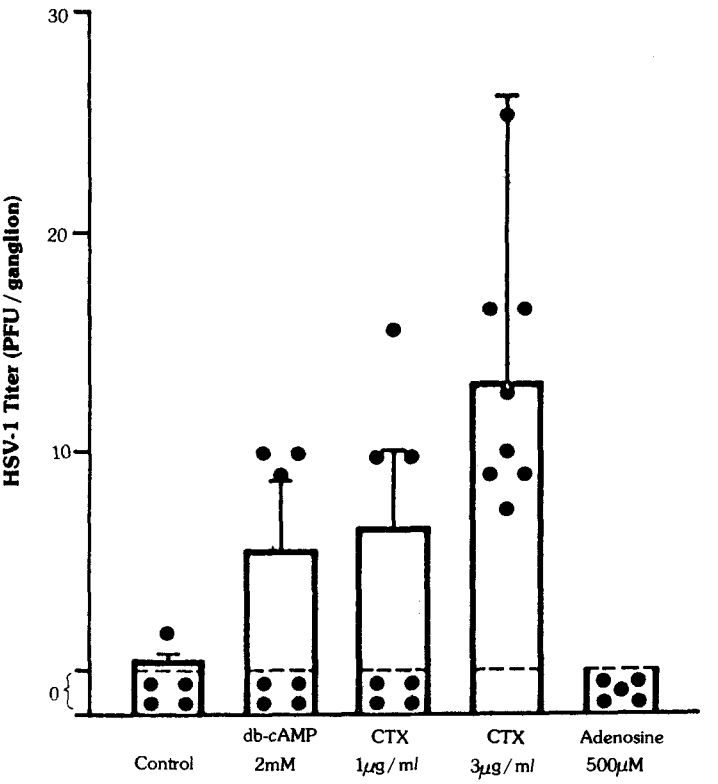

Fig. 1. Effect of db-cAMP, CTX and adenosine on the *in vitro* reactivation of latent HSV-1 in the murine trigeminal ganglia. The latently infected ganglia were removed, then incubated in the culture medium containing db-cAMP (2mM), CTX(1 or 3ug/ml) or adenosine (500uM) for 24hours. Ganglia were then processed as described in the text and the viral titers were determined. Each dot represents the viral titer from one ganglion.

que assay. New medium (chemical free) was then added. The cells were frozen and thawed three times, collected and centrifuged. Viral titers in the supernatant were assayed in CV-1 monolayers by an ordinary plaque assay technique(Rapp, 1963).

RESULTS

In the presence of CTX or db-cAMP in the culture medium, the reactivated infectious virus in the ganglion appeared earlier than controls and the amount of reactivated HSV was significantly increased ($p < 0.05$). CTX was much more effective than db-cAMP and 1 ug/ml CTX was apparently a near-maximal dose. The presence of adenosine in the culture medium notably delayed the appearance of reactivated HSV as compared to controls, and the titer of reactivated HSV was significantly lower in comparison to that of ganglia cultured in the medium only($p < 0.05$) (Fig. 1 & 2).

The cAMP level in the trigeminal ganglia was greatly increased by CTX, but unaffected by adenosine as compared to controls (Fig. 3); 1 ug/ml CTX was apparently a near-maximal dose. The presence of the phosphodiesterase inhibitor, IBMX, alone did not significantly alter the basal level of cAMP. As shown in Fig. 4, the time-course replication of HSV-1 in the CV-1 monolayers was not influenced by CTX, db-cAMP or adenosine at the concentration which affected the reactivation of latent HSV-1 in murine trigeminal ganglia (Fig. 4).

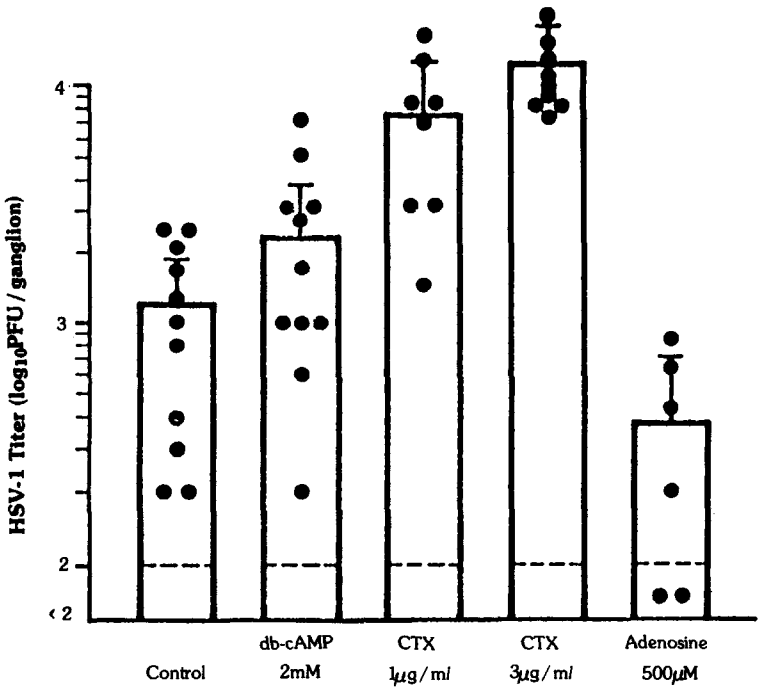

Fig. 2. Effect of db-cAMP, CTX and adenosine on the *in vitro* reactivation of latent HSV-1 in the murine trigeminal ganglia. The ganglia were incubated in the culture medium containing db-cAMP(2mM), CTX(1 or 3ug/ml) or adenosine (500uM) for 48hrs. Each dot represents the viral titer from one ganglion.

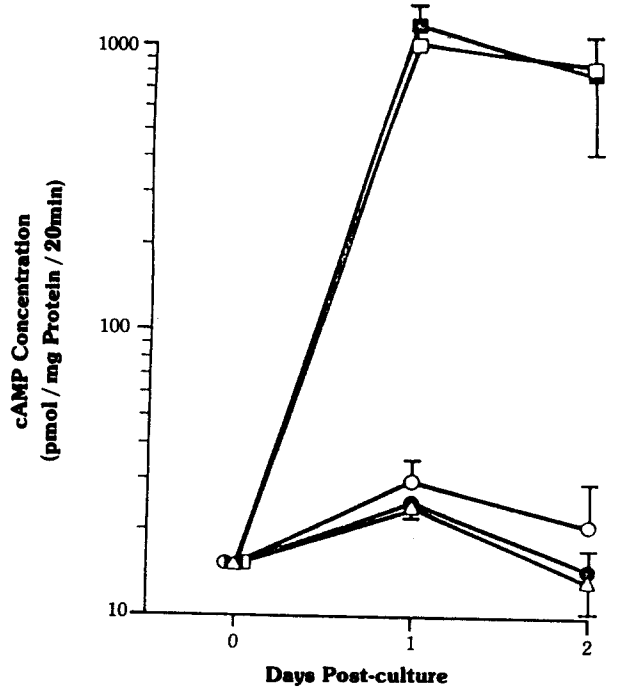

Fig. 3. Effect of CTX and adenosine on the cAMP concentration in the latently infected trigeminal ganglia. The latently infected ganglia were excised from mice, and then incubated in the culture medium containing no other chemicals (control group, ○), IBMX(0.5mM, ●), CTX(1ug/ml, □), CTX(3ug/ml, ■), or adenosine(500uM, △) for 0, 1, or 2 days. The ganglia were then processed to determine the cAMP concentration as described in the text. Each value represents the average of 10 samples.

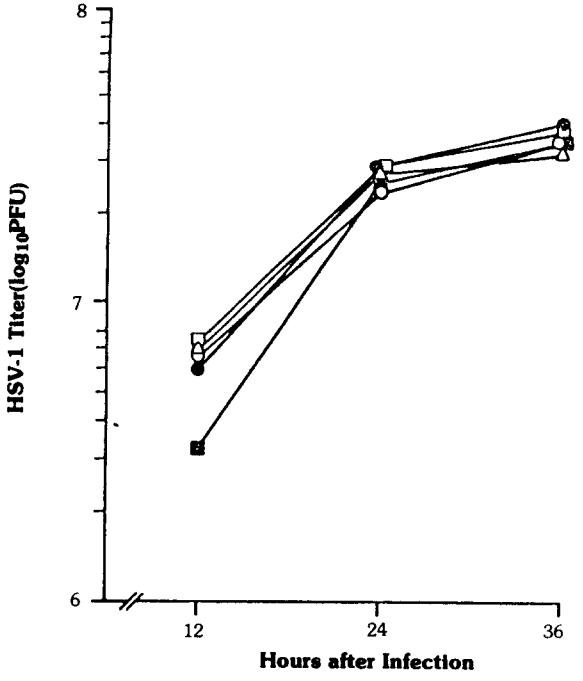

Fig. 4. Effect of CTX, db-cAMP and adenosine on the time-course *in vitro* replication of HSV-1 monolayers. Confluent CV-1 monolayers were inoculated with HSV-1 at a m.o.i. of 3 for 1hr., then washed with PBS. Medium containing no other chemicals(control group, ○), CTX(1ug/ml, ●), CTX(3ug/ml, □), db-cAMP(2mM, ■), or adenosine(500uM, △) was added to the inoculated cultures. All cultures were incubated for 12, 24, or 36hrs., and viral titers were determined as described in the text.

DISCUSSION

Our results imply that cAMP may accelerate the reactivation process of latent HSV in trigeminal ganglia. By directly activating adenyl cyclase, CTX causes marked stimulation of cAMP synthesis which may be associated with a massive production of reactivated HSV. The effect of db-cAMP was consistent with the results with HSV. Since we still do not know whether CTX and db-cAMP simply decrease the time necessary for HSV to undergo one cycle of replication, a time-course experiment was done for the further support of our results. It is possible that the increase in virus titer observed in ganglia explanted into CTX or db-cAMP containing media may be the result of more cycles of virus replication rather than an increase in the reactivation process. However, out data(Fig. 4) indicate that CTX and db-cAMP at the concentrations which increase the rate and number of infectious HSV from the latently infected ganglia, do not alter the time-course HSV-1 replication *in vitro*; thus, increased intracellular cAMP content does not directly affect viral replication in our experiment. In spite of the above result, the delay in appearance and the inhibition of the amount of reactivated HSV caused by adenosine are apparently not due to alteration of the intracellular cAMP level. Therefore, the intracellular mechanisms which initiate reactivation of latent HSV and the role of cAMP in the reactivation process need further study.

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