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Cyclic AMP Inhibits Developmental Regulation of *Chlamydia trachomatis*

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The effect of cyclic AMP (cAMP) on the chlamydial growth cycle was studied with *Chlamydia trachomatis*-infected HeLa cells. At concentrations of 1 mM, cAMP had a profound effect on the chlamydial developmental cycle, resulting in small, immature inclusions. Immunoblot analysis revealed the absence of elementary body (EB)-specific antigens in the cAMP-treated cells. This effect was observed only if cAMP was added within the first 12 h of incubation and continued thereafter. Its withdrawal at any time from the medium led to the reappearance of fully mature, infectious organisms. Analogs or breakdown products of cAMP exerted no inhibitory effect on chlamydial development. Intracellular inclusions from the cAMP-treated cells were unable to infect fresh HeLa monolayers, in contrast to the completely infectious nontreated inclusions. Protein profiles of the cAMP-treated organisms (at any time point) resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis very closely resembled reticulate bodies (RB) and did not possess characteristic EB-binding proteins. Collectively, these observations suggest an inhibitory role for cAMP at the RB stage of intracellular development. We also identified a cAMP receptor protein which is associated with RB and not with EB, further supporting a role for this system in the developmental regulation of chlamydiae.

Chlamydiae are obligate intracellular parasites of eucaryotic cells which have been implicated in a wide spectrum of human diseases (24). The developmental cycle of *Chlamydia trachomatis* proceeds by attachment to host cells, uptake, and enlargement of the infectious elementary bodies (EB) to form noninfectious, metabolically active reticulate bodies (RB). These RB divide within cytoplasmic inclusions by binary fission and then condense and subsequently rupture to release EB capable of infecting fresh host cells (16, 17).

The responsiveness of cells to a wide range of stimuli depends upon internal signalling systems such as cyclic AMP (cAMP) or cyclic GMP (cGMP) which regulate cellular activity. cAMP plays an important and multitudinous role in the regulation of both procaryotes and eucaryotes (6, 21). In procaryotes, cAMP mainly acts through a cAMP receptor protein (CRP) which is capable of binding two molecules of cAMP (2). Ward and Salari (30) postulated a bidirectional cyclic nucleotide control of chlamydial infections with low levels of cGMP acting as a stimulator and high levels of cAMP acting as an inhibitor. Prostaglandins E1 and E2 and other compounds known to alter cyclic nucleotide levels in the cell also affect the susceptibility of cells to chlamydial infections. The possible mechanisms of cAMP-mediated inhibition of chlamydial infection include: (i) inhibition of binding and uptake of chlamydiae by HeLa cells, (ii) interference with EB growth subsequent to uptake, or (iii) developmental inhibition at the RB stage.

To elucidate this mechanism, we added cAMP to the infectious system at different intervals after inoculation of HeLa cells with purified EB. cAMP was observed to act intracellularly by inhibiting developmental transformation of RB into EB. Further, CRP was found to be associated with RB and not with EB.

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MATERIALS AND METHODS

Organism. *C. trachomatis* serovar L_2 (L_2/434/Bu) was grown in HeLa 229 cells as described by Kuo et al. (13). Host-free intracellular RB and infectious EB were purified through Renografin (E. R. Squibb & Sons, Princeton, N.J.) at 18 and 48 h, respectively (13, 31). Infectivity of EB was determined by the method of Furness et al. (5).

^{3}H-labeled EB were prepared by growing serovar L_2 in the presence of 2 μCi of [^{3}H]isoleucine (New England Nuclear, Canada Ltd.) as described previously (31). Cells were harvested at 48 h postinfection, and EB were purified as described above.

Growth and inoculation of HeLa cells. HeLa monolayers were grown in 24-well tissue culture plates (Falcon) for 24 to 36 h in minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (FCS). The growth medium was aspirated, and the cells were washed with Hanks balanced salt solution (HBSS). Each adherent monolayer was inoculated with 2×10^5 inclusion-forming units of purified L_2 EB in a final volume of 200 μl of HBSS. The incubation was carried out at room temperature for 2 h with rocking. At the end of the incubation period, unbound EB were aspirated, and cells were refed with fresh medium containing 1 μg of cycloheximide per ml and 25 μg of gentamicin per ml and incubated at 37°C for 48 h.

Various treatments. cAMP or its breakdown products, viz., ATP, AMP, adenosine, or adenine (Sigma Chemical Co., St. Louis, Mo.) were added to the medium at different time intervals to give a final concentration of 10^{-3} M, unless otherwise stated. Ro 20-1724 (Roche Diagnostics, Div. Hoffmann-La Roche Inc., Nutley, N.J.), an inhibitor of phosphodiesterase, was added to the cells to a final concentration of 10 μg/ml with or without cAMP.

Replating of HeLa cells harboring chlamydiae. HeLa cells harboring EB infection for 24 h in the presence or absence of cAMP were removed by using 0.25% trypsin for 10 to 15 min at 37°C. The cells were washed, first with MEM supplemented with 10% FCS and then twice with HBSS. Cells

were spun at 300 × g for 5 min and suspended in MEM supplemented with 10% FCS followed by replating 2×10^5 to 3×10^5 cells in each well.

Isolation of intracellular inclusions for infectivity determination. Intracellular inclusions were harvested from infected HeLa cells at 40 to 48 h postincubation (inoculated with 2×10^5 inclusion-forming units of purified EB) in the presence or absence of cAMP. Essentially, each infected monolayer from eight wells was removed with glass beads, and the cells were broken by sonication. The whole suspension was spun down at 500 × g for 15 min to remove any unbroken cells and debris followed by further centrifugation at 15,000 × g for 30 min. The pellet was suspended in 500 μl of SPG (250 mM sucrose, 10 mM sodium phosphate, 5 mM glutamate buffer, pH 7.2) and dissociated by vigorous vortexing. An equal portion from each group (cAMP and non-cAMP) was used for determination of infectivity.

Inclusion counting. The effect of cAMP and its various breakdown products and analogs on inclusion development with a standard EB inoculum was determined after 48 h of incubation. Four different high-power microscopic fields were counted to score the number of inclusions in each field.

Preparation of chlamydial antisera. Hyperimmune rabbit antiserum to serovar L_2 purified EB was prepared as described earlier (12). Immunoglobulin G was purified by passing antisera through a protein A-Sepharose CL-4B (Pharmacia, Uppsala, Sweden) column and then eluting the bound immunoglobulin G with 0.1 N CH_3COOH. The specificity of antisera and immunoglobulin G was confirmed by immunoblot analysis. Both sera and immunoglobulin G were stored at −70°C.

Immunoblot analysis of EB antigens. Control and infected-cell monolayers with or without cAMP were washed twice with cold HBSS, dissolved in 200 μl of final sample buffer (containing 0.625 M Tris, 2% sodium dodecyl sulfate, 10% glycerol, 0.001% bromophenol blue, and 5% β-mercaptoethanol) and removed from microtiter wells. Samples were lysed by boiling for 3 to 5 min and then analyzed on 12.5% sodium dodecyl sulfate-polyacrylamide discontinuous gels (14). After electrophoresis the resolved polypeptides were transferred electrophoretically to nitrocellulose as described by Towbin et al. (28). The nitrocellulose blots were reacted with hyperimmune rabbit antiserum (1:100 dilution) to L_2 EB and then probed with ^{125}I-labeled protein A, washed, and autoradiographed.

Binding and uptake of ^{3}H-labeled EB. HeLa monolayers were grown in 24-well tissue culture plates in MEM supplemented with 10% FCS. After 48 h of incubation the growth medium was aspirated, and each adherent monolayer was inoculated with 5×10^3 dpm of ^{3}H-labeled EB in HBSS. Incubation was carried out at room temperature for 2 h with rocking followed by washing with HBSS and subsequent growth in MEM supplemented with 10% FCS and cycloheximide in the presence or absence of cAMP. At 24 h postincubation at 37°C, the incubation medium was aspirated, the cells were washed three times with phosphate-buffered saline, and the monolayer was removed by using 0.25% trypsin for 30 min at room temperature. The cells were removed from the wells, and then the wells were washed with 300 μl of 5% Triton X-100, and the combined cells and wash were counted in a scintillation counter.

Assay for [^{3}H]cAMP binding by CRP. The assay was carried out in 10 mM phosphate buffer (pH 7.7) at 1°C (1, 20). The assay involved the incubation of CRP samples (23,000 × g supernatant from French-pressed EB and sonicated RB) with [^{3}H]cAMP followed by precipitation with ammonium sulfate. Casein was added as a carrier for efficient precipitation. A solution containing 10 mM cold cAMP with [^{3}H]cAMP served as a control. The precipitates were collected on GF/C glass fiber filters and washed three times with 65% saturated ammonium sulfate. Each filter was transferred to a vial containing 10 ml of ethylene glycol monomethyl ether-Liquifluor (3:5, vol/vol) and then counted in a Beckman liquid scintillation counter. After subtraction of the control, 1 unit of cAMP-binding activity is defined as the binding of 1 pmol of cAMP by CRP.

RESULTS

Effect of cAMP on chlamydial development. cAMP at a concentration of 1 mM inhibited growth and development of intracellular inclusions in the HeLa cells as observed 48 h after inoculation with purified EB (Fig. 1a). The inclusions looked small and immature compared with those in control infected HeLa cells which contained fully developed inclusions (Fig. 1b). This inhibition was observed with between 0.5 and 1.5 mM cAMP.

The total number of inclusions in the control and treated groups revealed no significant differences; compared with 323 ± 50 inclusions per high-power field in controls, 320 ± 44 inclusions per high-power field were seen in the treated group. Surprisingly, a different picture emerged when these two groups were analyzed for EB-specific antigen expression. Figure 2 shows chlamydial antigen expression in the presence or absence of cAMP. In contrast to a normal antigenic expression at 48 h after inoculation in the control, the cAMP-treated group revealed no expression of chlamydial antigens by immunoblotting.

Antigenic expression as a function of addition of cAMP at different stages of the chlamydial growth cycle. Figure 3 shows the normal developmental profile of chlamydial specific antigens after inoculation of HeLa cells with EB. As observed, most of the major antigens are expressed only from 4 h onwards. In contrast, no antigen is expressed at any time if cAMP is added to the growth medium immediately after the inoculum (Fig. 4). Addition during different stages of the chlamydial growth cycle reveals that cAMP is effective only when added before 12 h postinoculation, after which it fails to block differentiation since most antigens are expressed normally when analyzed by immunoblotting (Fig. 4). Even chlamydial specific antigens that are expressed by 12 h during normal growth conditions fail to appear later (48 h) if cells are grown in the presence of cAMP from 12 h onwards.

Effect of cAMP analog, cAMP degradation products, and phosphodiesterase inhibitor on susceptibility of HeLa cells to EB infection. Dibutryl (db) cAMP, an analog of cAMP, failed to inhibit infection, as observed microscopically as well as antigenically. The possibility that degradation products of cAMP rather than cAMP itself are responsible for developmental inhibition of intracellular inclusions was explored by examining the effect of 5′-AMP, ATP, adenosine, and adenine at concentrations of 1 mM. No influence on infectivity was observed. Moreover, each treatment resulted in normal expression of chlamydial antigens, suggesting the modulatory effect of cAMP itself (data not shown).

Ro 20-1724, a powerful inhibitor of cyclic nucleotide phosphodiesterase, had no effect by itself (data not shown). Normal chlamydial infectivity of HeLa cells occurred. However, when added in conjunction with cAMP, no mature inclusions were observed, nor was any expression of chlamydial antigens seen, the same findings as observed with cAMP itself (data not shown).

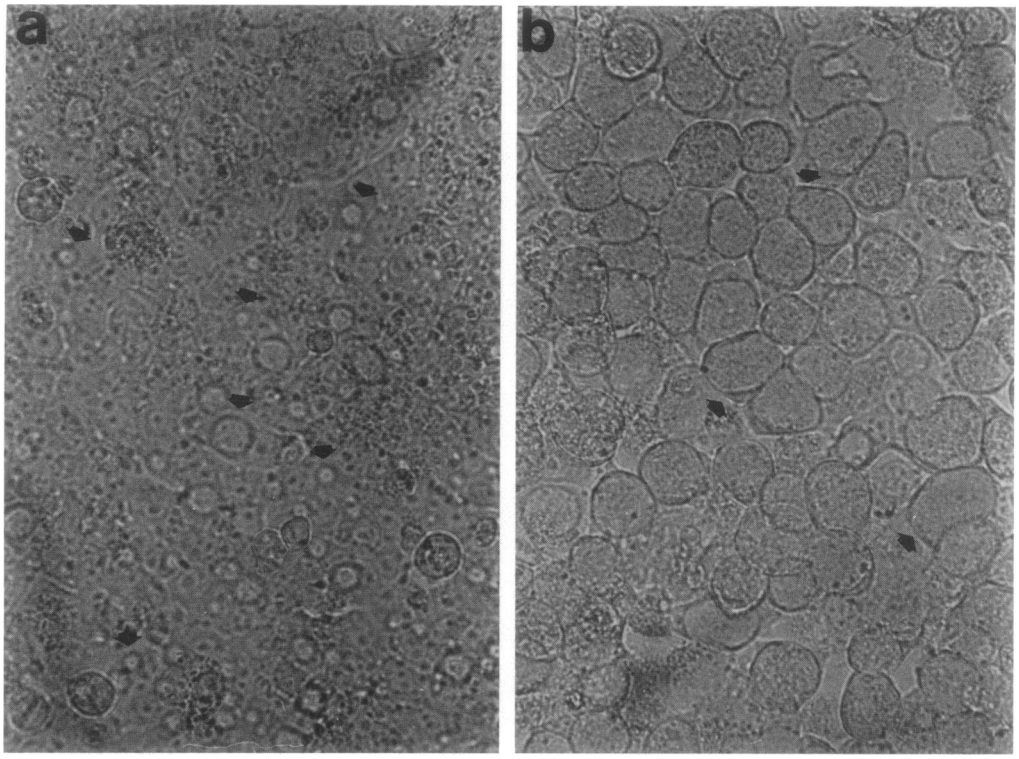

FIG. 1. Light microscopic view of HeLa cells infected with EB of *C. trachomatis* at 48 h after inoculation in the presence (a) or absence (b) of cAMP. Arrows indicate inclusions. Note the presence of small underdeveloped inclusions in panel a compared with the large, mature inclusions in panel b. (×375.)

Effect of cAMP withdrawal on subsequent infection and antigenic expression of chlamydia. cAMP withdrawal from the incubation medium at any time interval during development led to the resumption of normal infection. It required about 24 to 36 h after withdrawal before the reappearance of infection (Fig. 5). For example, in cells in which cAMP was present for the first 24 h and was withdrawn thereafter, infection appeared approximately 60 h from the time of initial inoculation (data not shown). However, no infection was observed in corresponding controls in which cAMP was present throughout (Fig. 5, lane 7).

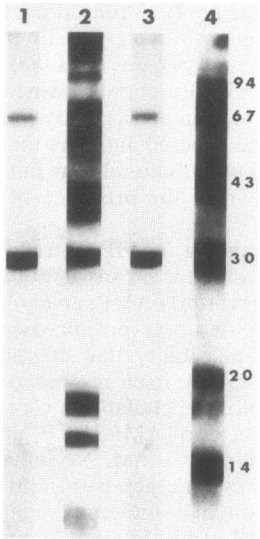

FIG. 2. Immunoblot analysis of *C. trachomatis*-specific antigenic expression 48 h postinoculation. Lanes 1 to 4 represent noninfected HeLa cells, infected HeLa cells in the absence of cAMP, infected HeLa cells in the presence of cAMP, and ^{125}I-labeled standard low-molecular-weight (LMW) markers, respectively. The standard markers were phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,000), and lysozyme (14,300).

Association of ^{3}H-labeled EB and effect of replating on preinfected HeLa monolayers grown in cAMP. To test the possibility that cAMP influences the binding and uptake of EB rather than intracellular development, we performed some association and replating experiments. Purified ^{3}H-labeled EB were incubated with HeLa monolayers for 2 h, and the infected monolayers representing bound and ingested EB were grown with or without cAMP. After 24 h both treated and nontreated groups revealed similar association values. Compared with 853 ± 40 dpm of ^{3}H-labeled EB associated with HeLa monolayers in controls, 869 ± 28 dpm were associated in the presence of cAMP.

For replating experiments, 24-h-old infected monolayers from treated and nontreated groups were trypsinized to remove the adherent monolayer and dissociate any noninternalized EB. Cells were replated on fresh plates, again in the presence or absence of cAMP. Cells which received cAMP continuously before and after replating showed the absence of any chlamydial antigens, while the ones which received cAMP before replating and not afterwards expressed a normal antigenic pattern similar to that of control cells.

Nature and characterization of intracellular inclusions grown in the presence or absence of cAMP. Intracellular inclusions (40 h old) were isolated from both the cAMP-treated and nontreated groups of HeLa cells. They were

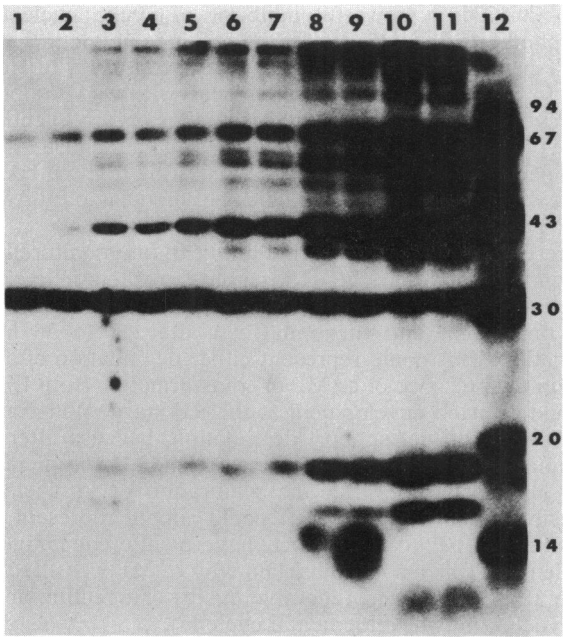

FIG. 3. Immunoblot analysis of *C. trachomatis* polypeptides expressed during the developmental cycle. Lanes 1 to 11 represent samples taken at 0, 2, 4, 6, 8, 10, 12, 18, 24, 36, and 48 h, respectively, while lane 12 represents ^{125}I-labeled LMW standards as described in the legend to Fig. 2.

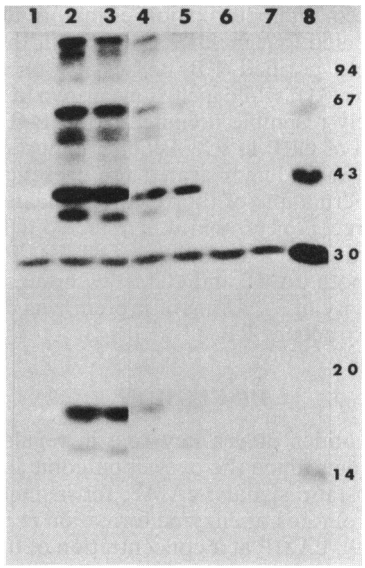

FIG. 5. Effect of cAMP withdrawal on antigenic expression. cAMP was added to the inoculated cells at 2 h, followed by withdrawal and replacement with fresh medium. Cells were allowed to grow until 48 h, and samples were then analyzed by immunoblotting. Lane 1 represents uninfected HeLa cells. Lanes 2 to 7 show withdrawal of cAMP at 0, 6, 12, 24, 36, and 48 h, respectively. Lane 8 represents ^{125}I-labeled LMW standards as described in the legend to Fig. 2.

tested for their ability to infect fresh HeLa cells. Mature inclusions isolated from nontreated HeLa cells were highly infectious. Nearly 300 inclusions per high-power field were visualized 48 h postinoculation. In contrast, equivalent inocula of immature inclusions from cAMP-treated HeLa cells were completely noninfectious.

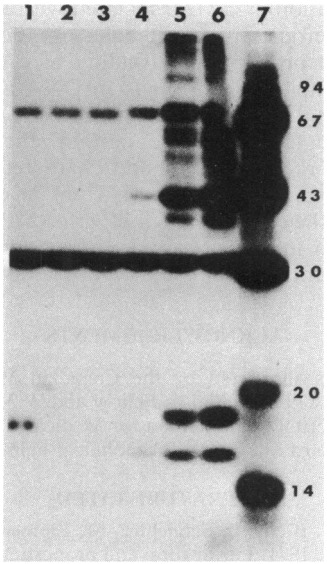

FIG. 4. Effect of addition of cAMP at different time intervals on the developmental cycle. Immunoblot analysis was carried out at 48 h on samples treated with cAMP during different time intervals. Lanes 1 to 6 represent cAMP addition at 0, 2, 6, 12, 24, and 48 h postinoculation, respectively, while lane 7 represents ^{125}I-labeled LMW markers as described in the legend to Fig. 2.

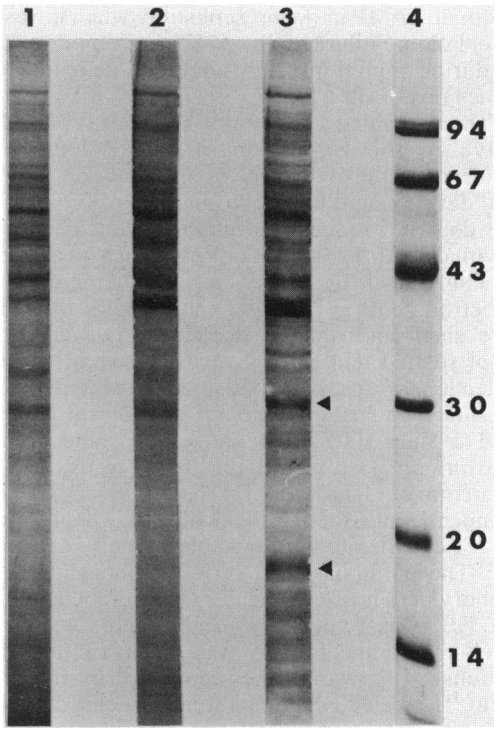

FIG. 6. Coomassie blue-stained sodium dodecyl sulfate-polyacrylamide gel analysis of isolated intracellular inclusions grown in the presence of cAMP. Lanes 1 to 4 represent purified RB, isolated intracellular inclusions grown in the presence of cAMP, purified EB, and LMW markers, respectively (LMW markers are described in the legend to Fig. 2). Note the presence of 31,000- and 18,000-molecular-weight binding proteins in lane 3 which are absent in lanes 1 and 2.

Further protein characterization of mature and immature inclusions revealed that inclusions grown in the absence of cAMP resemble purified EB possessing eucaryotic cell-binding proteins (31), while inclusions grown in the presence of cAMP closely resemble noninfectious RB (Fig. 6).

Identification of CRP in RB. Binding experiments carried out with [^{3}H]cAMP demonstrated the presence of CRP in 23,000 × *g* supernatants of thoroughly sonicated RB. However, no such receptor protein was detected in supernatants from either sonicated or French-pressed EB. This CRP was found to bind both cAMP and cGMP with the same affinity. A specific activity of 3.2 U/mg of protein was demonstrated in the crude extracts of RB.

DISCUSSION

Cyclic nucleotides play a key role in regulating cellular activity. They determine the overall outcome in response to various homeostatic signals; cAMP, for instance, has been shown to control rates of enzyme formation in both bacteria and fungi (2, 19). cAMP at a concentration of 0.5 to 1.5 mM inhibited developmental regulation of *C. trachomatis* when added to the culture medium up to the 12th hour of EB infection. Ward and Salari (30) postulated a bidirectional cyclic nucleotide control with low levels (10^{-6}) of cGMP acting as a stimulator and high levels (10^{-3}) of cAMP acting as an inhibitor of chlamydial infection. However, they observed an inhibitory effect only when HeLa cells were preincubated with db cAMP before inoculation with EB. Further, db cAMP failed to mimic the effect of cAMP if added during or after inoculation. Similar results were obtained with db cAMP in the present study when it was added after inoculation. Failure of db cAMP to inhibit development if added during or after infection could be due to inactivation of enzymes responsible for the breakdown of butyrate from cAMP. The butyrated compound has been reported to be ineffective in the regulation of cAMP-dependent β-galactosidase synthesis in *Escherichia coli* (22) and photosynthetic development in *Rhodospirillum rubrum* (27).

cAMP does not reduce the number of inclusions as observed with db cAMP (30), but morphological characteristics of intracellular inclusions are altered. Compared with the large, electron-dense vacuoles of control cells, treated inclusions are small and underdeveloped. However, antigenic profiles of treated and control inclusions may represent a true reflection of the chlamydial developmental process. Despite similar inclusion numbers, the control group demonstrated a fully developed antigenic profile, while the treated group revealed no EB-specific antigens.

Studies from our laboratory (11) have shown that by about 4 h postinoculation EB enter the host cell by dephosphorylation of host-cell membranes and subsequently express their antigens. The possibility that cAMP rephosphorylates the dephosphorylated host-cell membrane, thereby blocking the entry of EB, was refuted. No rephosphorylation of host HeLa cell membrane protein was observed (unpublished observations). This was further supported by the fact that equivalent ^{3}H-labeled EB counts were associated with HeLa cells 24 h after infection in both control and treated groups.

Trypsin-insoluble HeLa cells have been used as a measure of internalized EB (4, 26). Using the same approach we trypsinized 24-h-old infected HeLa cells and replated them on fresh plates. These plated cells were then grown in the absence of cycloheximide. Complete infection was observed after 48 h, confirming that EB were clearly lodged inside HeLa cells in the presence of cAMP.

Ro 20-1724, a powerful inhibitor of cyclic nucleotide phosphodiesterase (25), when added in conjunction with cAMP had an inhibitory effect on the susceptibility of HeLa cells to EB infection (as was observed with cAMP alone). However, Ro 20-1724 alone had no effect on the infectious process. Similarly, 1 mM AMP, ADP, ATP, adenosine, and adenine had no inhibitory effect, suggesting that the cAMP effect was a specific event and was not modulated by AMP, the degradation product of cAMP (10).

Interestingly, the EB-specific antigens (as monitored by immunoblotting) that were observed by 12 h in control infections disappeared if cells were treated with cAMP from that time point and then analyzed after 24 to 36 h of incubation. This could represent either degradation of antigens in the presence of cAMP or transformation from EB to RB and arrested development at the RB stage. Withdrawal experiments do not support degradation, because after removal of cAMP from the culture medium, EB-specific antigens became visible with the maturation of intracellular inclusions. The disappearance could simply represent the transition of EB to RB, which have a different antigenic profile (31). This may also explain why cAMP was effective up to 12 h, because of repetitive cycles of infection and a nonsynchronous developmental cycle (7).

Replating and withdrawal experiments have shown that as long as these immature inclusions are intracellular they can complete their developmental cycle when and if cAMP is withdrawn. However, chlamydiae isolated from cAMP-treated cells failed to infect fresh HeLa cells, and no infection was observed 40 h after inoculation. These cAMP-treated organisms behave like RB which are noninfectious and nonreplicative in the extracellular environment and replicative in an intracellular environment.

Recently, two groups (8, 31) have demonstrated the association of binding proteins with EB and not with RB. Protein profiles of intracellular inclusions isolated from the cAMP-treated group revealed the absence of either binding protein (18,000 and 31,000 molecular weight). Further, the protein pattern was identical to that of RB (9, 31).

The identification of CRP associated with RB is intriguing CRP has numerous important roles in the regulation of transcription in procaryotes. Binding of cAMP to a CRP results in a complex which binds to DNA at the promoter region of cAMP-dependent operons, thereby either stimulating or repressing transcription of certain genes (3, 15, 18, 23, 29). Further work on isolation and characterization of CRP from RB is currently being carried out in our laboratory. Once isolated, the question of whether cAMP really binds to this receptor to inhibit chlamydial development and what role it plays in the natural cycle can be addressed.

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