Both cytomegalovirus (CMV) and *Chlamydia pneumoniae* have been associated with atherosclerosis. CMV is believed to exist in host tissues in a latent state with periodic reactivation. This study was designed to determine whether *C. pneumoniae* infection stimulates the expression of CMV genes. Transactivation of the CMV major immediate early promoter (MIEP) is essential for viral gene expression and viral replication. HeLa cells were transfected with a construct containing a reporter gene (chloramphenicol acetyl transferase) controlled by the MIEP. The cells were then infected with *Chlamydia* at $10^2$–$10^6$ infection-forming units (IFU) per well at various times before assay of MIEP activity (72 h after transfection). Peak transactivation occurred 6 h after infection at $10^4$ IFU. *C. pneumoniae* increased MIEP activity in a dose-response manner; maximal increase was >2-fold. These results suggest that if CMV and *C. pneumoniae* do indeed contribute to atherosclerosis, their copresence may synergistically contribute to it.

In immunocompetent hosts, CMV persists in host tissues for life, presumably in a latent state in which no, or few, viral gene products are expressed. However, the virus could contribute to atherosclerosis if it undergoes periodic reactivation during which time either it expresses its full complement of gene products and replicates or it expresses only its immediate early genes (abortive infection); even the limited viral gene products expressed during an abortive infection have the capacity to elicit cellular changes that could be atherogenic [6, 7, 11]. It therefore becomes important to identify those factors that can reactivate latent virus or that can augment viral gene expression from existing low, and perhaps biologically inconsequential, levels of expression.

We reasoned that, if infection contributes to atherosclerosis, it would be highly unlikely that any single pathogen would be solely responsible; rather, multiple pathogens would contribute to the disease process. In this investigation, we tested the generic hypothesis that, when a host cell harboring, in a latent state, one of the several pathogens implicated in atherogenesis is newly infected with another such pathogen, the new infection will increase the capacity of the latent pathogen to express its gene products. This generic hypothesis was tested by transfecting cells with a plasmid construct containing a reporter gene controlled by the major immediate early promoter (MIEP) of CMV. Because activation of the MIEP is critical for CMV gene expression and thereby for viral activity, we were able to determine whether *C. pneumoniae* infection of such cells augmented MIEP activity.
Materials and Methods

Cells. The HeLa-229 cell line (American Type Culture Collection [ATCC], Rockville, MD) was used for transfection experiments. We chose this cell line because of its high efficiency for transfection and its permissivity for C. pneumoniae. HeLa cells were cultured in RPMI 1640 medium (BioWhittaker, Walkersville, MD) as described elsewhere [5], supplemented with 1% lysine (BioWhittaker), HEPES buffer 20 mM (Quality Biologics, Camden, NJ), mycostatin 12.5 IU/mL (Sigma, St. Louis), vancomycin 12.5 μg/mL (Sigma), gentamicin 5 μg/mL (Bethesda Research Laboratories, Gaithersburg, MD), and 10% fetal bovine serum (FBS; Bethesda Research Laboratories).

C. pneumoniae. TWAR strain (TW-183) of C. pneumoniae was purchased from ATCC and propagated in HEp-2 cells (ATCC). The infectivity titration of C. pneumoniae was performed on HEp-2 cells in 96-well microtiter plates by immunofluorescence staining. Infectivity was recorded according to the induction of positive cells in 96-well microtiter plates by immunofluorescence staining. The infectivity titration of C. pneumoniae was performed on HEp-2 cells in 96-well microtiter plates by immunofluorescence staining. Infectivity was recorded according to the induction of positive staining by serial 10-fold dilutions of the sample and expressed as infection-forming units (IFU). Stock C. pneumoniae (10^3–10^6 IFU/0.1 mL) was stored at −80°C until use.

Plasmids. We investigated the capacity of C. pneumoniae infection to stimulate the transcription of a chloramphenicol acetyl transferase (CAT) reporter construct controlled upstream by the CMV MIEP. The plasmid pHDI101CAT3, containing a 2.1-kb region of the human CMV MIEP fused to the CAT reporter gene, was provided by E. S. Huang (University of North Carolina, Chapel Hill, NC) [12].

Transfection. HeLa cells (2.5×10⁴ cells/well) were plated in 6-well plates and cultured in growth media for 24 h (~50% confluence). The cells were then washed with 2 mL Optimem media (Life Technologies, Gaithersburg, MD) and transfected in triplicate with 0.1 μg MIEP-CAT plus 0.9 μg carrier DNA (Bethesda Research Laboratories) and 7.5 μL lipofectamine reagent (Life Technologies) in 1 mL Optimem media per well for 5 h, according to the lipofectamine manufacturer’s instructions. The cells were then washed 3 times with Optimem media, and CMGA media containing 1% FBS were added to the cultures.

Stimulation of transfected cells with C. pneumoniae. After transfection with MIEP-CAT, the HeLa cells were infected with C. pneumoniae. The experiments were performed in 6-well plates, and each well contained ~8×10⁴ cells when the cells were infected with C. pneumoniae. To detect the maximal effect of infection on CAT activity, the cells were infected with C. pneumoniae at 10^5–10^7 IFU per well (in a volume of 1 mL) for 6 h (absorption was enhanced by centrifugation for 30 min). For a negative control, cells were mock infected with C. pneumoniae-free solution under the same conditions (C. pneumoniae was removed by filtering through a 0.1-μm filter system of the same C. pneumoniae stock). For a positive control, 10% FBS in CMGA media was added (FBS stimulates MIEP-CAT), and cells were cultured for another 48 h.

To detect the time course of changes in CAT activity after infection, the cells were infected with 10^4 IFU C. pneumoniae per well for 3, 6, 9, 12, and 24 h, at which points the CAT assay was performed. The controls described in the preceding paragraph were used.

CAT assay. Cells were gently washed 3 times with ice-cold PBS and then were exposed to 1 mL cell-lysing buffer (40 mM Tris [pH 7.5], 15 mM NaCl, 1 mM EDTA) for 10 min. The cells were then collected by scraping, transferred into Eppendorf tubes, and centrifuged for 4 min at 1000 g. The supernatant was discarded, and 100 μL of 0.25 M Tris (pH 8.0) was added to the tube to resuspend the cell pellet. The cell suspension was then subjected to 3 freeze-thaw cycles (ethanol–dry ice for 3 min followed by a water bath at 37°C for 3 min). Endogenous acetyl transferase was inactivated by heating at 60°C for 10 min. After spinning for 10 min at 14,000 g at 4°C, the lysate was then transferred into new microtubes. CAT activity was measured according to the published method [13]. In brief, the final reactive mixtures containing 5 μg protein lysate were mixed with 80 μL of reaction mixture (5 μL N-n-butyl CoA [Sigma], 3.5 μL 14C-chloramphenicol [0.1 μCi/mL; ICN, Costa Mesa, CA], and 71.5 μL Tris 0.25 M [pH 8.0]) and incubated for 30 min at 37°C. Each tube received 300 μL of 2:1 TMPD : xylene mixture and was vortexed for 15 min. After spinning at 14,000 g at room temperature for 10 min, 100 μL supernatant was transferred into new microtubes and then counted in a liquid scintillation counter for CAT activity. The results are the average counts per minute (cpm) of triplicate samples.

Statistical analysis. Standard 2-tailed Student’s t test was used for statistical analysis of the data.

Results

Dose response of C. pneumoniae infection on MIEP-CAT. In the dose-response experiment, we found that MIEP-CAT activity (indicated by cpm) increased as the number of IFU of C. pneumoniae increased from 10^2 to 10^6. The stimulation showed a linear dose response of 10^3–10^4 IFU per well, with peak stimulation occurring at 10^4 IFU per well (figure 1, column 3).
Discussion

The results of this investigation show that 2 infectious agents linked to the development of atherosclerosis, CMV and C. pneumoniae, have the capacity to interact in a host. Because of the interaction, infection with one pathogen has the capacity to increase gene expression of the other. CMV gene expression is critically controlled by the activity of the MIEP of the virus; stimulation of the MIEP is essential for reactivation of the virus from latency and for expression of all the gene products of the virus. Our data show that Chlamydia infection significantly increases MIEP activity in a cell previously transfected with a plasmid containing a reporter gene under the control of the MIEP.

The ability of one pathogen to affect the activity of another is not unique to C. pneumoniae and CMV. For example, previous studies have established that CMV infection of cells harboring latent HIV activates the latent virus [14]. It is therefore possible, on a more generic basis, that cells infected with latent pathogens, or with pathogens expressing genes at low and perhaps biologically insignificant levels, can be stimulated by infection with a second pathogen, which augments the activity of the resident pathogen. Such a paradigm could be one mechanism by which reactivation of latent viruses occurs.

These results also extend the concept generated by previous studies in which we showed that the “aggregate pathogen burden” contributes to increased risk of coronary artery disease [15]. The aggregate pathogen burden not only predicted risk of coronary artery disease but also was directly related to C-reactive protein levels, a marker of inflammation and a predictor of subsequent cardiovascular events. The current findings therefore suggest that multiple pathogens may contribute to the atherogenic process, not only by the direct interactions between the specific pathogen and the host but also by interactions among pathogens, whereby the copresence of one may augment the activity of the other. Such pathogen interactions may lead to synergistic effects on the atherogenic processes.

One limitation of the present study is that to simplify the transfection procedures, HeLa cells were used. These cells are easily transfected with plasmid DNA and are easily infected with C. pneumoniae. It would be important to determine whether such interactions occur in intact animals, because the intracellular milieu of HeLa cells may have facilitated an interaction that does not occur under more biologically relevant conditions. In addition, although we indicate throughout that the results we have observed on MIEP activation reflect the effects of Chlamydia infection, we should emphasize that infection might not be necessary; it is possible that exposure of the transfected HeLa cells to chlamydial antigens per se could have been responsible for triggering the signaling cascades ultimately leading to MIEP activation.

In summary, this investigation has shown proof of the concept that 2 pathogens commonly considered to contribute to atherogenesis have the capacity to interact, such that Chlamydia infection of a cell that harbors CMV can transactivate the MIEP of the virus and, presumably, can reactivate latent virus or augment existing levels of viral gene product expression or both. Insofar as these gene products facilitate atherogenesis, our results create a model showing how infection with one pathogen may exacerbate the vasculopathic effects of a second pathogen.

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References


