Aspirin Inhibits Chlamydia pneumoniae–Induced Nuclear Factor-κB Activation, Cytokine Expression, and Bacterial Development in Human Endothelial Cells

Andreas Tiran, Hans-Jürgen Gruber, Wolfgang F. Graier, Andreas H. Wagner, Ellen B.M. van Leeuwen, Beate Tiran

Objective—Chlamydia pneumoniae has been associated with atherosclerosis. Infection of vascular endothelial cells with C pneumoniae increases the expression of proatherogenic cytokines mediated by nuclear factor (NF)-κB, a transcription factor. The present study was designed to test the effect of aspirin on C pneumoniae–induced NF-κB activation, interleukin expression, and bacterial development in cultured human endothelial cells.

Methods and Results—Aspirin, its metabolite salicylic acid, and 2 other unrelated NF-κB inhibitors showed a strong concentration-dependent inhibitory effect on chlamydial growth, indicated by the reduction of bacterial inclusions and the titer of infectious progeny. Involvement of the transcription factor NF-κB was confirmed by electrophoretic mobility shift assay and by transfection experiments with appropriate decoy oligodeoxynucleotides. Attenuation of the C pneumoniae–induced activation of NF-κB by aspirin also reduced the secretion of interleukin-6 and interleukin-8, indicating efficient inhibition of NF-κB gene expression. Reduction of chlamydial growth was not caused by apoptosis of the host cell, as determined by monitoring characteristic chromatin condensation.

Conclusions—These data provide evidence that NF-κB–mediated gene activation represents a crucial step in the developmental cycle of C pneumoniae. Aspirin exerts an anti-chlamydial effect that is due to the inhibition of C pneumoniae–induced NF-κB activation, which might account for some of the cardioprotective activity of aspirin.

Key Words: aspirin ■ transcription factor ■ Chlamydia pneumoniae ■ nuclear factor-κB

The obligate intracellular bacterial pathogen Chlamydia pneumoniae causes acute and chronic respiratory disease. Recently, there has been increasing evidence that chronic or recurrent chlamydial infections of vessel walls might contribute to atherogenesis and, possibly, to decreased plaque stability and acute coronary syndromes. However, the molecular mechanisms by which C pneumoniae might initiate or promote plaque development are poorly understood. C pneumoniae is known to infect and replicate in cell types found within the atherosclerotic lesion, including endothelial cells (ECs), smooth muscle cells, and macrophages. Infection of these cells results in the increased expression of proinflammatory cytokines and adhesion molecules. The activation of nuclear factor (NF)-κB in the host cell obviously plays a crucial role in this bacteria-induced target cell activation. NF-κB contains a family of cellular transcription factors that get activated in response to a number of stimuli, including bacterial lipopolysaccharide, phorbol esters, interleukin (IL)-1, and tumor necrosis factor-α. NF-κB dimers, most commonly composed of the RelA (p65) and NF-κB1 (p50) or NF-κB2 (p52) subunits, are sequestered in an inactive cytoplasmic complex by binding to its inhibitory subunit, IκB. Phosphorylation by IκB kinase, a cellular kinase complex, on the stimulation and subsequent degradation of IκB leads to translocation of NF-κB to the nucleus, where it binds to specific DNA sequences and regulates the transcription of specific genes. Transcription factors of the NF-κB/Rel family are critical for the inducible expression of multiple genes involved in inflammatory responses and apoptosis.

The present study was designed to elucidate whether the activation of NF-κB represents a crucial step in the chlamydial growth cycle. The effect of aspirin and its metabolite sodium salicylate (both known inhibitors of NF-κB) on C pneumoniae–induced NF-κB activation, cytokine expression, and bacterial growth cycle was assessed in human ECs.
Methods

Materials
Aspirin (acetylsalicylic acid), sodium salicylate, and pyrroline dithiocarbamate (PDTC) were obtained from Sigma Chemical Co. Bay-117082 (BAY), an inhibitor of Ix-B-α phosphorylation, was from Alexis.

Cell Culture and Bacterial Strain
Generation and characterization of the EC line EVLC2, a cell line derived from human umbilical vein ECs by immortalization with simian virus 40 large T antigen, is described elsewhere. ECV-304 (American Type Culture Collection [ATCC] CRL-1998) and HEp-2 (ATCC CCL 23) cells, 2 epithelial cell lines, were obtained from ATCC. EVLC2, ECV-304, and HEp-2 cells were maintained in RPMI 1640 medium, medium 199, and MEM-α medium, respectively (GIBCO-BRL). All media were supplemented with 2 mmol/L L-glutamine, 10% FCS (PAA Laboratories), 1 mg/mL vancomycin (ICN), and 1 mg/mL streptomycin sulfate (Sigma). For EVLC2 cells, EC growth supplement (Technoclone) was added to a final concentration of 50 μg/mL. Cycloheximide (1 μg/mL) was added only for the propagation of bacteria in HEp-2 cells. Normal human umbilical vein ECs (HUVECs) were obtained from Technoclone and maintained in EC growth medium supplemented with EC growth supplement (Technoclone). Only cells up to the fourth passage were used for experiments.

*C pneumoniae* strain 2023, a kind gift from Dr J. Ossewaarde (National Institute of Public Health and the Environment, Bilthoven, the Netherlands) was propagated and titrated in HEp-2 cell monolayers, as described previously.

Growth and Staining of *C pneumoniae*
Cells were grown to near confluence in 12-well tissue culture plates. One-day-old monolayers were infected with *C pneumoniae* by centrifugation (1 hour, 10°C, 2400g) in a reduced volume of the respective cell-specific growth medium. The titer of inocula used was in the range of 1 to 5 inclusion-forming units (IFU) per cell, resulting in a multiplicity of infection of 0.7 to 1. After infection, the inocula were replaced by growth media containing the various test drugs at different concentrations. In a series of experiments, a possible effect of pretreatment on the outcome parameters investigated or an irreversible effect of the studied drugs was excluded (data not shown). Media were replaced every 24 hours. Positive control infections without added drug and negative controls with mock-infected cells were always run in parallel. Cultures were incubated for 72 hours at 35°C in a humidified atmosphere containing 5% CO2. Chlamydia inclusion were visualized by staining methanol-fixed ECs (HUVECs) from Technoclone and maintained in EC growth medium supplemented with EC growth supplement (Technoclone). Only cells up to the fourth passage were used for experiments.

Determination of Inclusion Count and of Titer of Infectious Progeny
The effect of inhibition of NF-κB on chlamydial growth was defined in terms of the reduction of visible inclusion bodies and in terms of the decrease in infectious progeny titers. The reduction of inclusions was expressed as percent inhibition, calculated as follows: [(inclusions of control)−(inclusions of treated sample)]/inclusions of control)×100. Inclusion counts represent average numbers of inclusion bodies per field at a magnification of ×400 determined from 10 fields per well from triplicate samples. To assess the increase or decrease in infectious progeny titers, infected ECs and their growth media were sonicated 72 hours after infection (hpi). This suspension was diluted 4-fold with fresh medium and inoculated in triplicate on indicator HEp2 cells. Inclusions were visualized and counted 72 hours later, as described above. Titers were expressed as IFU per milliliter.

Deconvolution Microscopy
The effect of aspirin and other NF-κB inhibitors on the morphology of chlamydial inclusions was studied by high-resolution image analysis with the use of deconvolution microscopy after staining with a genus-specific fluorescein-labeled monoclonal antibody. Briefly, at 72 hpi, *C pneumoniae*-infected cells were washed twice with PBS (pH 7.4) and fixed in methanol for 10 minutes. The cells were then incubated for 30 minutes with the monoclonal antibody (PathoDX, DPC) at a final dilution of 1:200 and counterstained with Evans blue.

Electrophoretic Mobility Shift Assay
For electrophoretic mobility shift assay (EMSA), cells were harvested at 6 hpi. Preparation of nuclear extracts, labeling, and binding reaction were carried out as described previously. The double-stranded gel shift oligonucleotides for NF-kB, NF-kBmut and activator protein (AP)-1, and the appropriate gel supershift antibodies were obtained from Santa Cruz Biotechnology. EMSAs were repeated 3 times, and representative gels are shown.

Decoy ODN Technique
Double-stranded decoy oligodeoxynucleotides (ODNs) were prepared from the complementary single-stranded phosphorothioate-bonded ODNs (Eurogentec) by melting at 95°C for 5 minutes, followed by a cool-down phase of 3 hours at ambient temperature. The efficiency of the hybridization was checked on 2.5% agarose gels containing 0.1% ethidium bromide and usually found to exceed 85%.

To measure the cytokine secretion from individual monolayers, the supernatant medium was removed at 48 hpi and stored at −70°C until tested. IL-6 and IL-8 were determined by enzyme immunoassay with signal detection by chemiluminescence, as described by the manufacturer (Quantiglo Immunoassay, R&D Systems).

Determination of IL-6 and IL-8 in Supernatant
To measure the cytokine secretion from individual monolayers, the supernatant medium was removed at 48 hpi and stored at −70°C until tested. IL-6 and IL-8 were determined by enzyme immunoassay with signal detection by chemiluminescence, as described by the manufacturer (Quantiglo Immunoassay, R&D Systems).

Apoptosis
Induction of apoptosis was shown by fluorescent staining of nuclear DNA with fluorescent bisbenzimide dye Hoechst 33342 (Calbiochem) at 12, 24, and 48 hpi with *C pneumoniae*. The cells grown on glass coverslips in 12-well plates were washed twice with HBSS and incubated with fixative buffer (5% formaldehyde in 145 mmol/L NaCl and 10 mmol/L HEPES-KOH, pH 7.5) for 20 minutes at room temperature. Next 33342 was added at a final concentration of 10 μg/mL for an incubation period of 20 minutes. Thereafter, the buffer was replaced by 50% glycerol in HBSS. Nuclear staining intensity and morphology were evaluated optically. As a control experiment for the test performance, EVLC2 cells were incubated for 6 to 8 hours with 2 μg/mL cycloheximide and 40 ng/mL tumor necrosis factor (Boehringer Biochemicals). This treatment induced morphological changes typical of apoptosis in a high percentage of ECs (data not shown). Nuclear morphology was assessed with the use of a fluorescence microscope. At least 300 nuclei per sample were counted in each of 4 independent experiments.
Statistical Analysis

Statistical analysis was performed on a personal computer with use of the SPSS software package (version 10.0). All values are expressed as mean±SEM; IC50 values represent mean and 95% CIs. The Student unpaired t test was used to determine differences between the means. For comparison between multiple drug concentrations, data were analyzed by ANOVA and the Dunnett post hoc test. All tests were 2-sided, and values of P<0.05 were considered significant.

Results

Effect of Aspirin on Chlamydial Growth

Figure 1 (top) shows infection of human EVLC2 cells at 72 hpi by conventional fluorescence microscopy. Large intracellular inclusion bodies are visible in the control infection; they contained actively replicating C pneumoniae. Treatment with a low concentration of aspirin (0.5 mmol/L) exerted a significant effect on the morphology of intracellular inclusion bodies. Inclusions were smaller (median [25% to 75% percentile] of inclusion area was 32 [16 to 49] μm2 versus 35 [19 to 53] μm2 in controls, P=0.017), sometimes irregularly shaped, and often of dull appearance. Sodium salicylate, PDTC, and BAY had similar effects (data not shown). At higher concentrations, the effect on the morphology of the inclusions became more distinct. Figure 1 (bottom) shows high-resolution images of C pneumoniae–infected ECs at 72 hpi. A well-developed inclusion body is visible in the control infection, whereas treatment with high concentrations of aspirin (2.5 mmol/L), salicylic acid (2.5 mmol/L), or PDTC (25 μmol/L) resulted in small, barely stained, disintegrated inclusions. All these drugs showed a clear concentration-dependent inhibitory effect on chlamydial growth (P<0.001). The number of inclusions and the titer in infectious progeny at 72 hpi were reduced significantly, as shown in Figure 2A and 2B. Inclusions were reduced, with IC50 values of 1.27 (0.88 to 1.83) mmol/L, 0.82 (0.71 to 0.96) mmol/L, and 25.3 (19.3 to 33.6) μmol/L for aspirin, salicylic acid, and PDTC, respectively. The titer of infectious progeny was inhibited with similar potency. This was not due to a direct toxic effect of the drugs on the host cells because the percentage of dead cells in our experiments did not differ between treated and untreated samples (Figure 2A). Essentially the same results were obtained in HUVECs (please see online Figure I, available at http://atvb.ahajournals.org) and ECV-304 cells (data not shown).

Growth-Inhibiting Effects of Aspirin Are Specific to NF-κB Inhibition

The effect of the drugs on transcription factor activation was investigated by EMSA. Results are shown in Figure 3. NF-κB
activity was increased at 6 hours after \textit{C pneumoniae} infection. Only weak NF-κB DNA binding activity was seen in mock-infected cells. Competitive inhibition by unlabeled NF-κB oligonucleotide, but not by an AP-1 DNA binding site, and the missing binding to the mutated NF-κB site suggest specificity of the interaction. Supershift experiments with p65 and p50 antibodies showed that these components of the NF-κB family were activated by \textit{C pneumoniae}.

All investigated drugs reduced activation of NF-κB in infected cells to a level comparable to that of mock-infected control cells. To further support the finding that NF-κB may contribute to the life cycle of chlamydial infection, the decoy ODN technique was used. Transfection of the infected ECs with the NF-κB–specific decoy ODN inhibited chlamydial growth by 50±2.6% (P<0.001), whereas the mutated decoy ODN had no effect on chlamydial growth (1±2.3% inhibition, P=NS). These data represent mean±SD values of 3 independent experiments.

**Aspirin Treatment Prevents \textit{C pneumoniae}–Induced IL Expression**

To confirm the ability of aspirin in the functional inhibition of NF-κB activation in infected ECs, we next investigated the effect of aspirin on the expression of IL-6 and IL-8, with both under the transcriptional control of NF-κB.

It has been shown previously that infection of ECs with \textit{C pneumoniae} induces secretion of IL-6 and IL-8, that maximal cytokine induction occurs by 48 hpi, and that it is sustained for the entire 72-hour study period.\textsuperscript{11} Figure 4 shows IL-6 and IL-8 concentrations in the supernatant of EC cultures at 48 hpi. \textit{C pneumoniae} infection induced IL-6 and IL-8 concentrations nearly 3- and 6-fold, respectively. Treatment of cell cultures with aspirin or sodium salicylate reduced IL-6 and IL-8 levels in a dose-dependent manner.

**Inhibition of Chlamydial Growth Is Not Caused by Apoptosis**

To investigate whether the inhibition of chlamydial growth was indeed related to reduced bacterial proliferation or whether the cells were driven to apoptosis, chromatin condensation was assessed by staining with the nuclear dye Hoechst 33342 in ECs with and without treatment with the various investigated drugs. The results for the highest drug concentrations at 12, 24, and 48 hpi are shown in the online Table (which can be accessed at http://atvb.ahajournals.org). The percentage of cells with nuclear changes indicative of apoptosis ranged between 6% and 15%, with a tendency toward higher rates at later time points. Compared with the untreated control cells, in the treated cells no significantly increased rates of apoptotic cells were seen after treatment with aspirin, salicylic acid, BAY, or decoy ODN. In contrast, treatment with PDTC efficiently induced apoptosis in up to 60% of cells at the highest concentration of 50 μmol/L only but not at concentrations ≤25 μmol/L. These data suggest that the described concentration-dependent effect of aspirin and the other inhibitors of NF-κB is not caused by apoptosis of the host cells.

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**Figure 2.** Inhibition of chlamydial growth and decrease of infectious progeny titers by aspirin (ASA), sodium salicylate (SSA), PDTC, and BAY in EVLC2 cells. A, EVLC2 cells were stained at 72 hpi with a genus-specific FITC-labeled antibody. Inclusions were counted, and percent inhibition was calculated as described in Methods. Mean±SEM values represent data of 5 independent experiments. B, EVLC2 cells were sonicated at 72 hpi, and growth titers were determined subsequently in HEp2 indicator cells to assess production of infectious progeny. Results were expressed as IFU per milliliter and represent mean±SEM of 3 independent experiments. *P<0.05 and **P<0.001 vs control by Dunnett test.

**Figure 3.** Effect of ASA, SSA, PDTC, and BAY on \textit{C pneumoniae}–induced NF-κB activation in EVLC2 cells at 6 hpi, analyzed by electromobility shift assay. Subunit composition of the NF-κB binding activity by supershift (SS) with antibodies against p65 and p50, by competition with AP-1 and unlabeled NF-κB oligonucleotide, and by binding to mutated NF-κB binding site is demonstrated.
triplicates. The experiment was repeated once.

**Discussion**

The present study demonstrates that aspirin and its metabolite salicylic acid counteract the intracellular development and maturation of chlamydial inclusions indicated by the reduction of bacterial inclusions and the titer of infectious progeny. The clear concentration-response relationship indicates a yet-unknown effect of these substances on chlamydial growth. The inhibitory effect of aspirin/salicylic acid on chlamydial development was accompanied by morphological changes of the inclusions that became smaller and less uniformly shaped. Our data suggest that the intracellular growth of *C. pneumoniae* is mediated by an activation of the transcription factor NF-κB. These findings apply not only to a single cell line but also to various endothelial and epithelial cell lines and to cultured HUVECs. It is tempting to speculate that the bacterium has evolved to benefit from cellular mechanisms of gene activation to promote its own replication.

Our data suggest that the effect of aspirin/salicylic acid on chlamydial growth is caused, at least in part, by its capacity to inhibit NF-κB. However, aspirin is a drug with many known mechanisms of action. First of all, aspirin is known to irreversibly inhibit cyclooxygenase (COX) by acetylation. Our findings that sodium salicylate, which lacks the respective acetyl group, mimics the inhibitory properties of aspirin on chlamydial growth indicate that the observed effect is not mediated by COX inhibition. Recently, it has been proposed that salicylates exert part of their anti-inflamatory action by the suppression of COX gene transcription. However, because PDTC and BAY, structurally unrelated inhibitors of NF-κB, had anti-chlamydial effects similar to those of the salicylates, it seems unlikely that the inhibitory effect of salicylates on chlamydial growth is mediated primarily by the suppression of COX gene transcription.

Because most of the chemical inhibitors lack exclusive specificity for NF-κB inhibition and also affect the activity of other transcription factors and signaling cascades, we used a highly specific decoy ODN technique to verify that NF-κB is causally involved in the observed inhibition of chlamydial growth. Indeed, reduction of chlamydial inclusions by 50% occurred after transfection of ECs with the consensus decoy ODN but not when the corresponding mutant decoy ODN was used. These data strongly suggest that activation of NF-κB constitutes an essential part of the host-pathogen relationship, with important implications for chlamydial growth. However, we cannot completely exclude the possibility that the effect of aspirin on chlamydial growth also involves other cellular signal transduction pathways. Besides its direct inhibitory effect on NF-κB, aspirin is known to be a scavenger of reactive oxygen species, which are involved as intracellular signal transduction messengers in the regulation of many transcription factors.

Therefore, part of the effects of aspirin that we have observed in this investigation might be due to this activity. Moreover, salicylates might also exert an additional direct toxic effect on the chlamydial developmental cycle that has not yet been recognized. Further studies are necessary to elucidate these issues.

In the next step, we investigated whether treatment of ECs with aspirin resulted in a decreased expression of genes under control of NF-κB to prove efficient NF-κB inhibition at the functional level. For this purpose, we determined the secretion of IL-6 and IL-8 into the supernatant medium. Both have been shown previously to be under the regulatory control of NF-κB and to be upregulated after the infection of ECs with *C. pneumoniae*. As with chlamydial growth, treatment with aspirin/salicylic acid resulted in a similar concentration-dependent inhibition of the secretion of IL-6 and IL-8 (Figure 4). We interpret this finding as another strong indication supporting the hypothesis that the growth inhibition of *C. pneumoniae* by aspirin is caused, at least partly, by inhibition of NF-κB.

Recent studies have broadened the role of NF-κB from that of a regulator of immune response and inflammatory responses to that of a regulator of apoptosis and cell growth. Therefore, we wondered whether the growth-inhibitory effect of aspirin reflected apoptotic cell death of the host cell rather than attenuation of chlamydial proliferation. This idea was supported by a recent study by Wahl et al., who showed that activation of NF-κB in *C. pneumoniae*-infected macrophages is associated with protection of these cells against apoptosis. When investigating the effect of treatment with aspirin and the other drugs on cell viability or the occurrence of cells with nuclear changes characteristic of apoptosis, we did not find any indication of a toxic or proapoptotic effect of these drugs (Figure 2 and online Table). The only exception was PDTC at >25 μmol/L, concentrations for which signs of induction of apoptosis were detected in a high percentage of cells, whereas the same cells were still classified as viable in the dye exclusion test. PDTC is known to be an unspecific inhibitor of NF-κB but a potent scavenger of reactive oxygen species with a high potential to induce apoptosis. Therefore, we conclude that the described growth-inhibiting effect of aspirin and the other inhibitors of NF-κB was not due to increased rates of necrosis or apoptosis of the host cells. This is consistent with other studies concerning the antiapoptotic activity...
conferred to the host cell on infection with \textit{C. pneumoniae}, resulting in the conclusion that no NF-\(\kappa\)B–dependent cellular factors were involved in the protection against apoptosis.\textsuperscript{32–34}

The presented results show that aspirin at concentrations as low as \(10^{-4}\) \text{mM} markedly decreased chlamydial growth. These findings may be of pharmacological relevance in vivo, inasmuch as serum concentrations of \(10^{-2}\) to \(10^{-3}\) \text{mM} are commonly achieved by therapeutic doses of aspirin in humans.\textsuperscript{23}

In coronary heart disease, aspirin has proven to reduce morbidity and mortality in primary prevention and secondary intervention. In addition to its antithrombotic effects, other mechanisms may contribute to these clinical benefits of aspirin.\textsuperscript{23} Therefore, it is tempting to speculate that mechanisms such as described in the present investigation play a role.

Taken together, these data strongly suggest that \textit{C. pneumoniae} has developed a strategy to exploit host cell biology to promote its own development. Our findings suggest that activation of NF-\(\kappa\)B plays a pivotal role in the developmental cycle of \textit{C. pneumoniae} and that aspirin and other inhibitors of NF-\(\kappa\)B activation might have previously unrecognized anti-chlamydial effects. The identification of NF-\(\kappa\)B as a critical transcription factor in \textit{C. pneumoniae} infection may provide insights into specific therapeutic strategies for the treatment of diseases associated with this organism.

\section*{Acknowledgments}
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**TABLE.** Effect of treatment of *C. pneumoniae* infected endothelial cells with various inhibitors of NF-κB on the percentage of apoptotic cells*

<table>
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<tr>
<th>Inhibitor</th>
<th>% cells (SD) with apoptotic nuclear morphology</th>
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<tbody>
<tr>
<td></td>
<td>12 hpi</td>
</tr>
<tr>
<td>untreated</td>
<td>8 (4.5)</td>
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<tr>
<td>Aspirin (5 mmol/L)</td>
<td>8 (2.7)</td>
</tr>
<tr>
<td>Salicylic acid (5 mmol/L)</td>
<td>7 (4.8)</td>
</tr>
<tr>
<td>Bay 117082 (4 µmol/L)</td>
<td>8 (3.7)</td>
</tr>
<tr>
<td>PDTC (25 µmol/L)</td>
<td>9 (4.2)</td>
</tr>
<tr>
<td>PDTC (50 µmol/L)</td>
<td>14 (1.9)†</td>
</tr>
<tr>
<td>NF-κB decoy ODN</td>
<td>8 (2.9)</td>
</tr>
<tr>
<td>NF-κBmut decoy ODN</td>
<td>6 (1.8)</td>
</tr>
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*C. pneumoniae* infected EVCL2 cells were incubated in medium containing the various inhibitors or were left untreated as a control. At the indicated time points, cells were stained with Hoechst 33342 and nuclear morphology was assessed by fluorescence microscopy. At least 300 nuclei per sample were counted. Mean percentages represent data from 4 independent experiments. † *P*<0.05, ‡ *P*<0.001 by Student’s *t* test as compared to the untreated control.
**Figure I.** Inhibition of chlamydial growth and decrease of infectious progeny titers by aspirin (ASA), sodium salicylate (SSA), and PDTC in HUVECs. 

A, HUVECs were stained at 72 hpi with a genus-specific FITC-labeled antibody. Inclusions were counted and percent inhibition was calculated as given in “Methods”. Means ± SEM represent data of 2 independent experiments. 

B, HUVECs were sonicated at 72 hpi and growth titers were determined subsequently in HEp2 indicator cells to assess production of infectious progeny. Results were expressed as inclusion forming units (IFU) per milliliter and represent means ± SEM of 2 independent experiments. *P<0.05, by Dunnett’s test vs control.