Effect of PEX, a Noncatalytic Metalloproteinase Fragment with Integrin-Binding Activity, on Experimental 
Chlamydia pneumoniae Infection

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Chlamydia pneumoniae is a pathogen that is involved in acute and chronic respiratory infections and that is 
associated with asthma and coronary artery diseases. In this study, we evaluated the effects of PEX, a noncatalytic metalloproteinase fragment with integrin-binding activity, against experimental infections caused 
by C. pneumoniae. Moreover, we investigated the relationships between C. pneumoniae and α,β3 integrin 
functions in order to explain the possible mechanism of action of PEX both in vitro and in vivo. For the in vitro 
experiments, HeLa cells were infected with C. pneumoniae and treated with either PEX or azithromycin. The 
results obtained with PEX were not significantly different (P > 0.05) from those achieved with azithromycin. 
Similar results were also obtained in a lung infection model. Male C57BL/J6 mice inoculated intranasally with 
106 inclusion-forming units of C. pneumoniae were treated with either PEX or azithromycin plus rifampin. 
Infected mice treated with PEX showed a marked decrease in C. pneumoniae counts versus those for the 
treatments; this finding did not differ significantly (P > 0.05) from the results observed for the antibiotic-treated 
group. Integrin α,β3 plays an important role in C. pneumoniae infection. Blockage of integrin activation led to 
a significant inhibition of C. pneumoniae infection in HeLa cells. Moreover, CHO-DHER α,β3-expressing cells 
were significantly (P < 0.001) more susceptible to C. pneumoniae infection than CHO-DHER cells. These results 
offer new perspectives on the treatment of C. pneumoniae infection and indicate that α,β3 could be a promising 
target for new agents developed for activity against this pathogen.

Chlamydia pneumoniae is responsible for respiratory in-
fec tions, such as sinusitis, bronchitis, and pneumonia (8). The 
ability of C. pneumoniae to cause persistent infections, com-
bined with its capacity to spread throughout the vascular 
system, has raised questions about the role of this bacterium in 
chronic human diseases, such as atherosclerosis. In 1988, 
Saikku et al. reported evidence of an association between acute 
myocardial infarction and C. pneumoniae (20). Since then, 
several studies have suggested a potential role of C. pneumoniae 
in the pathogenesis of atherosclerosis. Increased anti-
chlamydia antibody (Ab) titers have been correlated with cor-
ony artery diseases (14). C. pneumoniae has been reported to 
infest a large number of vascular or circulating cells, such as 
endothelial cells, aortic smooth muscle cells, and macrophages 
(7). In addition, chlamydial infection has been observed to 
induce a series of events involved in plaque formation and 
evolution. Infected macrophages produce matrix-degrading 
metalloproteinases (MMPs), which bind to integrins and which 
promote plaque rupture (6). Moreover, cells infected with C. pneumoniae are resistant to apoptosis induced by various stim-
uli (18). These data suggest that the inhibition of proapoptotic 
pathways by C. pneumoniae might be a key factor in its persisten-
tce in host cells.

The adhesion and invasion of infectious agents and tumor cells 
via integrins have been studied for several years. The cyta-
herence of host cells, through recognition of the RGD-containing 
peptide (that is, a soluble synthetic peptide containing an argi-
nine, glycine, and aspartic acid, which possess high-affinity α,β3 
trin binding), RGDS, has been observed in Treponema palli-
dium and Treponema cruzi (17, 23). Moreover, attachment of 
Borreia burgdorferi mediated by integrins α,β1 and α,β5, as well 
as RGD peptide sequences, has been reported, and the appro-
priate receptor-specific antibodies have been shown to inhibit this 
process (4). Integrins, which are important for the adhesion and 
invasion of several infectious agents, might also be involved in C. pneumoniae infection.

The aim of the present study was to assess whether PEX, 
which is a peptide with integrin-binding activity, is active 
against C. pneumoniae. Moreover, we investigated the relation-
ship between C. pneumoniae and α,β3 integrin functions. A 
link between α,β3 integrin expression and C. pneumoniae 
infection might explain its selective localization in the plaques 
and help to explain the mechanism of action of PEX.

MATERIALS AND METHODS

C. pneumoniae in vitro infection model. C. pneumoniae stock suspensions were 
prepared from the CWL-029 strain (CWL; ATCC VR-1310). Infection was 

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established in HeLa 229, CHO<sub>DM8</sub>, and CHO<sub>DM2</sub> cells transfected with human α<sub>3</sub>β<sub>3</sub> integrin cDNA (CHO<sub>DM8</sub>α<sub>3</sub>β<sub>3</sub>), by centrifuging (2,000 x g for 2 h at 35°C). 1.5 x 10<sup>6</sup> inclusion-forming units (IFU) onto confluent cell monolayers in 12-well culture plates (10, 12). HeLa cell monolayers were exposed to C. pneumoniae elementary bodies and were treated for 30 min, before centrifugation, with one of the following reagents: monoclonal Ab (MAb) against human integrin α<sub>3</sub>β<sub>3</sub> (MAβ LM609; 0.01 ng/ml to 1 μg/ml); or unspecific Ab (P0399 [Dako]; 0.01 ng/ml to 1 μg/ml); or bovine albumin (0.01 ng/ml to 1 μg/ml). In additional experiments, C. pneumoniae elementary bodies were pretreated for 30 min with soluble human integrin α<sub>3</sub>β<sub>3</sub> purified protein (0.01 ng/ml to 1 μg/ml), provided by Chemicon Int. (Temecula, CA); or bovine albumin (0.01 ng/ml to 1 μg/ml). In all experiments, C. pneumoniae elementary bodies were treated for 30 min with soluble human integrin α<sub>3</sub>β<sub>3</sub> purified protein (0.01 ng/ml to 1 μg/ml), centrifuged at 30,000 x g for 45 min at 4°C, and resuspended in chlamydia growth medium; and then 1.5 x 10<sup>6</sup> IFU was used to infect HeLa cell monolayers.

In all experiments, after centrifugation, the supernatant was replaced with fresh chlamydia growth medium (treated as described above), and HeLa 229 cells were incubated for 72 h (37°C; 5% CO<sub>2</sub>). CHO<sub>DM8</sub> and CHO<sub>DM2</sub> cell monolayers were exposed to C. pneumoniae elementary bodies and treated for 30 min, before centrifugation, with either MAb against human integrin α<sub>3</sub>β<sub>3</sub> (MAβ LM609; 0.01 ng/ml to 1 μg/ml) or an unspecific Ab (P0399 [Dako]; 10 μg/ml to 1 μg/ml). After centrifugation, the supernatant was replaced with 1.5 ml chlamydia growth medium (treated as described above), and the cell monolayers were cultured for 72 h (37°C; 5% CO<sub>2</sub>).

C. pneumoniae was detected by indirect immunofluorescence. The monolayers were initially incubated for 2 h with a specific MAb to C. pneumoniae major outer membrane protein (1:50), followed by 1 h of incubation with a secondary fluororeactive isothiocyanate-conjugated Ab (1:100; Dako). Images were taken from four randomly chosen fields for each slide. The numbers of cells and C. pneumoniae IFU were counted for each field by two separate double-blinded observers. Statistical analysis was performed by using the Prism software package (GraphPad; San Diego, CA). Data were analyzed by one-way analysis of variance (ANOVA).

Binding of C. pneumoniae to α<sub>3</sub>β<sub>3</sub> integrin. Next, 96-well polystyrene enzyme-linked immunosorbent assay (ELISA) plates were coated with 100 ng/well of either integrin α<sub>3</sub>β<sub>3</sub> or bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and were incubated overnight at 4°C. The plates were washed with PBS containing 0.5% Tween 20 (PBS-Tween) and blocked with 200 μl of 1% BSA in Tris-buffered saline (TBS; 25 mM Tris-HCl [pH 7.5] and 150 mM NaCl) containing 0.5 mM CaCl<sub>2</sub> and 0.9 mM MgCl<sub>2</sub> (blocking buffer) for 2 h at room temperature. C. pneumoniae cells that had been diluted in TBS containing 0.1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, and 0.1% BSA were added; and the plates were incubated for 1 h at room temperature. The plates were washed with PBS-Tween, and 100 μl primary Ab (mouse anti-C. pneumoniae Ab diluted 1:1,500 in blocking buffer) was added to each well. The plates were incubated for 1 h at room temperature and washed with PBS-Tween, 100 μl horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (1:1,000 in blocking buffer) was added, and the mixture was incubated for 30 min at room temperature. After this, the mixture was washed with PBS-Tween, the substrate (tetramethylbenzidine) was added, and the mixture was incubated for 5 to 15 min at room temperature. The reaction was stopped with 50 μl of 2 M H<sub>2</sub>SO<sub>4</sub> and the plates were read at 450 nm.

C. pneumoniae in vivo infection model. The study protocol was reviewed and approved by the Committee on Ethics in Animal Experiments of the Italian National Institutes of Health. Male C57BL/6J mice (age, 6 weeks) were purchased from Charles River (Como, Italy), fed a normal mouse diet ad libitum, housed under biosafety level 2 conditions, and cared for according to the standard and specific procedures outlined by the Italian National Institutes of Health. All mouse experiments were performed under barrier conditions, with sentinel mice monitored by a quality assurance program.

Mice were anesthetized by ether inhalation to induce hyperventilation, and each animal was inoculated intranasally with 10<sup>6</sup> IFU in 10 μl sucrose phosphate glutamic-acid buffer. Delivery of the inoculum was timed with the inhalation phase of respiration. Mice were divided into three treatment groups: those receiving an injection of normal saline, those receiving an injection of azithromycin (10 mg/kg of body weight/day) plus rifampin (20 mg/kg/day), and those receiving an injection of human PEX (2 mg/kg/day). The treatments started 3 days before the C. pneumoniae inoculation, and all animals were injected subcutaneously once a day for 10 consecutive days. The animals were killed on day 7 after C. pneumoniae inoculation. The lungs were removed and were processed for culture and inclusion counting, as described previously (13, 16). The viable count was expressed as the log<sub>10</sub> IFU/lung.

Other reagents. Bovine albumin, azithromycin, and rifampin were obtained from Sigma (St. Louis, MO).

RESULTS

PEX reduces infection of host HeLa cells. To examine the effect of PEX on C. pneumoniae infection, HeLa cells were exposed to C. pneumoniae (1.5 x 10<sup>6</sup> IFU) in the presence of increasing concentrations of peptide (0.01 ng/ml to 1 μg/ml). The effect on C. pneumoniae entry and proliferation was evaluated and expressed as the number of IFU/field. For each treatment, four different experiments were performed, and the results are shown as the means ± standard deviations (SDs).

After 72 h, treatment with PEX led to a significant decrease in the number of IFU/field (Fig. 1) in comparison with the number for the untreated control cells or nonspecific peptide-treated cells (maximum inhibitory concentration, 79.43 ± 10.4 ng/ml; 50% maximum inhibitory concentration [IC<sub>50</sub>] 4.16 ± 2.34 ng/ml; P < 0.001; R<sup>2</sup> = 0.981).

HeLa cells were also exposed to C. pneumoniae in the presence of increasing concentrations of azithromycin; this resulted (Fig. 1) in a significant decrease in the number of IFU/field (maximum inhibitory concentration, 73.32 ± 6.44 ng/ml; IC<sub>50</sub> 5.32 ± 3.73 ng/ml; P < 0.001; R<sup>2</sup> = 0.989). The results obtained by treating the cells with azithromycin were not significantly different from those achieved by treating the cells with PEX (P > 0.05).

Inhibition of α<sub>3</sub>β<sub>3</sub> integrin reduces C. pneumoniae infection of host cells. To examine the effect of α<sub>3</sub>β<sub>3</sub> inhibition on C. pneumoniae entry and proliferation, HeLa cells that tested positive for α<sub>3</sub>β<sub>3</sub> expression were exposed to C. pneumoniae

![Concentration (ng/ml)](image)

**FIG. 1.** Inhibitory effects of human PEX and azithromycin on the infectivity of C. pneumoniae. HeLa cells were inoculated with 1.5 x 10<sup>6</sup> IFU/well and were treated with either human PEX or azithromycin at various concentrations. The number of IFU per field was evaluated. The results of four different experiments are shown (mean ± SD). ★, human PEX; ▼, bovine albumin; ■, azithromycin.
C. pneumoniae the entry and proliferation of a dose-dependent manner. Incubation with anti-\(\alpha_\beta_3\) antibodies on HeLa cells (for comparison with the number for the untreated control cells or nonspecific Ab-treated cells (maximum inhibitory concentration, 0.55 ± 0.21 \(\mu g/ml; IC_{50} 0.43 ± 0.15 \mu g/ml; P < 0.001; R^2 = 0.973\)).

To investigate the effect of soluble \(\alpha_\beta_3\) integrin on the entry and proliferation of C. pneumoniae in host cells, the cells were exposed to bacteria in the presence of increasing concentrations of soluble integrin \(\alpha_\beta_3\) (0.01 ng/ml to 1 \(\mu g/ml\)). As shown in Fig. 2, this resulted in a significant decrease in the number of IFU/field (Fig. 2) in comparison with the number for the untreated control cells or nonspecific Ab-treated cells (maximum inhibitory concentration, 182 ± 15.7 ng/ml; IC\(_{50}\) 13.8 ± 10.2 ng/ml; \(P < 0.001; R^2 = 0.980\)).

A similar trend was obtained by pretreating elementary bodies with soluble \(\alpha_\beta_3\) integrin (maximum inhibitory concentration, 97.6 ± 12.3 ng/ml; IC\(_{50}\) 8.92 ± 3.24 ng/ml; \(P < 0.01; R^2 = 0.970\)) (Fig. 3), while soluble \(\alpha_\beta_3\) integrin coincubated with PEX was unable to prevent the infection of HeLa cells (for soluble \(\alpha_\beta_3\) integrin, 25.5 ± 4.65 IFU/field; for soluble \(\alpha_\beta_3\) integrin coincubated with PEX, 45.75 ± 5.32 IFU/field; \(P < 0.001; R^2 = 0.925\)) (Fig. 4).

Infection in CHO\(_{DHFR}\) cells expressing human \(\alpha_\beta_3\) integrin. To explore the specific role of \(\alpha_\beta_3\) as a receptor of C. pneumoniae, CHO\(_{DHFR}\) \(\alpha_\beta_3\)-negative and CHO\(_{DHFR}\) \(\alpha_\beta_3\)-positive cells were exposed to C. pneumoniae and the numbers of IFU/field were evaluated. Four different experiments were performed, and the results are shown as the means ± SD.

CHO\(_{DHFR}\) \(\alpha_\beta_3\)-expressing cells were significantly \((P < 0.001)\) more susceptible to C. pneumoniae than CHO\(_{DHFR}\) cells. In fact, the proportion of infected cells in CHO\(_{DHFR}\) monolayers versus the proportion in CHO\(_{DHFR}\) \(\alpha_\beta_3\)-transfected control monolayers, evaluated 72 h after infection, was only 8%. C. pneumoniae infection of CHO\(_{DHFR}\)
αβ3-positive cells was inhibited by increasing concentrations of anti-αβ3 blocking antibodies (maximum inhibitory concentration, 12.4 ± 8.4 ng/ml; IC50, 2.3 ± 1.1 ng/ml; R2 = 0.99) (Fig. 5).

C. pneumoniae binds to αβ3 integrin. To determine whether C. pneumoniae binds directly to αβ3 integrin, ELISA plates coated with either αβ3 integrin or BSA were incubated with increasing amounts of C. pneumoniae, and the amount of the microorganism that bound to each plate was detected.

C. pneumoniae was found to bind to purified integrin αβ3 in a concentration-dependent manner. This differed significantly (P < 0.001) from the binding observed in BSA-coated wells (Fig. 6).

Inhibition of C. pneumoniae infection by systemic administration of PEX in vivo. To evaluate the effect of human PEX on C. pneumoniae in vivo infectivity, male C57BL/6 mice were infected with Chlamydia by intranasal inoculation of 10⁶ IFU of the pathogen. Three days before inoculation, the animals were preventively treated with a systemic injection of either human PEX or an antibiotic. The experiment compared the effect of integrin inhibition by PEX on C. pneumoniae infection with the effects of standard antibiotic regimens. Seven days after C. pneumoniae inoculation, the animals were killed and the number of IFU in each lung was calculated (Fig. 7). All animals belonging to the infected control group, and those treated with vehicle only were positive for C. pneumoniae (16/16 animals). The mean number of IFU/lung in the PEX-treated group was 3.37 (range, 0 to 2.5 × 10⁷ IFU/mouse), which was significantly different (P < 0.001) from that for the vehicle-treated mice. Only 11 of the 16 animals receiving systemic PEX were positive for C. pneumoniae on day 7 after inoculation. The mice belonging to the PEX group showed a lower number of IFU than both the control group and the antibiotic-treated group. The number of IFU/lung in the PEX-treated group was 3.90 × 10³ (range, 0 to 2.5 × 10⁴ IFU/mouse), which was not significantly different from that for the antibiotic-treated group.

DISCUSSION

Some previous studies have shown that prolonged treatment with antibiotics, such as macrolides or fluoroquinolones, sig-
nificantly reduces but does not eliminate \textit{C. pneumoniae} both in vitro and in vivo (11).

Following treatment with moxifloxacin or levofloxacin, eradication rates of only 70 to 80\% have been found in adults with culture-documented \textit{C. pneumoniae} infection (9). Antibiotic therapy, which reduces the bacterial load during the acute phase of chlamydial infections, leads to clinical recovery. Nevertheless, the lack of eradication can be responsible for chronic or latent infections.

New therapeutic approaches are therefore necessary to counteract \textit{C. pneumoniae} infections and to elucidate the role of this pathogen in the development and/or progression of atherosclerotic plaques or asthma.

For bacterial infection to take place, the adhesion of elementary bodies, which leads to invasion into cells, must occur. This process involves ligand-mediated adhesion of the bacterium, in which a receptor on the host cell surface is bound by a ligand on the bacterial surface. The presence of receptors and ligands leads to bacterial entry into specific tissues (15).

The mechanism of \textit{C. pneumoniae} entry into host cells is largely unknown (21). In particular, the surface molecules that are responsible for \textit{C. pneumoniae} attachment and invasion have not been identified, and the reason for the preferential localization of this pathogen within the atherosclerotic plaque is not understood. In the current study, we present experimental evidence that integrins are critically involved in \textit{C. pneumoniae} infection and that PEX (an MMP fragment with integrin-binding activity) is highly effective against experimental infection by this pathogen.

In our in vitro model, infection of HeLa host cells was inhibited by human PEX. These results were similar to those obtained by treating cells with azithromycin or RGD peptides (data not shown) or with anti-\(\alpha_\beta_3\) integrin blocking antibodies or soluble \(\alpha_\beta_3\). In addition, human PEX, which is a ligand for \(\alpha_\beta_3\) (2, 3), reduces the infection in the lungs of treated animals, decreasing the number of inclusion bodies to an extent comparable to that produced by standard antibiotic treatments.

Thus, the main findings of the present study were that human PEX displayed dose-dependent activity against \textit{C. pneumoniae} in vitro and, more importantly, was highly effective against this pathogen in an experimental lung infection model.

Moreover, the PEX-mediated inhibition of infection supports the concept that \(\alpha_\beta_3\) plays a major role in the infectious mechanism of \textit{C. pneumoniae}. In fact, PEX, which lacks an RGD sequence (2, 3), is a more selective inhibitor of \(\alpha_\beta_3\) activation and binding than RGD peptides, which block several integrins (19).

Several other integrins are probably involved in the pathogenesis of \textit{C. pneumoniae} infection in vivo. Here, we focused on \(\alpha_\beta_3\) integrin, because activation of this surface molecule is involved in atherosclerotic plaque destabilization and progression (1). Our findings demonstrated the specific involvement of \(\alpha_\beta_3\) in \textit{C. pneumoniae} infection, as determined by the use of CHODHPR \(\alpha_\beta_3\)-positive and \(\alpha_\beta_3\)-negative cells.

These results suggest that \(\alpha_\beta_3\) integrin is a key molecule in \textit{C. pneumoniae} infection. Integrin ligation leads to the activation of both the Ras–PI-3-kinase–Akt pathway and the Ras–Raf–MEK–ERK cascade (22). These two pathways have both been identified as essential in the \textit{C. pneumoniae} invasion of epithelial cells (5).

A previous paper showed that monoclonal antibodies that bind to the alpha and/or beta 1 subunit of classic integrin receptors on HEC-1B cells were unable to prevent the colonization and infection of epithelial cells by a genital isolate of \textit{Chlamydia trachomatis} (25). This suggests that the mechanism of entry into the cell by integrins could be specific for \textit{C. pneumoniae}.

Further studies will be necessary to evaluate the benefit of human PEX in different cellular and animal \textit{C. pneumoniae} infection models. The activity of PEX should also be tested against other agents, such as adenosivirus or \textit{B. burgdorferi}, both of which are \(\alpha_\beta_3\) integrin dependent, as they are inhibited by blocking monoclonal antibodies and RGD peptides (4, 24).

Our current objective was not to compare the antibacterial and anti-inflammatory properties of PEX and azithromycin. Rather, we aimed to demonstrate that blockage of the \(\alpha_\beta_3\) receptor with PEX results in a reduction of infection similar to that obtained with a traditional anti-inflammatory agent, such as azithromycin, even if the mode of action is different.

Moreover, we can speculate that effective preventative agents, such as PEX (or similar, as yet undiscovered drugs), could be combined with antibiotics that act on intracellular bacteria in order to eradicate \textit{C. pneumoniae}.

In summary, our results highlight a new role of human PEX in \textit{C. pneumoniae} infection and provide the necessary link by identifying the cell surface binding sites involved. In addition, our findings suggest that human PEX could be used for the treatment of \textit{C. pneumoniae} infections, as shown by both in vitro and in vivo experiments. This peptide might therefore represent a therapeutic tool for the development of new strategies for the treatment of diseases related to acute or chronic \textit{C. pneumoniae} infections.

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