

## Glucocorticoids Increase In Vitro and In Vivo Activities of Antibiotics against *Chlamydomphila pneumoniae*

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**The in vitro and in vivo antichlamydial activities of dexamethasone and beclomethasone alone and in combination with an antibiotic were tested. In vitro, dexamethasone and beclomethasone decreased the number of inclusion-forming units versus the control number ( $P < 0.001$ ). The combination of glucocorticoids with azithromycin, telithromycin, or levofloxacin was more active than antibiotics used alone ( $P < 0.001$ ). The combination, tested in a murine *Chlamydomphila pneumoniae* infection model, produced similar results.**

*Chlamydomphila pneumoniae* is an obligate intracellular pathogen responsible for respiratory infections such as sinusitis, bronchitis, and pneumonia (9). Moreover this pathogen can cause chronic, persistent, and often asymptomatic infections (10). Persistent *C. pneumoniae* infection has been associated with chronic human diseases, including atherosclerosis and asthma (3, 4). Several studies have reported that, in an in vitro model of chronic *C. pneumoniae* infection, treatment with antibiotics like macrolides or fluoroquinolones at concentrations of up to four times the MIC reduced, but did not completely eliminate, the organism (8).

Recently, modulation of host cell apoptosis has been discussed as a survival strategy of *C. pneumoniae* (7). The transcription factor NF- $\kappa$ B may contribute to infection with and systemic spread of *C. pneumoniae* and is associated with protection of host cells against apoptosis (5).

Some researchers have investigated the effects of aspirin on *C. pneumoniae* infection and demonstrated that it has an antichlamydial activity at high doses and inhibits NF- $\kappa$ B activation induced by *C. pneumoniae* (17).

Glucocorticoids, anti-inflammatory drugs inhibiting NF- $\kappa$ B (1), are drugs commonly used against respiratory inflammatory diseases. In the present study, the effects of dexamethasone (Dex) and beclomethasone (Becl) on *C. pneumoniae* infection in vitro, as well as in vivo, were evaluated. Glucocorticoids were tested alone or in combination with azithromycin (AZM), telithromycin (TEL), or levofloxacin (LVX), antibiotics commonly used against *C. pneumoniae* infections. An in vitro Hep-2 cell infection model was used as previously described (11).

Monolayers of cells were cultured on coverslips, and when the cells reached confluence, each monolayer was inoculated with 1.5 ml of *Chlamydia* growth medium with 10<sup>4</sup> inclusion-forming units (IFU) of *C. pneumoniae* CWL 029 (ATCC VR-1310) per ml, supplemented with Dex or Becl at various concentrations. Afterwards, *C. pneumoniae* was forced onto

the cell surface by centrifugation (2,000  $\times$  g for 2 h at 35°C). The supernatant was then replaced with 1.5 ml of *Chlamydia* growth medium supplemented once more with Dex or Becl alone or in combination with AZM, TEL, or LVX at their MICs (0.05, 0.0156, and 0.5  $\mu$ g/ml, respectively). Sub-MICs were also tested. After 72 h of incubation (37°C, 5% CO<sub>2</sub>), *C. pneumoniae* was detected by immunofluorescence assay with a fluorescein isothiocyanate-conjugated monoclonal antibody specific for *C. pneumoniae* (Dako Ltd., Glostrup, Denmark).

*C. pneumoniae* IFU were counted in a double-blind manner by two independent observers using a Zeiss Axioplan microscope with a Zeiss Plan-Neofluar  $\times$ 40 ocular. Statistical analysis was performed with the Prism software package (GraphPad, San Diego, Calif.). Data were analyzed by one-way analysis of variance with the Newman-Keuls posttest.

In vivo *C. pneumoniae* infections were performed with 6-week-old male BALB/c mice purchased from Charles River (Como, Italy), fed ad libitum with a normal mouse diet, housed under biosafety level 2 conditions, and cared for by standard and specific procedures, as outlined by the Italian National Institutes of Health. The study protocol was approved by the Committee on Ethics in Animal Experiments, Italian National Institutes of Health.

Mice were anesthetized by ether inhalation to induce hyperventilation, and each mouse was inoculated intranasally with 10<sup>6</sup> IFU in 10  $\mu$ l of sucrose-phosphate-glutamic acid buffer. Only AZM and Dex, administered so as to produce blood concentrations similar to those reached in humans after routine treatment (6, 14), were tested in our in vivo experiments.

Three groups of 10 infected mice each were treated with AZM (40 mg/kg/day), Dex (120  $\mu$ g/kg/day), or AZM-Dex, respectively. Appropriate uninfected or infected-untreated control groups were also prepared. Four days after *C. pneumoniae* inoculation, animals were injected subcutaneously (once a day for 4 consecutive days). Twenty-four hours after the last injection, the animals were sacrificed and their lungs were removed. Serial 20- $\mu$ m sections of lungs were processed for inclusion evaluation as described above.

In vitro at 1 and 5  $\mu$ M, both glucocorticoids decreased the number of IFU versus the control number ( $P < 0.001$ ). Dex and Becl reduced the number of IFU versus the control

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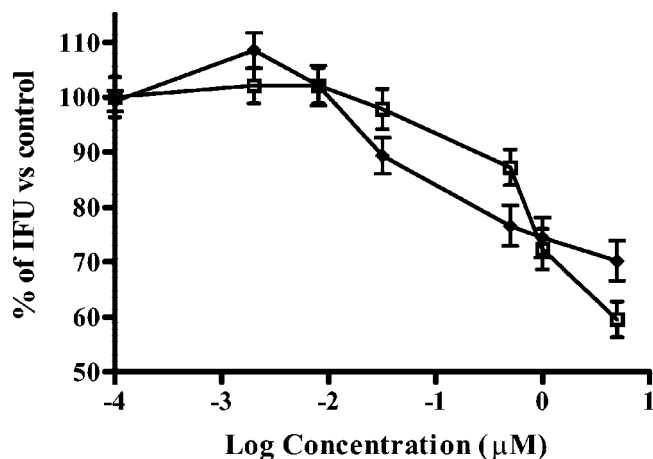


FIG. 1. Hep-2 cells were inoculated with *C. pneumoniae* ( $1.5 \times 10^4$  IFU/well) and treated with Dex or Becl at various concentrations. The number of IFU versus that in untreated Hep-2 monolayers was evaluated and is presented as a percentage  $\pm$  the standard deviation. One result representative of four different experiments is shown. Symbols:  $\blacklozenge$ , Becl;  $\square$ , Dex.

number by about 25% at 1  $\mu$ M and by about 40 and 30%, respectively, at 5  $\mu$ M. At lower Dex and Becl concentrations, the numbers of IFU of *C. pneumoniae* in the glucocorticoid-treated monolayers were not different ( $P > 0.05$ ) from those in control cell monolayers (Fig. 1).

AZM, TEL, and LVX used alone significantly decreased the number of IFU versus the control number ( $P < 0.001$ ) at all of the concentrations tested (Fig. 2). AZM reduced the number of IFU versus the control number by about 34% at 1/100 of the MIC, by 45% at 1/10 of the MIC, and by 92% at 1/2 of the MIC. TEL reduced the number of IFU by about 36% at 1/100 of the MIC, by 38% at 1/10 of the MIC, and by 96% at 1/2 of the MIC. LVX reduced the number of IFU by about 20% at 1/100 of the MIC, by 22% at 1/10 of the MIC, and by 91% at 1/2 of the MIC.

Combination of corticosteroids with AZM, TEL, or LVX at sub-MICs produced a significant decrease in the number of infected cells versus that achieved by using the antibiotics alone. Combination of AZM (at 1/100 of the MIC) and Dex (1  $\mu$ M) reduced the number of IFU versus the control number by about 47%, which is significantly different from AZM or Dex used alone ( $P < 0.001$ ). Combination of AZM (at 1/10 of the MIC) with Dex reduced the number of IFU versus the control number by about 53%, which is significantly different from the effect of using Dex alone ( $P < 0.001$ ).

In addition, the combination of AZM (at one-half of the MIC) and Dex, which was significantly more active than the antibiotic alone ( $P < 0.001$ ), produced effects similar to those of AZM at the MIC, reducing the antibiotic concentration necessary to satisfy the requirements of the MIC definition (15). Similar results were obtained with TEL and LVX. No significant differences were observed when the antibiotics were combined with Becl instead of Dex.

Corticosteroids evidently potentiate antibiotic activity when the latter are used at sub-MICs. At the MICs of the antibiotics, the combination produced results similar to those obtained with the antibiotics alone.

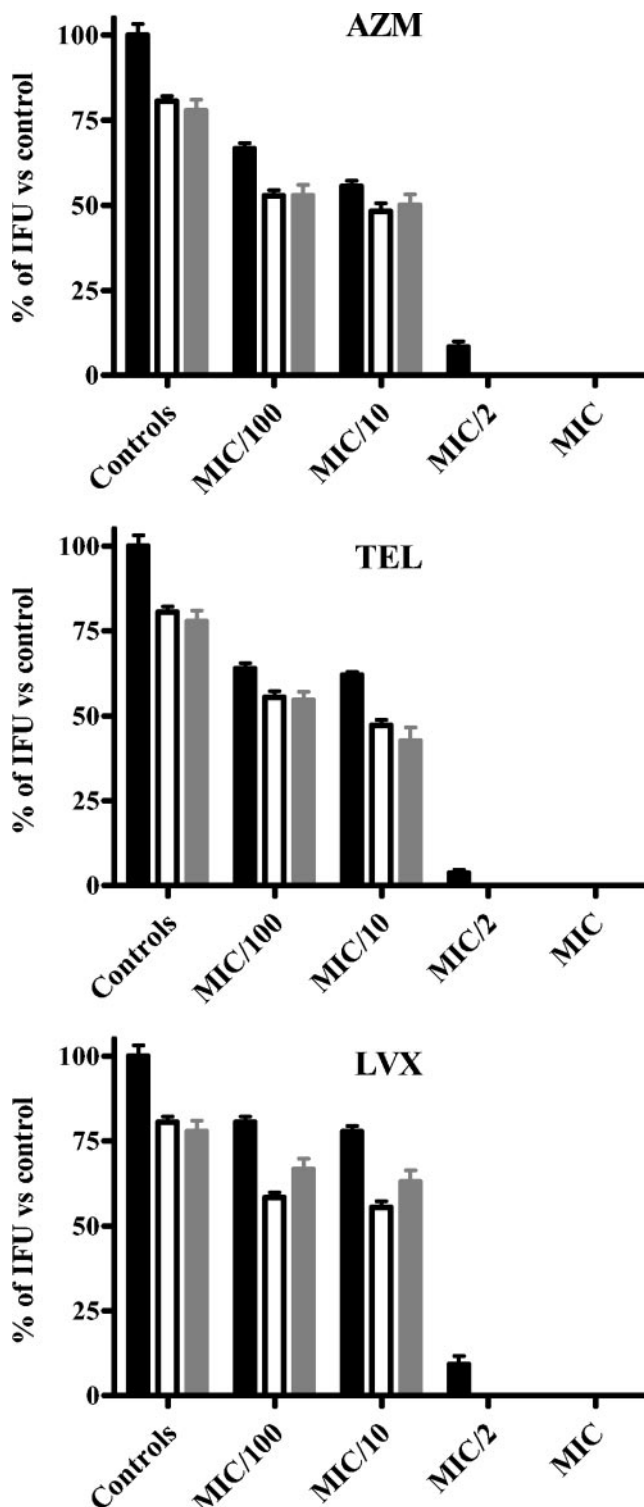


FIG. 2. Hep-2 cells were inoculated with *C. pneumoniae* ( $1.5 \times 10^4$  IFU/well) and treated with a combination of Dex or Becl (1  $\mu$ M) and AZM, TEL, or LVX at various fractions of the MIC. The number of IFU versus that in untreated Hep-2 monolayers was evaluated and is presented as a percentage  $\pm$  the standard deviation. One result representative of four different experiments is shown. Closed bars, antibiotic alone; open bars, antibiotic plus Dex; grey bars, antibiotic plus Becl.

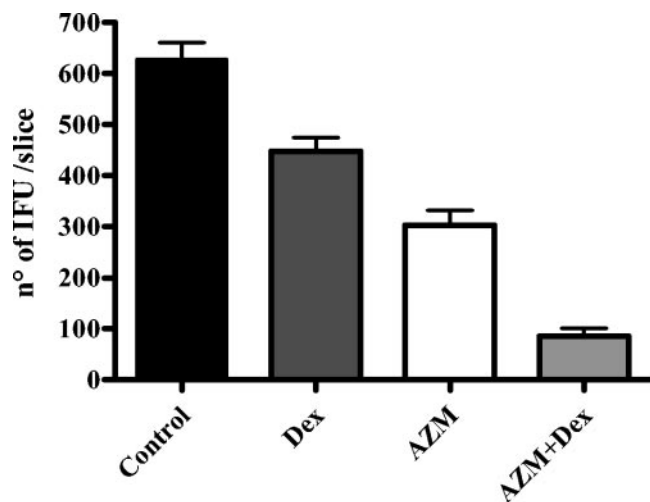


FIG. 3. Mice at 6 weeks of age were sham inoculated or inoculated with *C. pneumoniae* ( $1.0 \times 10^6$  IFU/mouse). One result representative of two different experiments is shown. Data are presented as the number of IFU per slice  $\pm$  the standard deviation. Closed bars, phosphate-buffered saline; dark grey bars, Dex; open bars, AZM; light grey bars, AZM plus Dex.

In the in vivo infection model, Dex and AZM significantly ( $P < 0.05$  and  $P < 0.001$ , respectively) reduced the number of IFU per slice versus that of untreated controls. The combination of AZM and Dex was significantly ( $P < 0.001$  versus the control and Dex;  $P < 0.01$  versus AZM) more active than the other treatments (Fig. 3).

In our in vitro infection model, Dex and Beclomethasone inhibited *C. pneumoniae* growth in Hep-2 cells. Moreover, at a concentration commonly achieved in the lung after inhalation ( $1 \mu\text{M}$ ), they increased the antichlamydial activity of antibiotics. These positive results were confirmed in vivo.

Our data are apparently in contrast to those of some previous studies, which reported enhanced in vitro growth of *C. pneumoniae* (12) or in vivo reactivation of *Chlamydia* infection (13) in the presence of glucocorticoids. There are several reasons for these differences.

In our in vitro experiments, the glucocorticoids were used to supplement the medium at the time of *C. pneumoniae* inoculation, while in the previous studies glucocorticoids were added after the entrance of chlamydiae into the cells. Moreover, the glucocorticoid concentrations that were active in our experiments are higher than those tested in the previous in vitro studies. The previous in vivo studies showing that cortisone reactivates *C. pneumoniae* in vivo via an immunosuppressant effect (13) are not in disagreement with our data. We used an in vivo dose of Dex and Beclomethasone ( $120 \mu\text{g/kg/day}$ ) significantly lower than the cortisone acetate dose tested in the past ( $125 \text{ mg/kg/day}$ ). Our dose of Dex might mainly lead to an anti-inflammatory effect, while the dose of cortisone leads to an immunosuppressant effect. Moreover, cortisone acetate was used in infected mice carrying a latent infection while we treated a primary infection.

Our data are indirectly in agreement with a recent study in which, in subjects with severe asthma, anti-inflammatory doses of steroids were found to be associated with lower titers of

immunoglobulin G and A antichlamydial antibodies, compared with those of untreated controls, as well as with those of patients treated with high doses of the same steroids (2).

In a primary infection, an anti-inflammatory and/or a light immunosuppressant effect might be required to counteract the evolution of *C. pneumoniae* in latent or persistent forms, keeping *C. pneumoniae* inclusions in an antibiotic-susceptible form.

In conclusion, our results suggest that glucocorticoid therapy, in addition to traditional antibiotic chemotherapy, may be useful against acute *C. pneumoniae* infections. Combinations of antibiotics and Dex (or others glucocorticoids) could be clinically evaluated as an appreciable therapeutic opportunity for the treatment of *C. pneumoniae* infection.

We do not know the mechanisms involved in the antichlamydial effect of glucocorticoids. *C. pneumoniae* promotes its entrance and/or its proliferation and persistence in host cells by enhancing NF- $\kappa$ B activation (16); therefore, we speculate that inhibition of this transcription factor might explain, at least in part, the antichlamydial effects of glucocorticoids.

Nevertheless, other mechanisms may be involved. Further preclinical and clinical studies are necessary to elucidate the effect shown by corticosteroids, with primary-infection models and chronic- or latent-infection models, as well as different doses.

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