Doxycycline suppresses Chlamydia pneumoniae-mediated increases in ongoing immunoglobulin E and interleukin-4 responses by peripheral blood mononuclear cells of patients with allergic asthma

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Objectives: Chlamydia pneumoniae, an obligate intracellular bacterium, has been associated with asthma and the induction of immunoglobulin E (IgE) responses. Whereas tetracyclines have anti-chlamydial activity, their effect on human IgE responses to C. pneumoniae has not been studied.

Methods: Peripheral blood mononuclear cells (PBMCs) from serum IgE+ allergic asthmatic subjects (n=11) and healthy controls (n=12) were infected with C. pneumoniae and cultured for 12 days with or without doxycycline (0.01–1.0 mg/L). IgE, interferon (IFN)-γ and interleukin (IL)-4 levels in supernatants were determined on days 1–12 post-infection, and C. pneumoniae DNA copy numbers in PBMC culture were measured on day 2 (quantitative PCR).

Results: C. pneumoniae-infected PBMCs from allergic asthmatic individuals had increased levels of IgE in supernatants compared with uninfected PBMCs (520% on day 10 post-infection, P=0.008). IgE levels in PBMC cultures from controls were undetectable (<0.3 ng/mL). Increases in C. pneumoniae-induced IgE in asthmatics correlated with those of C. pneumoniae-induced IL-4 (r=0.98; P<0.001), but not with IFN-γ. The addition of doxycycline (1.0 mg/L) to the culture strongly suppressed the production of IgE (>70%, P=0.04) and IL-4 (75%, P=0.018), but not IFN-γ. The suppressive effect on IL-4 production remained significant even at concentrations of doxycycline that were subinhibitory (0.01 mg/L) for C. pneumoniae. In both asthmatic participants and controls, no significant effect of doxycycline on DNA copy numbers of C. pneumoniae was observed.

Conclusions: Doxycycline suppressed the C. pneumoniae-induced production of IgE and IL-4, but not IFN-γ, in PBMCs from IgE+ allergic asthmatic subjects. These findings resulted from the immunomodulatory anti-allergic properties of tetracyclines.

Keywords: tetracyclines, inflammation, immunomodulation, cytokines

Introduction

Chlamydia pneumoniae, an obligate intracellular bacterium, is a frequent cause of respiratory infection in adults and children and has been implicated in exacerbations of asthma. C. pneumoniae is also capable of causing prolonged respiratory infections in asthmatic and non-asthmatic individuals. Studies in our laboratory have also described an increased prevalence of anti-C. pneumoniae immunoglobulin E (IgE) in children with wheezing compared with healthy controls and children with pneumonia who were not wheezing. Treatment with antibiotics active against atypical bacteria may be beneficial for patients with asthma because of the eradication of acute or persistent infection with C. pneumoniae. The commonly used macrolide and tetracycline antibiotics have anti-inflammatory properties in addition to anti-chlamydial activity. We have previously reported on the steroid-sparing effects of minocycline when given as an add-on to steroid treatment in adults with severe asthma, which also resulted in improved lung function and quality of life. It is possible that the positive effects may
be due to suppression of inflammation in addition to eradication of the bacterium.1,11

It is well established that tetracyclines have anti-inflammatory properties, and they have been used for that purpose in periodontitis, rosacea and acne.12,13 Targeting inflammatory mediators leading to IgE production is an important approach to improving asthma symptoms. We have demonstrated isotype-specific IgE mediators as well as a steroid-sparing effect of minocycline in patients with allergic asthma independent of their infection status.10 Other studies in our laboratory have also described decreased IgE production in vitro by peripheral blood mononuclear cells (PBMCs) from asthmatic subjects in the presence of minocycline or doxycycline.14 However, the effects of tetracyclines on the C. pneumoniae-induced inflammatory responses of leucocytes from adults with persistent asthma without acute C. pneumoniae infection are unknown.

In this study, we investigated the effect of doxycycline on C. pneumoniae-induced IgE responses, type 1 T helper cell (Th1)/Th2-type cytokine production and chlamydial growth using an in vitro human PBMC model.

**Methods**

**Subjects**

The study was approved by the institutional review board of State University of New York (SUNY) Downstate Medical Center, Brooklyn, NY, USA (study number 08-027). Adult allergic asthmatic subjects and healthy adult controls (18–65 years of age), receiving care in the outpatient department at SUNY Downstate Medical Center, were consecutively enrolled after written informed consent had been obtained. Inclusion criteria for enrolled patients included a clinical diagnosis of allergic asthma without current or recent symptoms of an asthma exacerbation or acute respiratory infection (within the previous 3 months).16,17 Controls were defined as subjects without a history of atopic diseases or asthma, with normal serum IgE levels and without current or recent symptoms suggestive of acute respiratory infection (within the previous 3 months).16,17 Exclusion criteria for all subjects were pregnancy, a history of smoking or immunodeficiency and the use of systemic corticosteroids within 30 days prior to enrolment. The C. pneumoniae infection status of the study subjects was characterized by quantitative PCR (qPCR) of nasopharyngeal swabs (as described below) to determine current infection and by serology to determine past infection (cut-off titre >1:16) using microimmunofluorescence according to the manufacturer’s protocol (Ani Labsystems, Finland).

**Preparation of C. pneumoniae**

C. pneumoniae TW-183 (ATCC 53592) was propagated as previously described.18 Briefly, HEp-2 cell (ATCC LCL-23) monolayers were inoculated with C. pneumoniae and grown to high titres by serial passage in HEp-2 cells. C. pneumoniae elementary bodies (EBs) were purified by Urogafin (Schering, Berlin, Germany) density gradient centrifugation and were suspended in sucrose phosphate glutamate buffer. Titres were determined by infecting HEp-2 cells with serial dilutions of EB suspension aliquots, fixing cells at 72 h post-infection (p.i.), staining with fluorescein-conjugated murine monoclonal genus-specific anti-lipopolysaccharide antibodies (Pathfinder, Bio-Rad, Hercules, CA, USA) and counting the inclusions per well. Aliquots were frozen at −80°C until use.

**PBMC culture**

PBMCs were isolated from EDTA whole blood on Ficoll-Paque (GE Healthcare, Sweden) and then washed in RPMI 1640 (Sigma, St Louis, MO, USA) with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, CA, USA), and resuspended in complete RPMI 1640 (c-RPMI). c-RPMI contained RPMI 1640 Medium HEPES Modification (Sigma) supplemented with 5 mM l-glutamine (Sigma) and 10% FBS. Cells were counted and cell viability evaluated with trypan blue dye using a haemocytometer (Spencer, Buffalo, NY, USA). For each experimental condition, PBMCs (1.5×10^7/mL) were cultured in duplicate in 24-well flat-bottom plates (1 mL/well) for up to 12 days in absolute humidification and 5% CO2. Cell viability was determined at 0, 48 and 240 h (>98%, 95% and 90%, respectively). Following a 2 h incubation to allow adherence, PBMC cultures were infected or stimulated in the presence or absence of doxycycline (0.01, 0.1 and 1.0 mg/L) (Sigma) for up to 12 days.

**In vitro infection with C. pneumoniae and treatment with doxycycline**

PBMCs were infected by adding purified EBs for 1 h, followed by further incubation in the presence or absence of physiological concentrations of doxycycline, 0.01–1 mg/mL, for up to 12 days p.i. at 37°C in c-RPMI in a humidified 5% CO2 atmosphere. Doxycycline stock solutions were added as 1 μL per 1 mL of culture to achieve the desired final concentration. The multiplicity of infection (moi) of 0.1 and timepoints (48 h p.i. for cytokines and 10 days p.i. for IgE) used for analysis were selected by kinetic and dose–response studies (using mois of 0.01–10) for optimization of the assay, which revealed peak concentrations and clear distinctive profiles for the respective outcome variables at these timepoints. Adherent cells were stained with fluorescein-conjugated murine monoclonal genus-specific anti-lipopolysaccharide antibodies to confirm and quantify infection with C. pneumoniae at 72 h p.i. Two types of controls were used in infection experiments: identical volumes of heat-inactivated purified C. pneumoniae and identical volumes of HEp-2 cell cultures not containing any bacteria processed in the same way as the purified C. pneumoniae.

**ELISA: cytokines [interleukin (IL)-4 and interferon (IFN)-γ] and IgE**

Cell culture supernatants were collected at 48 h p.i. (cytokines) and 10 days p.i. (IgE), centrifuged to remove cellular debris and stored at −80°C until analysis. Supernatants collected for IgE measurement were frozen and thawed three times to release intracellular IgE before centrifugation and freezing. IL-4, IFN-γ and IgE were determined in duplicate by solid-phase sandwich ELISA according to the manufacturers’ protocols (BioLegend, San Diego, CA, USA for IL-4 and IFN-γ and Bio-Quant, San Diego, CA, USA for IgE). The IgE ELISA was modified for in vitro use by using a low-range standard curve, and the sensitivity of the assay was determined by using two standard deviations above the mean of 10 negative control measurements (0.3 ng/mL). Plates were read using a 450 nm measurement filter, and optical densities were converted into IU/mL, ng/mL or pg/mL (1 IU of IgE = 2.4 ng of IgE protein) according to the standard curve prepared fresh for each run.

**qPCR**

Extraction of DNA from nasopharyngeal swabs and PBMCs was performed using a commercial kit (QIAamp DNA Mini Kit) according to
the manufacturer’s recommendations (Qiagen Inc., Valencia, CA, USA). For PBMC cultures, supernatants with adherent and non-adherent cells were collected and DNA extracted using the same kit. Detection and quantification of *C. pneumoniae* and *Mycoplasma pneumoniae* DNA were performed according to Apfalter et al.¹ and Waring et al.,² respectively, using TaqMan-technology-based qPCR on the LightCycler 2.0 platform (software version 4.0; Roche, USA). Three replicates of each dilution of the standards and sample extracts were tested; an additional replicate was checked for qPCR inhibitors, and inhibited specimens were retested at a dilution of 1:10. qPCR analyses were considered negative if the crossing-point values exceeded 45 cycles.

The effect of doxycycline on chlamydial growth in PBMC culture was determined by comparing the number of DNA copies at different timepoints in the absence or presence of different concentrations of the antibiotic.

### Statistical analysis

Data are expressed as means with standard deviations unless otherwise indicated. The Student’s t-test and non-parametric Wilcoxon signed rank test were used to compare differences in the means of normally and non-normally distributed data, respectively. The Pearson correlation test was used to assess the degree of correlation for continuous variables. A P-value of <0.05 was considered statistically significant for all comparisons. Statistical analyses were performed using SPSS, version 12.0 software (Chicago, IL, USA).

### Results

#### Subject characteristics

Allergic asthmatic adults (*n*=11, female/male 7/4, mean±SD age =46±17 years) were enrolled. All subjects were serum IgE positive (>100 IU/mL) at the time the blood specimens were obtained (mean 614±512 IU/mL). The mean Juniper Asthma Quality of Life Questionnaire score of asthmatic subjects was 150±44 and their mean exhaled nitric oxide was 38±41 parts per billion; mean forced expiratory volume in 1 s (FEV₁) (% predicted) was 70±17 and mean forced vital capacity (FVC) (% predicted) was 79±17. All patients were classified as having moderate persistent asthma and all were treated with inhaled corticosteroids. A total of 55% of the allergic asthmatic subjects had *C. pneumoniae* IgG titres ≥1:16, with a median titre of 1:32. Healthy adult controls (*n*=12, female/male 7/5, mean±SD age =36±12 years) were also enrolled. None of the healthy subjects had a history of asthma or allergic rhinoconjunctivitis; serum IgE levels were low in all controls (<100 IU/mL). A total of 50% of control subjects had *C. pneumoniae* IgG titres ≥1:16 (median titre of 1:32).

All subjects, both asthmatics and healthy controls, tested negative for *C. pneumoniae* and *M. pneumoniae* by qPCR of nasopharyngeal swabs.

### Effect of doxycycline on production of IgE in PBMCs

When PBMCs from patients with allergic asthma were cultured in the presence of *C. pneumoniae* (moi=0.1), we observed increased IgE production on day 10, compared with uninfected PBMCs (4.7±5.4 ng/mL versus 0.9±0.7 ng/mL, *P*=0.008) (Figure 1). No significant differences in IgE production between conditions were seen at 48 h p.i. Culturing PBMCs with heat-inactivated *C. pneumoniae* did not result in significant changes in IgE (<0.3 ng/mL; *P*=0.2). The effect of doxycycline on cytokines

PBMC culture supernatants from subjects with allergic asthma had increased IL-4 levels on day 2, but not on day 10, in response to *C. pneumoniae* (moi=0.1) infection (mean 9.5±16.9 pg/mL) compared with uninfected PBMCs from asthmatic subjects (mean 2.3±4.1 pg/mL, *P*=0.019) (Figure 2). IL-4 concentrations in PBMC cultures from healthy control subjects were low in both uninfected cells (0.1±0.3 pg/mL) and infected cells (0.2±0.3 pg/mL, *P*=not significant). We observed higher IFN-γ levels in PBMC cultures from asthmatic subjects on day 2 in response to *C. pneumoniae* (moi=0.1) infection (mean 311±414 pg/mL), compared with uninfected PBMC cultures (mean 9.4±6.6 pg/mL, *P*=not significant) (Figure 2), while the IFN-γ concentration in control PBMC cultures increased from 12.5±8.3 pg/mL in uninfected PBMCs to 498±552 pg/mL in infected PBMC cultures (*P*=0.048).

*C. pneumoniae* infection-induced increases in IL-4 level at 48 h p.i. correlated with increases in IgE production at 10 days p.i. in PBMC cultures (*r*=0.98, *P*<0.001), whereas IFN-γ production did not correlate with *in vitro* IgE production.

To determine the effect of different concentrations of doxycycline, we treated PBMCs with 10-fold dilutions of drug concentrations from 1.0 mg/L to 0.01 mg/L. When doxycycline (1.0 mg/L) was added to infected PBMCs, IL-4, but not IFN-γ, levels were markedly decreased (Figure 2) with a mean percentage decrease of

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**Figure 1.** Effect of *C. pneumoniae* (Cpn) and doxycycline on the concentration of IgE. IgE in PBMC culture from serum IgE+ allergic asthmatic subjects (*n*=11) on day 10 of culture. Data are expressed as means±SD. The broken line separates the results of unstimulated from those of stimulated PBMC cultures. *Statistically significant increase compared with uninfected PBMCs (P<0.05).* #Statistically significant inhibition compared with infected untreated PBMCs (P<0.05).
was observed with either of the lower doxycycline concentrations used (0.01 and 0.1 mg/L). There were $3.83 \times 10^3 \pm 1.43 \times 10^2$ and $1.45 \times 10^4 \pm 5.33 \times 10^2$ bacterial genomes in infected PBMCs in the presence of 0.01 and 0.1 mg/L, respectively, versus $8.73 \times 10^4 \pm 2.66 \times 10^3$ in the absence of doxycycline ($P$ = not significant for both concentrations).

### Discussion

The present studies have demonstrated the ability of doxycycline to suppress the *C. pneumoniae*-induced production of IgE and IL-4 in PBMCs from IgE+ allergic asthmatic subjects. The association between *C. pneumoniae* infection and asthma symptoms has been well established in clinical and epidemiological studies. However, the use of antibiotics as anti-inflammatory agents in asthma treatment remains controversial.

Ongoing inflammation following *C. pneumoniae* infection may contribute to the severity and progression of asthma. In this study, we observed a significant increase in the secretion of IL-4, a critical cytokine for the induction of IgE production, and low levels of IFN-γ from *C. pneumoniae*-infected cultures of PBMCs obtained from allergic asthmatics when compared with controls. The results suggest that *C. pneumoniae* infection may promote allergic inflammation and the ongoing production of IgE. Both groups (asthmatic subjects and controls) had similar seroprevalence rates and titres of *C. pneumoniae* IgG (1:32), indicating that both probably had similar rates of past infection with *C. pneumoniae*. This suggests that the differences in overall cytokine and IgE responses between groups cannot be attributed to differences in exposure alone. The magnitude of the measured in vitro concentration of cytokines and IgE, which is correlated with IL-4, within the asthma group is likely to be affected by the presence of memory lymphocytes following prior infection, as well as the length of time since infection. The anti-inflammatory effects of doxycycline, as shown in our PBMC infection model, are therefore greatest in the presence of memory responses to *C. pneumoniae*. However, similar anti-inflammatory effects of doxycycline have been demonstrated in studies using other kinds of PBMC stimulation, indicating a broader mechanism not specific to *C. pneumoniae*-induced inflammation.

There is additional concern about the ability to clear chlamydial infections in the presence of a Th2-biased T cell response and the development of persistent infection. Suppression of IFN-γ production may therefore inhibit clearance of *C. pneumoniae* through cell-mediated immunity, in particular Th1 responses (IFN-γ), which are crucial in the clearance of infections caused by *C. pneumoniae*. Persistent infections have been described as occurring in asthmatics and may be associated with the lack of a strong Th1 response. Animal studies have demonstrated Th2-associated late-phase inflammation and a lack of control of infection with *C. pneumoniae* in IFN-γ knockout mice, suggesting a critical role for the balance between Th1 and Th2 cytokines. In the absence of documented persistent infection from culture or PCR, no human biomarker of chronic infection with *C. pneumoniae* currently exists to further characterize our subjects in that respect. The other major potentially treatable bacterial pathogen associated with asthma is *M. pneumoniae*. Our subjects were all tested and found to be negative by qPCR of nasopharyngeal swabs; in addition, PBMC cytokine and IgE production observed in our study were...
elicited by and specific to in vitro infection with C. pneumoniae. Hence, infection with M. pneumoniae should not have interfered with the observed results.

Antimicrobial agents have received renewed attention as a treatment strategy for asthma and allergy. However, Sutherland et al. reported a randomized controlled trial of clarithromycin in asthma that failed to show an improvement in the selected primary clinical outcomes for patients treated with the macrolide clarithromycin. It may therefore prove critical: (i) to identify the most effective class of antibiotics with anti-inflammatory properties; and (ii) to study biomarkers to identify the subgroups of patients with asthma, who may benefit most from this treatment.

The immunomodulatory effect of the tetracycline doxycycline in our study is consistent with earlier studies in our laboratory that demonstrated the ability of minocycline to reduce serum IgE and provide a steroid-sparing effect for allergic asthmatic individuals. Minocycline, as well as doxycycline, also suppresses the in vitro production of IgE by IL-4/anti-CD40-stimulated PBMCs from allergic asthmatic subjects in a dose-dependent fashion. The use of an inexpensive class of drugs such as tetracyclines with a known safety profile and the ability to decrease the de novo production of IgE may be advantageous.

The mechanisms by which tetracyclines suppress IgE responses in allergic asthmatic individuals remain to be identified; both anti-infectious and anti-inflammatory effects may play a role. Doxycycline has been shown to suppress immunoglobulin secretion and class-switching by activated murine B cells in vitro. In addition, there is evidence for effects on T lymphocyte function. Doxycycline and tetracycline inhibited the ability of mice to mount delayed-type hypersensitivity responses and diminished lymphoproliferative responses.

Our present findings for C. pneumoniae-infected human PBMCs may help to clarify some of these suspected mechanisms—concurrent suppression of IL-4 and IgE, while maintaining IFN-γ responses, suggests that suppression of Th2 lymphocyte functions plays a role in the effects of doxycycline. In related studies, we have identified the suppression of p38 MAP kinase as a potential mechanism for T cell-mediated IgE suppression.

The studies of Ingham et al. reported that doxycycline and minocycline suppressed the mitotic responses of human peripheral blood lymphocytes at high (superphysiological) concentrations, but not at physiological concentrations similar to the ones used in our experiments. In contrast, we found a selective suppression of Th2 and IgE responses at low doses, with preservation of IFN-γ production, which is protective against intracellular pathogens. IFN-γ responses are critical for in vivo protection from infection with C. pneumoniae.

The results of our study help to answer the question of whether the beneficial effects of doxycycline are due to anti-chlamydial or anti-inflammatory properties. Primary monocyte-derived cells naturally restrict the growth of C. pneumoniae, leading to metabolically active, intracellular organisms, but a lack of infectious progeny. Hence it would not be possible to determine the MIC or chlamydial concentration of doxycycline in primary monocyte-derived cells by the most commonly used method—quantification of C. pneumoniae in respiratory epithelial cells by immunofluorescence staining and counting of inclusions, followed by the removal of antibiotics and passage onto fresh respiratory epithelial cells for quantification. Instead, we applied a method for quantifying the number of C. pneumoniae in cell cultures of monocytic cells by qPCR as suggested by Poikonen et al. Our results suggest that alterations in the inflammatory response to the intracellular pathogen rather than restriction of its growth by doxycycline is the primary effect in our in vitro model.

The in vitro anti-inflammatory effect was observed over a wide range of physiological concentrations (0.01–1.0 mg/L), including those commonly achieved in the serum of patients treated with standard doses of doxycycline. The lowest concentration (0.01 mg/L) is well below the reported range of MICs (0.06–0.25 mg/L) and the MIC90 (0.25 mg/L) of doxycycline for C. pneumoniae. In addition, the number of chlamydial DNA copies in PBMC culture was not significantly suppressed by any of the concentrations (0.01–1.0 mg/L) of doxycycline added to the cultures. Therefore it can be suggested that, in our model of human PBMCs, doxycycline did not inhibit chlamydial infection, but rather it suppressed C. pneumoniae-mediated IgE responses and cytokine production. This appears to be, in part, related to a selective suppression of IL-4, which plays an important role in producing and maintaining inflammation in allergic asthma. These findings are consistent with studies of subantimicrobial-dose doxycycline in the treatment of acne, which have demonstrated an improvement in inflammatory lesions, but no effects on skin flora.

Mukouyama et al. have reported a spontaneous production of IgE by PBMCs from asthmatic children (n = 26), with IgE supernatant secretion levels ranging from 0.1 to 15.0 IU/mL. While we also observed spontaneous IgE production, there was a significant enhancement of IgE production along with significant increases in IL-4 and a correlation between levels of C. pneumoniae-induced IgE and IL-4, suggesting that C. pneumoniae mediates IgE increases through Th2 lymphocyte activation. In our in vitro model, active infection was required to stimulate both cytokine and IgE production, thus indicating a role for signals from antigen-presenting cells present in PBMCs. Monocytes are the primary cell type present in PBMCs that are susceptible to C. pneumoniae infection and appear to be involved in providing inflammatory signals required for the lymphocyte response observed in our culture model. Reversal of the cytokine imbalance by doxycycline may indicate beneficial effects for patients in two ways: (i) by suppressing IgE responses; and (ii) by allowing effective clearance of the intracellular bacterium C. pneumoniae from the patient’s airways.

In this study, we have demonstrated potent anti-inflammatory and IgE-suppressive effects of doxycycline in an in vitro model of C. pneumoniae infection, lending further support to the use of and further research into tetracyclines as immune modulators and anti-allergic medications. Additional clinical studies involving subjects with allergic asthma who are infected with C. pneumoniae are indicated to study the effects of tetracyclines in relation to asthma symptoms, clearance of infection and correlation with changes in cytokines and IgE production.

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Transparency declarations
None to declare.
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