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N-acetyl-cysteine increases the replication of *Chlamydia pneumoniae* and prolongs the clearance of the pathogen from mice

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Abstract

Purpose. Within the community, 10 % of acquired pneumonia is caused by *Chlamydia pneumoniae*. *N*-acetyl-cysteine (NAC) is one of the most commonly used mucolytics in respiratory diseases, but its effect on *C. pneumoniae* infection has not yet been investigated. In this study, our aim was to investigate whether NAC influences the replication of *C. pneumoniae*. After determining that NAC does have an effect on *C. pneumoniae* replication, the effect of an alternative drug called Ambroxol (Ax) was investigated.

Methodology. The *in vitro* effect of NAC and Ax was studied on *C. pneumoniae*-infected A549 and McCoy cells. Furthermore, the influence of NAC and Ax was examined in mice infected intranasally with *C. pneumoniae*.

Results. NAC treatment resulted in approximately sixfold more efficient *C. pneumoniae* growth in tissue culture compared to the untreated control cells, and this effect was shown to be based on the increased binding of the bacterium to the host cells. The *C. pneumoniae*-infected mice to which NAC was given had prolonged and more severe infections than the control mice. Ax decreased *C. pneumoniae* replication *in vitro*, which was partially associated with the increased expression of indolamine 2,3-dioxygenase. In animals, using the adapted usual human dose, Ax did not alter the number of recoverable *C. pneumoniae*.

Conclusion. Based on our results, it might be recommended that a mucolytic agent other than NAC, such as Ax, be used in respiratory diseases suspected to be caused by *C. pneumoniae*.

INTRODUCTION

N-acetyl-cysteine (NAC) is a commonly used agent in the healthcare profession. It has multiple therapeutic uses in psychiatry and is useful in the case of an acetaminophen overdose. Moreover, NAC is used as a mucolytic agent in respiratory diseases, where its free thiol group breaks down the disulphide bonds in mucus, thereby decreasing its viscosity. NAC is mostly taken *per os*, except when it is taken to combat acetaminophen intoxication [1]. Ambroxol (Ax) is another mucolytic and expectorant drug that is used to treat different respiratory diseases. In addition to the effects of Ax on mucus regulation and its local anaesthetic effects, a wide range of pharmacological anti-inflammatory properties of Ax have been described *in vitro* and *in vivo* [2, 3].

Chlamydia pneumoniae, belonging to the family *Chlamydiaceae*, is a Gram-negative obligate intracellular bacterium. It is a common cause of acute respiratory infection, including community-acquired pneumonia, sinusitis, pharyngitis, bronchitis and exacerbations of chronic bronchitis. *C. pneumoniae* is responsible for approximately 10 % of pneumonia cases [4]. The infectivity of *Chlamydia* species depends on the reduced state of a cysteine-rich protein [5]. There is a cysteine-rich, strongly disulphide cross-linked protein of the family *Chlamydiaceae* that is called OmcB, and this is indispensible for binding [6]. *C. pneumoniae* and *Chlamydia trachomatis* carry OmcB protein in their outer membrane, where the reduction of its disulphide bonds can be achieved by glutathione (GSH) [7] and by the host cell's protein disulphide isomerase [5]. The influence of NAC, a potent reducing agent, on *C. pneumoniae* replication has not yet been investigated. On the basis of our hypothesis, the reduction of OmcB (which has the highest disulphide content among the structural proteins in *C. pneumoniae*) by NAC can increase binding and consequently increase the replication of *C. pneumoniae*. In this study, we found that

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Abbreviations: Ax, ambroxol; GSH, glutathione; IDO 1,2, indolamine 2,3-dioxygenase 1,2; NAC, *N*-acetyl-cysteine.

NAC promotes *C. pneumoniae* growth *in vitro* and aggravates the severity of pneumonia in mice.

METHODS

Propagation of *C. pneumoniae*

In our experiments, the CWL029 strain of *C. pneumoniae* from the ATCC (Manassas, VA, USA) was used. *C. pneumoniae* was propagated in HEp-2 cells (ATCC), as described previously [8, 9]. The titre of the infectious elementary bodies (EBs) was determined by indirect immunofluorescence assay. Serial dilutions of the EB preparation were inoculated onto McCoy cells (ECACC, London UK), and after being cultured for 48 h, the cells were fixed with acetone and stained with a monoclonal anti-*Chlamydia* lipopolysaccharide antibody (AbD Serotec, Oxford, UK) and FITC-labelled anti-mouse IgG (Sigma, St Louis, MO, USA). The number of *C. pneumoniae* inclusions was counted under a UV microscope, and the titre was expressed as inclusion-forming units/ml (i.f.u. ml^{-1}).

In vitro effect of NAC and Ax

McCoy and A549 (ATCC) cultures were grown in 24-well tissue culture plates containing a 13 mm cover glass in minimum essential medium Eagle with Earle's salts (Sigma), supplemented with 10 % vol/vol foetal calf serum, 0.5 % wt/vol glucose, 0.3 mg of L-glutamine ml^{-1}, 4 mM HEPES and 25 µg of gentamycin ml^{-1}. Five parallel wells of semi-confluent cultures were infected with 2×10^3 $well^{-1}$ *C. pneumoniae* or treated simultaneously with NAC (0.01–10 mg ml^{-1}) or Ax (0.002–0.05 mg ml^{-1}) at the time of infection. Separate cells were infected with *C. pneumoniae* pretreated (1 h) with different concentrations of NAC. Subsequently, *C. pneumoniae* EBs were pretreated with NAC or Ax by continuous shaking in the presence of NAC (0.1 mg ml^{-1}) or Ax (0.05 mg ml^{-1}) at room temperature, and after 1 h these drugs were washed out using a culture medium and centrifugation at 13 800 ***g*** for 15 min (Heraus Fresco 17) or left unwashed. NAC- or Ax-treated *C. pneumoniae* or non-treated *C. pneumoniae* were inoculated onto cells on cover glasses in 24-well plates and centrifuged at 800 ***g*** for 1 h. After incubation for 48 h, the cells on the cover glasses were fixed with acetone and stained as described above in the 'Propagation of *C. pneumoniae*' section to visualize the inclusions of *C. pneumoniae*. In order to detect the effect of NAC or Ax on the attachment of chlamydial EBs to host cells, McCoy or A549 cells were infected with NAC- or Ax-treated *C. pneumoniae* EBs and after a 1 h incubation period and centrifugation the cells were washed and fixed and the bound EBs were stained by indirect immunofluorescence, as described above.

Fluorescence signals were analysed via Olympus UV microscopy. The immunofluorescence of non-infected or *C. pneumoniae*-infected cells, or cells infected with drug-treated *C. pneumoniae* was analysed quantitatively by ImageQuantTL 8.1 software as follows: 6–6 equally sized circular areas covering the cells were randomly selected on each image, and then the background signals of the selected areas were eliminated by a threshold set-up and the fluorescence intensity/pixel values of the randomly selected cells were quantified.

Mice and infection conditions

Pathogen-free 6-week-old female BALB/c mice were obtained from Charles River Laboratories (Hungary). The mice were maintained under standard husbandry conditions at the animal facility of the Department of Medical Microbiology and Immunobiology, University of Szeged, and were provided with food and water *ad libitum*. Before infection, the mice were mildly sedated with an intraperitoneal injection of 200 µl of sodium pentobarbital (7.5 mg ml^{-1}). They were then infected intranasally with 2×10^5 i.f.u. *C. pneumoniae* in 20 µl sucrose/phosphate/glutamic acid (SPG) buffer, and from the second day post-infection they were treated with 0.2 mg NAC (Sigma) in a volume of 50 µl drinking water *per os* daily. Mice were anaesthetized and sacrificed 7 days, or in another experiment 20 days, after the infection. Blood was then taken by cardiac puncture. In a separate study, *C. pneumoniae*-infected mice were treated in a similar way as above with 25 µg Ax (Sigma) and sacrificed 7 days after infection. The control mice received the same amount of tap water by oral administration using a Gilson pipette to mimic the stress of the watering process. After euthanization, the lungs of the mice were removed and homogenized with acid-purified sea sand (Fluka Chemie AG, Buchs, Switzerland). The homogenized lungs were suspended in 1 ml of SPG for the detection of viable *C. pneumoniae*. The experiments were approved by the Animal Welfare Committee of the University of Szeged and they conformed to Directive 2010/63/EU of the European Parliament.

The culturing of *C. pneumoniae* from the lungs of mice

After two freeze-thaw cycles, the homogenized lungs from individual mice were centrifuged (10 min, 400 ***g***) and serial dilutions of the supernatants were inoculated onto McCoy cell monolayers. These samples were then centrifuged (1 h, 800 ***g***), and after a 48 h culture the cells were fixed with acetone and stained as described above in the 'Propagation of *C. pneumoniae*' section to visualize the inclusions of *C. pneumoniae*.

Total RNA extraction and cDNA synthesis

One-day-old semi-confluent McCoy cells in six-well plates were infected with *C. pneumoniae* using a multiplicity of infection (m.o.i.) of 4. The cells were then left untreated or NAC (0.1 mg ml^{-1}) or Ax (0.05 mg ml^{-1}) were added to the medium. Total RNA was extracted after a 1-day incubation from three parallel wells of each condition with Tri Reagent according to the manufacturer's protocol (Sigma). RNA concentrations were measured at 260 nm via a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). The purity of the RNA samples was given as the ratio of the RNA absorbance at 260 and 280 nm, which was higher than 2 in each sample. Afterwards, 1 µg of total RNA

was reverse-transcribed using Maxima Reverse Transcriptase according to the manufacturer's protocol with random hexamer priming (Thermo Fisher Scientific, Inc. Waltham, MA, USA).

Quantitative PCR (qPCR) of the indolamine 2,3-dioxygenase 1,2 (*IDO1,2*)

qPCR was performed in a Bio-Rad CFX96 real-time system by using a SsoFast EvaGreen qPCR Supermix (Bio-Rad, Hercules, CA, USA) master mix and the murine-specific primer pairs IDO1 : 5′-GCTTCTTCCTCGTCTCTCTA TTG-3′, 5′-TCTCCAGACTGGTAGCTATGT-3′; IDO2 : 5′-CCTGGACTGCAGATTCCTAAAG-3′, 5′-CCAAGTTCC TGGATACCTCAAC-3′; beta-actin: 5′-TGGAATCCTG TGGCATCCATGAAAC-3′, 5′-TAAAACGCAGCTCAG TAACAGTCCG-3′. All of the primers were designed using PrimerQuest Tool (IDT) software and synthesized by Integrated DNA Technologies, Inc. (Montreal, Quebec, Canada). To check the amplification specificity, the qPCR was followed by a melting curve analysis. Threshold cycles (Ct) were calculated for the *IDO1*, *IDO2* and beta-actin genes, and the relative gene expressions were calculated by the ΔΔCt method. Student's *t*-test was used to compare the statistical differences of ΔCt values between the infected and control samples, as described previously [10], with a significance level of $P<0.05$.

Statistical analysis

The data are expressed as mean±standard deviation (SD). Student's *t*-test was applied using Microsoft Office Excel and a *P* value of less than 0.05 indicated a statistically significant difference.

RESULTS

NAC increases the *in vitro* replication of *C. pneumoniae*

Initially, we wanted to check the potential anti-chlamydial effect of NAC under *in vitro* conditions. During our experiments, different concentrations of NAC were applied using two approaches. First, a decreasing concentration of NAC was mixed directly with *C. pneumoniae,* and the host cells were immediately infected. Among the concentrations applied, doses of 10 mg ml^{-1} and 2 mg ml^{-1} NAC were toxic to the cells. Surprisingly, 0.1 mg ml^{-1} NAC resulted in a nearly sixfold increase in the number of *C. pneumoniae* inclusions in McCoy cells as compared to the number observed after infection with untreated *C. pneumoniae* ($P<0.05$; Fig. 1). Accordingly, we chose this concentration in later studies. In the second approach, *C. pneumoniae* was preincubated with NAC at the respective three concentrations by shaking the mixture of *C. pneumoniae* and NAC continuously for 1 h before infecting the cells. As shown in Fig. 1, there was no significant difference in the infectivity of *C. pneumoniae* pretreated for 1 h with NAC and *C. pneumoniae* treated with 0.1 mg ml^{-1} NAC immediately before inoculation of the cells ($P>0.05$).

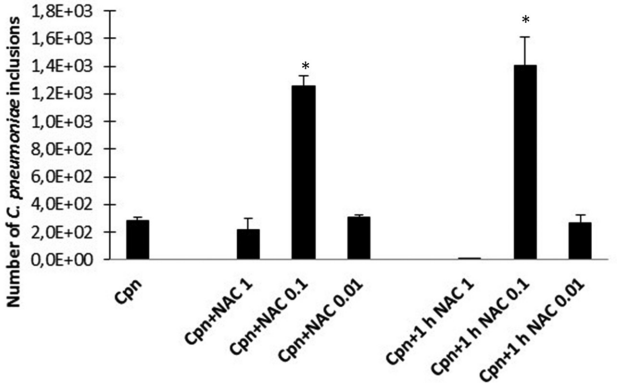

Fig. 1. The effect of different concentrations of NAC and different treatment conditions on *C. pneumoniae* replication. McCoy cells were infected with *C. pneumoniae* treated with different concentrations (0.01–1 mg ml^{-1}) of NAC at the time of inoculation or infected with *C. pneumoniae* pretreated with NAC. *C. pneumoniae* inclusions were counted under a UV microscope after indirect immunofluorescence staining. Here, the bars denote the means and SDs of the results on five parallel tissue wells (*$P<0.05$).

NAC increases the binding of *C. pneumoniae* to the host cell

To investigate whether NAC is able to influence the attachment of *C. pneumoniae*, McCoy and the more relevant A549 epithelial cells of human respiratory origin were infected with NAC-pretreated *C. pneumoniae* or with untreated *C. pneumoniae*. As shown in Fig. 2(a, b, d), NAC-treated *C. pneumoniae* produced a significantly higher fluorescence intensity as compared to the untreated *C. pneumoniae* in McCoy cells. A similar effect was observed in the A549 cells (Fig. 2e, f, j). The control cells did not display any fluorescence (Fig. 2c, g).

Subsequent experiments indicated that the removal of NAC with the culture medium from *C. pneumoniae* did not significantly modify the number of replicating *C. pneumoniae* as compared to the outcome of the infection with unwashed *C. pneumoniae* ($P>0.05$; Fig. 3). Adding NAC to the cells 6 or 24 h after *C. pneumoniae* infection did not change the number of inclusions formed by *C. pneumoniae* (Fig. 3). Furthermore, preincubation of the host cells with NAC for 48 h before infection with *C. pneumoniae* did not modify the replication of *C. pneumoniae* (data not shown).

Exposure to NAC not only increases the chlamydial lung burden, but also prolongs the infection in mice

Here, we investigated whether NAC might aggravate the chlamydial infection in the short term. Twenty mice were infected with *C. pneumoniae*, and 10 were treated with 10 mg kg^{-1} NAC *per os* at the same concentration as that applied for human respiratory infections as a mucolytic drug for 6 days. On the seventh day, the mice were sacrificed; the lungs were removed for the determination of recoverable *C. pneumoniae*. We found that after 6 days of

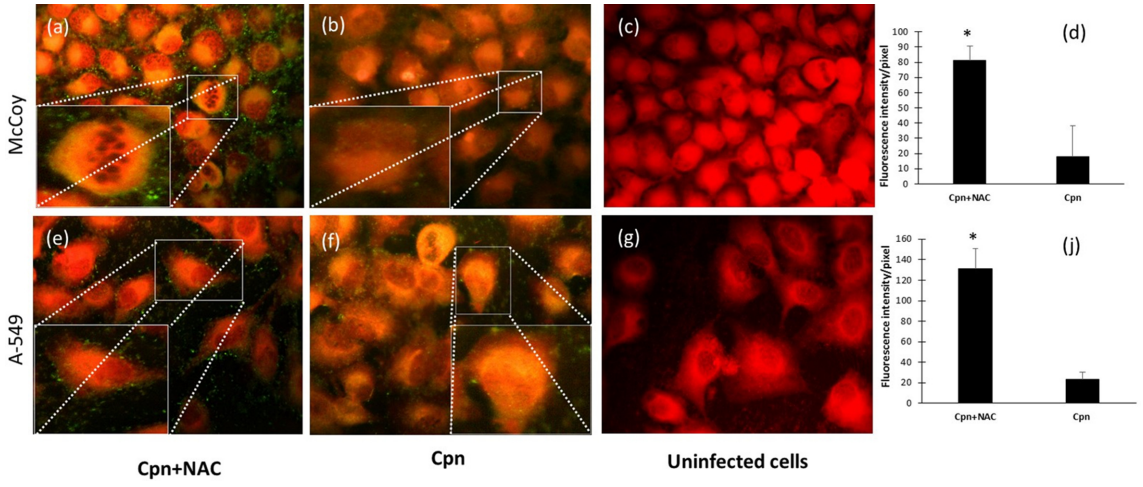

Fig. 2. The effect of NAC treatment on *C. pneumoniae* attachment. McCoy or A549 cells were infected with NAC-treated (0.1 mg ml^{-1}) (a, e) or untreated *C. pneumoniae* (Cpn) (b, f). After the incubation period, the cells were stained as described above in the Methods section. Fluorescence signals were analysed via UV microscopy, and the immunofluorescence of non-infected, *C. pneumoniae*-infected or drug-treated *C. pneumoniae*-infected cells was analysed quantitatively by ImageQuantTL 8.1 software. The results are expressed as the mean±SD of the data from three independent experiments, *P<0.05 (d, j).

exposure to NAC the severity of the *C. pneumoniae* infection increased. The number of recoverable chlamydial inclusions was approximately three times higher in the NAC-treated group than in the *C. pneumoniae*-infected mice without NAC treatment (Fig. 4a). Next, we investigated whether NAC prolongs the clearance of *C. pneumoniae* from the lungs. Sixteen mice were infected with *C. pneumoniae* and eight mice were treated with NAC for 19 days. On the twentieth day, the mice were sacrificed and their lungs were removed for the detection of viable *C. pneumoniae*. All of the mice were *C. pneumoniae* culture-positive in the NAC-treated group and the number of *C. pneumoniae* inclusions was 2.5 times higher than that in the control group. Moreover, in the control group two mice became culture-negative (the sensitivity of our method was <40 i.f.u./lung), suggesting recovery from the disease. The data for these mice were not included in Fig. 4(b).

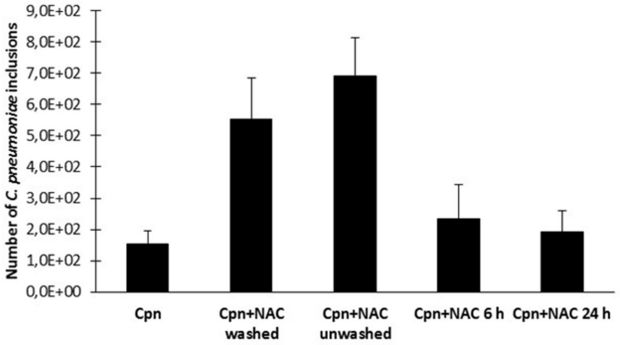

Fig. 3. The effect of pre- or post-infection exposure to NAC on *C. pneumoniae* replication. The number of *C. pneumoniae* inclusions were counted in McCoy cells infected with untreated *C. pneumoniae* (Cpn), cells infected with *C. pneumoniae* preincubated for 1 h with (0.1 mg ml^{-1}) NAC and subsequently washed via high-speed centrifugation (Cpn+NAC washed), cells infected with NAC-treated but unwashed *C. pneumoniae* (Cpn+NAC unwashed) and cells infected with untreated *C. pneumoniae* to which NAC was added 6 h (Cpn+NAC 6 h) or 24 h (Cpn +NAC 24 h) post-infection. The *C. pneumoniae* inclusions were revealed by indirect immunofluorescence days post-infection. The means of the titres are expressed as i.f.u. ml^{-1} in five parallel cultures and the SDs are shown.

Ax does not increase the number of infective *C. pneumoniae*

To look for a better alternative to NAC, the effect of Ax on *C. pneumoniae* replication was tested in *in vitro* and *in vivo* systems. Using concentrations of 0.002 and 0.01 mg ml^{-1}, Ax did not cause any significant changes in bacterial replication in McCoy cells, but 0.05 mg ml^{-1} Ax had a strong antimicrobial effect and this reduced the number of *C. pneumoniae* to approximately one-fifth of that observed with untreated cells (P<0.05; Fig. 5a). As shown in Fig. 5(b), none of the applied concentrations of Ax influenced host cell viability. Increasing the dose to 0.25 mg ml^{-1} Ax proved to be toxic to the cells (data not shown). We then wanted to determine the reason for the antimicrobial activity of Ax. An identical experiment to that performed with NAC was conducted. Ax-treated and untreated *C. pneumoniae* were inoculated into McCoy and A549 cells, and the cells were stained using the immunofluorescence method. An increase in immunofluorescence was not observed in this case, and we inferred that the Ax treatment did not modify the number of *C. pneumoniae* EBs attached to the cell membrane

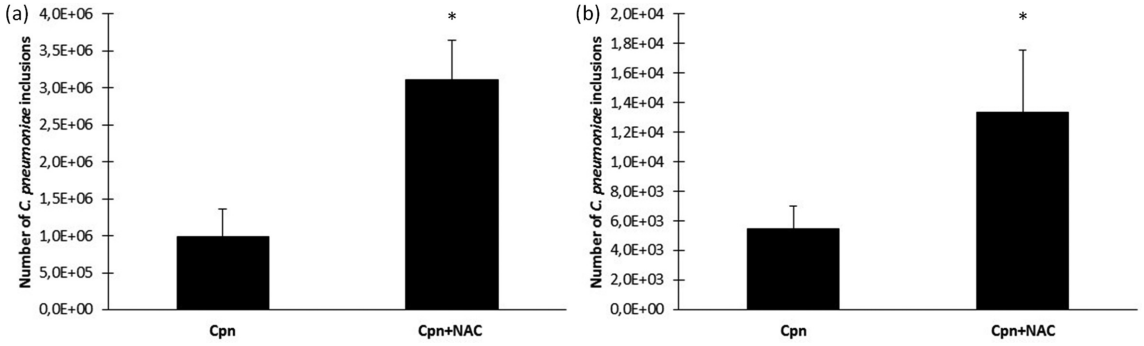

Fig. 4. Recoverable i.f.u. in *C. pneumoniae*-infected mice with or without oral NAC treatment. The lung homogenates of infected (Cpn) or infected and NAC-treated (10 mg kg^{-1}) (Cpn+NAC) mice on day 7 (a) or on day 20 (b) post-infection were inoculated onto McCoy cell monolayers, and *C. pneumoniae* inclusions were detected by indirect immunofluorescence. Here, the data are the means±SD of the number of *C. pneumoniae* inclusions (i.f.u./lung) in the lung homogenates of individual mice (*P<0.05).

(data not shown). It is well documented that IDO1,2 shows antimicrobial activity that metabolizes the tryptophan, which is essential in chlamydial replication. We then tested whether Ax might influence the expression of IDO1,2. Ax caused a 2.5-fold increase in IDO2 expression in *C. pneumoniae*-infected cells as compared to the expression in untreated cells. In our experiments, NAC treatment did not influence the expression of IDO1,2 in *C. pneumoniae*-infected cells (data not shown). To further investigate the role of Ax during *C. pneumoniae* infection, our mice were infected with *C. pneumoniae* and from the second day were treated with tap water-diluted Ax at the same concentration as is applied for human respiratory infections when it is used as a mucolytic drug (1.25 mg kg^{-1}). The control mice were given tap water. During the 7-day period, the behaviour (activity and appetite) and weight of the animals did not change significantly between groups (data not shown). The mice were sacrificed 7 days after the infection and the number of recoverable *C. pneumoniae* was counted from the lungs by direct immunofluorescence. As shown in Fig. 5 (c), the number of recoverable *C. pneumoniae* did not change significantly in the Ax-treated and un-treated *C. pneumoniae*-infected group of mice.

DISCUSSION

NAC is a multifaceted drug that is used in the treatment of different diseases, mainly as a mucolytic agent. It is relatively inexpensive and commercially available as an over-the-counter (OTC) medicine. It has been shown to increase the level of GSH, the body's major antioxidant, by increasing glutathione S-transferase activity. It is a powerful antioxidant and has the potential to treat diseases characterized by the generation of free oxygen radicals [1]. For instance, NAC is a therapeutic option in chronic obstructive pulmonary disease. In an open-label study of 1392 patients, NAC reduced the viscosity of expectorated sputum, reduced cough severity and improved the ease of expectoration in patients after 2 months of treatment [11]. Furthermore, NAC dramatically attenuated influenza symptoms in a group of patients as compared with a placebo-treated group [12].

The direct antimicrobial role of NAC is not well defined, but there are data regarding its inhibitory effect on biofilm formation. NAC displayed a direct antimicrobial effect against extracellular pathogens, but the concentrations applied by different authors varied greatly (0.003–80 mg ml^{-1}) [13–15]. In the case of tuberculosis, NAC was found to play a part in inhibiting the growth of intracellular *Mycobacterium tuberculosis* through bacteriostatic mechanisms [16]. In our study, we found that instead of decreasing bacterial replication, NAC actually increased the number of replicating *C. pneumoniae* in both *in vitro* and *in vivo* infections. Based on our *in vitro* experiment, we disclosed that NAC increased the attachment of the pathogens to the host cells (Fig. 2). Lazarev *et al.* investigated the role of intracellular GSH in *C. trachomatis* infection, and they found that the treatment of cells with buthionine sulfoximine, which causes the irreversible inhibition of GSH biosynthesis or hydrogen peroxide-induced oxidation of GSH, decreased the number of *C. trachomatis* inclusions. In contrast with this finding, the treatment of cells with NAC increased the number of chlamydial inclusions. The researchers concluded that GSH plays a crucial role in chlamydial replication. In their experiments NAC was used as a GSH precursor, and they did not attempt to investigate the anti-chlamydial effect of NAC [7]. However, it is well known that the infectivity of *Chlamydia* species depends on the reduced status of the cell membrane protein OmcB [5]. In agreement with our hypothesis, NAC treatment of the EBs increases the attachment directly, probably by reducing OmcB, which could not have been from an increased level of GSH, because NAC was removed from the culture medium in some of our experiments. It would be worth examining other reducing agents, such as ascorbic acid (vitamin C), to find out whether they influence the binding of *C. pneumoniae* EBs onto host cells.

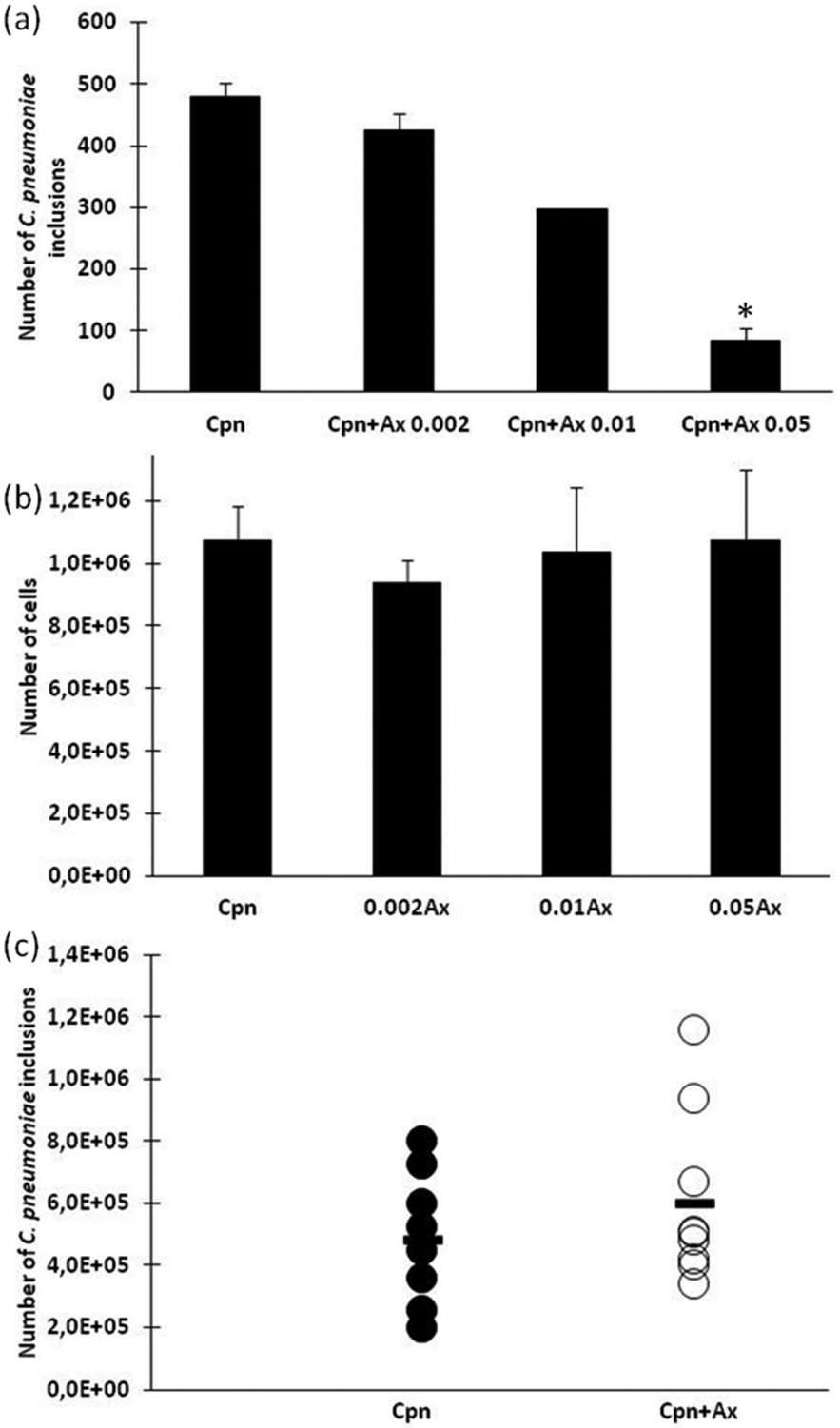

Fig. 5. The effect of Ax treatment on *in vitro* and *in vivo C. pneumoniae* infection. (a) McCoy cells were infected with *C. pneumoniae* (2×10^3 i.f.u.) and simultaneously treated with different amounts of Ax (0.002–0.05 mg ml^{-1}). *C. pneumoniae* inclusions were detected by indirect immunofluorescence. Here, the data are presented as the means±SD of the results in five parallel cultures. (b) The cell numbers for different concentrations of Ax. Five parallel wells of viable cells were counted under a light microscope using trypan blue dye. (c) A group of 10 *C. pneumoniae*-infected mice were treated *per os* with Ax (1.25 mg kg^{-1}). The mice were sacrificed 7 days after infection and the number of infective *C. pneumoniae* was detected in the homogenized lungs by inoculation into McCoy cells and staining using indirect immunofluorescence. The symbols (●, *C. pneumoniae*-infected; ○, *C. pneumoniae*-infected+Ax-treated) stand for individual mice. The bars represent the means of the recoverable *C. pneumoniae* inclusions in the groups.

Aside from the negative effect of NAC on acute *C. pneumoniae* infection, we need to take into account another possible effect of this drug. *C. trachomatis*, which belongs to the family *Chlamydiaceae*, is one of the most common sexually transmitted pathogens. Unfortunately, the infection is asymptomatic in up to 70 % of the cases. This means that the infections usually go unrecognized. The severe consequences of chronic *C. trachomatis* infection are ectopic pregnancy, infertility, or a pelvic inflammatory disease [17]. In the worst-case scenario, NAC applied simultaneously in the treatment of ongoing respiratory diseases may not only increase the growth of *C. pneumoniae,* but stimulate the growth of *C. trachomatis* as well.

In order to circumvent NAC's aggravating effect on *C. pneumoniae* infection, we looked for a better mucolytic agent that does not increase the severity of the respiratory disease. In our *in vitro* study, Ax displayed significant anti-chlamydial activity that was not associated with decreased binding of the pathogen to the host cells. The complete antimicrobial mechanism of Ax was not analysed, but Ax treatment increased the expression of the anti-chlamydial IDO2, which may in part cause a reduction in the number of pathogens. In our *in vivo* experiment, mice were infected with *C. pneumoniae* and then treated with Ax. Using a human equivalent Ax/body weight dose we found no significant difference in the number of recoverable *C. pneumoniae* between the treated and the untreated groups. The limitation of our study is that despite the promising *in vitro* results, we did not check to see whether a higher dose of Ax has an anti-chlamydial effect *in vivo*. Yang *et al*. found that 10 mg kg^{-1} day^{-1} (which is eight times higher than the normal dose in a human) significantly reduced the mortality of mice infected with a lethal dose of H3N2 influenza virus [18]. Further experiments are needed to investigate the possible anti-chlamydial effects of Ax at a higher dose.

In Germany, NAC is the second most popular drug for acute coughs, with 23.5 % of the OTC expectorant market share in 2015 (Ax is first with 24 %) [19]. *C. pneumoniae* is a common respiratory pathogen and unfortunately it is not always diagnosed correctly, and if a doctor suggests NAC as a mucolytic agent it might worsen and delay the patient's recovery. Based on the prevalence of *C. pneumoniae,* many patients could suffer prolonged respiratory disease because of NAC.

Overall, on the basis of our results, we can state that NAC can aggravate and prolong the infection caused by *C. pneumoniae* in an animal model. This information will be useful for physicians who recommend NAC as a mucolytic drug in respiratory diseases with a non-identified aetiology. In the case of a *C. pneumoniae* infection, a correct laboratory diagnosis is imperative, because in its absence the use of NAC may worsen the patient's chance of recovery. It is important that clinical studies to prove our results are implemented. Instead of NAC, Ax might be recommended, as it does not support the growth of *C. pneumoniae* in the mouse model.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical statement
The experiments were approved by the Animal Welfare Committee of the University of Szeged and they conformed to Directive 2010/63/EU of the European Parliament.

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