Non-thermal argon plasma is bactericidal for the intracellular bacterial pathogen *Chlamydia trachomatis*

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Non-thermal plasma (NTP) is a flow of partially ionized argon gas at an ambient macroscopic temperature and is microbicidal for bacteria, viruses and fungi. Viability of the Gram-negative obligate intracellular bacterial parasite *Chlamydia trachomatis* and its host cells was investigated after NTP treatment. NTP treatment of *C. trachomatis* extracellular elementary bodies (EBs) diminished the concentration of infectious bacteria by a factor of $9 \times 10^4$, as established by the parallel infection of murine fibroblast McCoy cells with treated and control EBs. NTP treatment of infected McCoy cells caused disruption of membrane-restricted vacuoles (inclusions), where *C. trachomatis* intracellular reticulate bodies (RBs) multiply, and a $2 \times 10^6$-fold reduction in the concentration of infectious bacteria. When the samples were covered with magnesium fluoride glass to obstruct plasma particles and UV rays alone were applied, the bactericidal effect was reduced $1.4 \times 10^1$-fold and $5 \times 10^4$-fold for EBs and RBs, respectively. NTP treatment caused the viability of host McCoy cells to diminish by 19%. Therefore, the results obtained demonstrated that (i) both extracellular and intracellular forms of *C. trachomatis* are sensitive to NTP treatment; (ii) the reduction in concentration of infectious bacteria after NTP treatment of infected cells is superior to the reduction in viability of host cells; and (iii) the effect of NTP on intracellular bacteria does not depend on UV rays.

INTRODUCTION

Non-thermal (low temperature) physical plasma is the flow of a partially ionized neutral gas obtained at atmospheric pressure. The non-thermal plasma (NTP) torch has a macroscopic temperature of approximately 30–40 °C and includes ions, electrons, active species and photons (Laroussi, 2005). There has been considerable interest in NTP among physicians, biologists, hygienists and food specialists over the past decade due to its increased number of medical and industrial applications (Isbary et al., 2010; Joaquin et al., 2009; Kong et al., 2009; Laroussi, 2005; Moreau et al., 2008; Nosenko et al., 2009). In particular, NTP has shown to be microbicidal for Gram-positive and Gram-negative bacteria, bacterial spores and fungi (Ermolaeva et al., 2011; Joshi et al., 2010; Kayes et al., 2007; Lee et al., 2006; Venezia et al., 2008). Moreover, bacteria in biofilms and on wound surfaces are susceptible to plasma treatment (Ermolaeva et al., 2011; Isbary et al., 2010; Joaquin et al., 2009; Joshi et al., 2010). In contrast, plasma treatment does not cause immediate mortality in mammalian cells. Depending on the cell type, plasma torch characteristics and treatment duration, effects on eukaryotic cells range from stimulation of migration to apoptosis within 1–3 days (Kalghatgi et al., 2010, 2011; Sensenig et al., 2011; Volotskova et al., 2011).

The differences in susceptibility between prokaryotic (bacterial) and eukaryotic (particularly, human) cells suggests that

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**Abbreviations:** EBs, elementary bodies; IFU, inclusion-forming units; NTP, non-thermal plasma; RBs, reticulate bodies.
the application of plasma could be used to eradicate intracellular parasitizing bacteria. Pathogenic Gram-negative bacteria of the family Chlamydiaceae are obligate intracellular pathogens that infect epithelial and endothelial cells and circulating macrophages (Beagley & Timms, 2000; Hammerschlag, 2002). Species of the genus Chlamydia are associated with chronic infections in humans including infections of the urogenital tract, eyes and lungs (Adderley-Kelly & Stephens, 2005; Beagley & Timms, 2000; Bébéré & de Barbeyrac, 2009; Kayes et al., 2007). Recent findings demonstrate that Chlamydia species are opportunistic pathogens that can be detected in chronic skin ulcers and other inflammatory skin conditions (King et al., 2001). The Chlamydia life cycle includes two forms: infectious, metabolically inert, extracellular elementary bodies (EBs); and vegetative, intracellular, reticulate bodies (RBs). The developmental cycle of C. trachomatis begins when infectious EBs attach to and stimulate uptake by host cells. Internalized EBs are located within a host-derived vacuole, termed an inclusion, and differentiate into larger, metabolically active RBs. RBs multiply by binary fission, and after eight to twelve rounds of multiplication, the RB progeny asynchronously differentiate into EBs. Release of EBs from host cells allows another cycle to be initiated (Cocchiaro & Valdivia, 2009; Dautry-Varsat et al., 2004; Fields & Hackstadt, 2002; Hackstadt et al., 1997; Hammerschlag, 2002). Chlamydia interacts with signalling pathways of the host cell in order to survive (Byrne & Ojcius, 2004; Cocchiaro & Valdivia, 2009; Dong et al., 2005).

The bactericidal action of NTP is a result of the concerted action of ions, electrons, active species and UV rays (Dobrynin et al., 2009; Nosenko et al., 2009). The complex interactions between plasma and biological objects are responsible for its effectiveness against various types of vegetative micro-organisms and spores. These features are particularly important for micro-organisms that, like Chlamydiae, exist in various forms.

In the present study, the bactericidal action of non-thermal argon plasma on Chlamydia trachomatis and the plasma toxicity for eukaryotic cells were investigated. In addition, we studied the relative contribution of UV rays to the total plasma action on intracellular C. trachomatis. A bactericidal effect exerted by argon plasma on extracellular and intracellular bacteria was evident and superior to the reduction in viability of host cells. The effect of UV rays was relatively low.

**METHODS**

*Cell lines, bacterial strains and growth conditions.* Mouse fibroblasts, McCoy cells, were used in this study and were grown in DMEM medium supplemented with 10% HiClone fetal calf serum, 2 mM glutamine, 4.0 mg ml⁻¹ gentamicin and 5.0 mg ml⁻¹ amphotericin B in a 5% CO₂ atmosphere.

*C. trachomatis* strain Bu 434/L2 was routinely propagated in McCoy cells and EBs were purified by Renografin gradient centrifugation (Caldwell et al., 1981). Purified EBs were resuspended in a sucrose/phosphate/glutamic acid buffer (SPG) and stored at −70 °C. Titres were determined by infecting cell monolayers with decimal dilutions of the stock suspension.

**Intracellular infection with C. trachomatis.** Subconfluent McCoy cell monolayers grown on glass coverslips in 24-well plates were infected with the thawed C. trachomatis EB suspension at an m.o.i. of 2 and cultivated at 37 °C in a 5% CO₂ atmosphere for a maximum of 48 h post-infection.

Intracellular C. trachomatis cells were visualized with fluorescence microscopy using the following procedure. Infected McCoy cells were fixed with 50% methanol, permeabilized with 1% Triton X-100 and stained with FITC-conjugated mAbs specific for the C. trachomatis MOMP protein (NearMedic Plus). Fluorescence of inclusion-containing cells was examined and photographed using a Nikon Eclipse 50i fluorescent microscope at ×1350 magnification.

**Non-thermal argon plasma source.** All experiments were performed using a MicroPlaSter β device, which was developed at the Max Planck Institute for Extraterrestrial Physics, Germany, in collaboration with ADTEC Plasma Technology, Japan (Shimizu et al., 2008). The MicroPlaSter β device produces a flow of argon gas ionized with a high-frequency electromagnetic field. The device uses two regimens, the argon plasma regimen and the placebo regimen, which includes the flow of non-ionized argon gas. The argon plasma torch has a diameter of 3.5 cm and a length of about 5 cm. During all experiments, the distance of the treated surfaces from the plasma source was equal to 2 ± 0.2 cm. At this distance, the torch temperature was 36 ± 2 °C.

**C. trachomatis treatment and assessment.** To evaluate the bactericidal effect of the plasma on extracellular C. trachomatis forms, the EB suspension was titrated to the concentration required for an m.o.i. of 2 in DMEM medium. The final suspension was placed into a Petri dish to form a layer of approximately 2 mm and then treated with NTP or with non-ionized argon gas (Fig. 1). The suspension was then carefully removed from the dish to be used for McCoy cell infection. Infection was allowed to develop for 48 h. Intracellular C. trachomatis cells were then visualized as described above. Infection with untreated EBs was used as a control.

To evaluate the effect of NTP on C. trachomatis invasion, EB suspensions were added to McCoy cells grown in 4 cm Petri dishes and then centrifuged for 60 min at 4 °C. This allowed EB adhesion but restricted invasion (Carabeo et al., 2002). McCoy cells with adhered bacteria were treated with NTP as described above, fresh medium was added and the infected cells were incubated at 37 °C in a 5% CO₂ atmosphere for 48 h. Intracellular C. trachomatis cells were visualized as described earlier. Non-treated samples were used as a control.

To evaluate the bactericidal effect of NTP on intracellular bacteria, infected cells were treated at 24 h post-infection with NTP as described below. Cells were then incubated for a further 24 h and sonicated. Serial dilutions of lysate were used for infection of intact McCoy cells. Infection was determined at 48 h post-infection for all dilutions.

**NTP treatment of infected cells.** Cells were grown in 4 cm Petri dishes in DMEM medium for 24 h and infected with C. trachomatis as described earlier. Just before NTP treatment, cultivation medium was removed and a 1 mm layer of medium was left to preserve cells from desiccation during the treatment. Cells were treated with plasma for 2 min and fresh DMEM medium was added immediately after treatment. Cell viability was determined by cell staining with 0.5% methylene blue dye 24 h post treatment. Cells were then lysed with 0.5% SDS and OD₅₄₀ was measured.

**Sample treatment with UV rays.** To exclude plasma particles, magnesium fluoride glass with a diameter of 4 cm was placed between the plasma torch and the sample. The factual transmission spectrum
The paired errors were calculated with Excel software (Microsoft Office 2007). and repeated at least three times. The mean values and standard assessment of statistical significance.

Non-thermal argon plasma is bactericidal for

RESULTS

All experiments were performed using duplicate samples and repeated at least three times. The mean values and standard errors were calculated with Excel software (Microsoft Office 2007). The paired t-test included in the same software was used for assessment of statistical significance.

Non-thermal argon plasma is bactericidal for C. trachomatis EBs

The bactericidal effect of the NTP was studied during the major stages of the C. trachomatis life cycle, including infectious extracellular EBs in DMEM medium, EBs attached to the cell surface during the process of internalization into cells, and actively multiplying intracellular bacteria (RBs) that form 24 h post-infection. These stages were designated I, II and III, respectively (Fig. 1).

To study the effect of argon plasma on extracellular EBs (stage I in Fig. 1), a suspension of EBs was treated with NTP for 2 min. We have found previously that this plasma dose is effective in killing 99.9% of 10^5 c.f.u. of Gram-negative bacteria belonging to different species (Ermolaeva et al., 2011). Control samples were treated with non-ionized argon for 2 min or left untreated. Treated and non-treated EBs were used to infect McCoy cells. Intracellular inclusion bodies were visualized using FITC-labelled mAbs 48 h post-infection. Approximately 90% of cells were infected with control EBs (Fig. 2). Plasma-treated EBs caused minimal infection, with less than 0.01% of cells being infected (Fig. 2, P<0.001). Non-ionized argon treatment of EBs did not result in significant changes to the level of infection when compared to the untreated control (Fig. 2).

In order to study the effect of Chlamydia invasion into eukaryotic cells, EBs were allowed to adhere to the surface of McCoy cells at 4 °C (stage II in Fig. 2), at which they are able to adhere to but not invade host cells (Carabeo et al., 2002). The cells with attached bacteria were treated with NTP. Formation of intracellular inclusions was visualized 48 h post-infection as described above. Approximately 90–95% of cells were infected in the control (Fig. 2). Plasma treatment of attached EBs almost completely prevented the development of intracellular infection. Bacteria treated at the stage of adhesion did not form the expected inclusions. The majority of observations were individual bacterial cells or minor formations. Only individual cells contained regular inclusions (Fig. 2).

To confirm the effect of plasma treatment on Chlamydia invasion into eukaryotic cells, samples were lysed at 48 h

The plasma emission spectrum measurements. The plasma emission spectrum was measured with an Ocean Optics USB4000 spectrophotometer with a TCD1304AP detector, which operates in a range of wavelengths from 200 to 1100 nm and has an optical resolution of 0.3 nm. The integration time was 10 s.

Statistics. All experiments were performed using duplicate samples and repeated at least three times. The mean values and standard errors were calculated with Excel software (Microsoft Office 2007). The paired t-test included in the same software was used for assessment of statistical significance.

Non-thermal argon plasma affects intracellular Chlamydia

Fig. 2. The effects of NTP on extracellular C. trachomatis EBs. EBs in suspension (stage I) or attached to the cell surface (stage II) were treated with NTP (black bars) or with non-ionized argon (grey bars). The control samples (white bars) were left untreated. The median ± SEM of inclusion-forming units (IFU) per well are shown. The data were derived from at least three independent experiments made in duplicate. ** P<0.001.
post-infection and the lysates were used for the secondary infection of the intact McCoy cells. The plasma treatment reduced the concentration of newly formed infectious EBs by a factor of 5.6 × 10^4. Therefore, extracellular Chlamydia forms are highly sensitive to plasma treatment, as has been demonstrated for other extracellular bacteria (Kayes et al., 2007; Lee et al., 2006; Venezia et al., 2008; Ermolaeva et al., 2011).

Plasma is bactericidal for intracellular C. trachomatis

The total reproductive cycle of C. trachomatis takes 48 h to complete in McCoy cells. Halfway through the life cycle (24 h post-infection), the intracellular C. trachomatis population is predominantly composed of actively dividing bacteria (RBs) located within membrane restricted inclusions (Fig. 1, stage III).

Infected McCoy cells were treated with NTP or non-ionized argon for 2 min 24 h post-infection and allowed to complete the 48 h reproductive cycle. To quantify the concentration of viable infectious bacteria, cell lysates were subjected to decimal dilutions for infection of intact McCoy cells. Secondary infections were significantly lower when cells were infected with lysates obtained from plasma-treated samples. Plasma treatment had reduced the concentration of EBs by a factor of 1.9 × 10^6, suggesting that normal development of RBs was prevented by plasma treatment (Fig. 3). Non-ionized argon did not affect the concentration of infectious EBs (Fig. 3).

Bactericidal plasma effects on intracellular bacteria do not depend on UV

The spectrum of argon plasma includes UV-A, -B and -C rays (Nosenko et al., 2009). To evaluate the role of UV rays in the overall plasma bactericidal action, magnesium fluoride glass that detained plasma particles but was essentially transparent (about 60%) to UV rays was utilized (data not shown). The UV effect was studied in EBs and intracellular RBs 24 h post-infection. The concentration of infectious EBs was evaluated by counting cells infected with treated or control EBs as described earlier. The amount of infected cells dropped by factors of 6 × 10^4 and 4.2 × 10^3 after NTP and UV treatment, respectively (Fig. 4; P<0.05). Therefore, placing magnesium fluoride glass between the plasma torch and the sample diminished the bactericidal effect on EBs up to 14-fold.

Dividing RBs were treated with plasma or UV rays alone at 24 h post-infection and the bacteria were allowed to form intracellular EBs for a further 24 h, after which the concentration of intracellularly formed infectious bacteria was evaluated using the infection assay on intact McCoy cells as described earlier. The number of observed inclusions was reduced 3 × 10^6-fold after plasma treatment while the concentration of extracellular EBs in comparison with the control untreated samples (P<0.001) (Fig. 4). Therefore, whilst NTP had the greatest bactericidal effect on both EBs and RBs, UV still had a significant bactericidal effect on EBs but not RBs.

Interestingly, plasma treatment of infected cells resulted in an inclusion morphology of discontinuous aggregates of bacteria without distinct borders 48 h post-infection (Fig. 5c). When further passaged, these inclusions were unable to produce infectious EBs (Fig. 4). In contrast, UV-treated cells carried inclusions with a normal morphology, similar to control non-treated cells (Fig. 5a and b).

Argon plasma has low toxicity towards eukaryotic cells

The noticeable effects of plasma treatments on intracellular bacteria raised questions concerning the susceptibility of
host eukaryotic cells to the treatment. Plasma treatment for 2 min did not cause immediate mortality to cells (data not shown). After 24 h, a 19 ± 1.5 % drop in the number of viable fibroblast McCoy cells was observed compared with control cells (P<0.05). Non-ionized argon gas, therefore, did not change cell viability.

DISCUSSION

To our knowledge, this is the first study to investigate the bactericidal effect of NTP on intracellular bacteria. We demonstrated that the Gram-negative obligate intracellular pathogen C. trachomatis is highly susceptible to treatment with NTP. Both extracellular (EBs) and intracellular (RBs) forms of C. trachomatis were sensitive to treatment with argon plasma for 2 min. Plasma-treated bacteria failed to start a new intracellular cycle or finish a current cycle.

The metabolic inertness of EBs helps preserve them from antibiotics and treatments that exert a bactericidal effect via interruption of bimolecular synthesis (Hammerschlag, 2002). Treating extracellular EBs with argon plasma for 2 min resulted in a ~4-log10 reduction in infection. These results are comparable to those obtained for other Gram-negative bacteria (Ermolaeva et al., 2011).

NTP treatment includes charged and metastable particles, free radicals and UV rays. EB inactivation could have been due to damage of lipid and other surface structures caused by active particles of the plasma torch or by the action of UV rays. A direct interaction of NTP with biological polymers has been shown to cause mechanical damage to polymers including those on the cell surface. The damage, termed ‘etching’, is caused by active plasma particles (Dobrynin et al., 2009; Fricke et al., 2011). In addition, UV may also cause damage to both DNA and surface structures. Therefore, it was important to determine whether the bactericidal effect of NTP on C. trachomatis EBs was due to UV rays alone, active particles alone, or a combination of the two emitted by the torch. Magnesium fluoride glass was used to prevent the action of active particles as the transmission spectrum of the magnesium fluoride glass is uniform and in the range of 130–7500 nm. The measured spectrum demonstrated that more than half of the UV rays passed through the glass. The mortality of EBs due to UV treatment alone was <10 %, relative to whole plasma. Therefore, the role of UV in terms of the plasma bactericidal effect on EBs was significant but not predominant. Additional bactericidal activity against extracellular EBs could be due to surface lesions caused by bombardment with plasma particles. Recent evidence suggests that cell-surface structures are the primary targets of NTP (Laroussi et al., 2003; Venezia et al., 2008). C. trachomatis EBs were shown to be highly sensitive to surface-damaging substances (Yasin et al., 1996). Therefore, it is possible that surface damage by plasma particles, with the help of UV rays, is responsible for the bactericidal effect of NTP on EBs.

The mechanisms by which NTP affects intracellular RBs are less evident. The obtained results suggest that the role of UV, in terms of the overall effect on intracellular bacteria, is very minimal (<0.1 %), suggesting that plasma particles are primarily responsible for the bactericidal effect of NTP on intracellular C. trachomatis. Direct interaction of NTP particles with intracellular RBs, as well as indirect effects mediated by host cell components, could be responsible for
the elimination of intracellular bacteria. Recent evidence suggests that there may be a direct effect of NTP on intracellular bacteria via plasma particles entering the host cell through the membrane electroporation that occurs during NTP treatment (Kolb et al., 2006; Laroussi et al., 2003; Leduc et al., 2009). Alternatively, indirect effects mediated by host-cell components may also be responsible for the interruption of the intracellular Chlamydia life cycle. Intracellular replication of Chlamydia within inclusions is strictly dependent on host resources. Maintaining an intact inclusion and a whole host cell is vitally important for bacterial viability (Hackstadt et al., 1997; Hammerschlag, 2002). Treatment of infected cells with NTP resulted in noticeable changes in inclusions; isolated bacterial cells were observed intracellularly rather than inside regular inclusion bodies (Fig. 5c). Deregulation of inclusion maintenance could be the cause of mortality among intracellular bacteria. Such deregulation could be a component of the response to plasma action by the host cell.

Taken together, our results demonstrate that NTP treatment causes a significant reduction in the infectious capability of both extracellular and intracellular forms of C. trachomatis, in vitro. The most important issues that should be addressed in the future are whether these effects could be applied to bacterial elimination in vivo and whether NTP is applicable as a therapy for intracellular infections. Intracellular pathogenic bacteria are often associated with infections of the respiratory system and the urogenital tract. Intracellular bacteria including Mycobacterium tuberculosis, Legionella pneumophila, C. pneumoniae, C. psittaci and Coxiella burnetii are common causes of acute and chronic lung infections. C. trachomatis is often associated with chronic genital infections and infertility. Recently developed endoscopic plasma sources could be used to treat infections of pathogens in these niches (Robert et al., 2009). The effectiveness of NTP as an antibacterial therapeutic tool was demonstrated by the decontamination of wound surfaces both in experimental conditions and in the clinic (Ermolaeva et al., 2011; Isbary et al., 2010). Intracellular bacteria are often part of wound communities in naturally contaminated wounds. Whether NTP treatment can affect intracellular pathogens during the wound healing process requires further investigation.

Overall, the results of this study demonstrate the potential of non-thermal argon plasma for use in the elimination of extracellular and intracellular C. trachomatis. Further research is ongoing to establish the mechanisms responsible for the effects of plasma on intracellular bacteria and, particularly, to characterize signalling events initiated in the host cell and their impact on the host fate and host-pathogen interactions.

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