A domino-like chlamydial attachment process: concurrent *Parachlamydia acanthamoebae* attachment to amoebae is required for several amoebal released molecules and serine protease activity

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*Parachlamydia acanthamoebae* is an obligate intracellular bacterium that infects free-living amoebae (*Acanthamoeba*), and is a potential human pathogen associated with hospital-acquired pneumonia. The attachment mechanism of this bacteria to host cells is crucial in bacterial pathogenesis, yet remains undetermined. Hence, we obtained monoclonal antibodies (mAbs) specific to either *P. acanthamoebae* or amoebae in an attempt to elucidate the attachment mechanism involved. Hybridomas of 954 clones were assessed, and we found that four mAbs (mAb38, mAb300, mAb311, mAb562) that were reactive to the amoebae significantly inhibited bacterial attachment. All mAbs recognized amoebal released molecules, and mAb311 also recognized the amoebal surface. mAbs reacted with the bacteria not only within amoebae, but also when they were released from amoebae (except mAb311). Furthermore, a serine protease inhibitor had an inhibitory effect on the bacterial attachment to amoebae, although none of the mAbs had any synergistic effect on the inhibition of attachment by the protease inhibitor. Taken together, we conclude that concurrent *P. acanthamoebae* attachment to amoebae is required for several amoebal released molecules and serine protease activity, implying the existence of a complicated host–parasite relationship.

INTRODUCTION

Chlamydiae, which are obligate intracellular bacterial pathogens, have been reclassified into the order Chlamydiales, which includes four families, *Chlamydiaceae*, *Parachlamydiaceae*, *Waddliaceae* and *Simkaniaceae* (Horn, 2008). The family *Chlamydiaceae*, which is broadly distributed among mammals, including humans, includes two major human pathogens designated the pathogenic chlamydiae. *Chlamydophila pneumoniae* is a causal agent of common respiratory infection (Bartlett, 2008) and is also suspected of being involved in certain chronic diseases, such as asthma (Sutherland & Martin, 2007) and atherosclerosis (Watson & Alp, 2008), while *Chlamydia trachomatis* is responsible for sexually transmitted disease and preventable blindness (Jordan et al., 2011). Furthermore, *Parachlamydiaceae*, *Waddliaceae* and *Simkaniaceae* have only recently been recognized as environmental chlamydiae, and exhibit a wide distribution in natural environments, such as in rivers and soil (Greub, 2009; Horn et al., 2004). These species can grow and survive within the free-living amoeba *Acanthamoeba*, which is the most abundant genus of amoebae (Khan, 2006).
Parachlamydia acanthamoebae and Simkania negevensis are potentially associated with human lower respiratory tract infections (Greub, 2009; Horn, 2008; Nascimento-Carvalho et al., 2009), and Waddlia chondrophila, which was originally isolated from an aborted bovine fetus, is considered a potential abortogenic agent (Baud et al., 2011; Greub, 2009; Horn, 2008). There is also accumulating evidence that supports the pathogenic role of P. acanthamoebae in humans. Several studies have reported that parachlamydial DNA was detected by PCR in mononuclear cells of sputum and bronchoalveolar lavage samples from a patient with bronchitis (Casson & Greub, 2006; Corsaro et al., 2002). Other studies have suggested that P. acanthamoebae may cause hospital-acquired pneumonia (Greub, 2009; Greub et al., 2003), and it has been identified as a risk factor in HIV-infected patients and organ transplant recipients receiving immunosuppressive therapy (Casson & Greub, 2006; Corsaro et al., 2002). In addition, we recently demonstrated that P. acanthamoebae could inhabit and spread between amoebae within a hospital environment (Fukumoto et al., 2010). However, the biological features of these bacteria that relate to host cell attachment, which is a critical step for successful infection, still remain unknown.

We therefore aimed to obtain cultured P. acanthamoebae and amoeba-specific mouse monoclonal antibodies (mAbs) in an attempt to analyse bacterial attachment to host amoebae. We here show that concurrent bacterial attachment to amoebae is required for several amoebal released molecules and serine protease activity.

**METHODS**

**Amoebae.** Free-living amoebae (Acanthamoeba castellanii C3) were used for our study. A. castellanii C3 (ATCC 50739) was purchased from the American Type Culture Collection (ATCC). Amoebae were maintained at 30 °C in PYG broth that included 0.75 % (w/v) peptone, 0.75 % (w/v) yeast extract and 1.5 % (w/v) glucose (Matsuo et al., 2008).

**Bacteria.** P. acanthamoebae Bn9 (VR-1476) was also purchased from the ATCC, and was propagated in the amoeba culture system according to methods described previously (Matsuo et al., 2008). Bacteria were further purified by density-gradient ultracentrifugation with Percoll (Sigma) (Weiss et al., 1989), and then the preparation was used to immunize mice. Numbers of infectious progenies in the infected amoebae were also determined by the method described previously (Matsuo et al., 2008) (see below).

**Obtaining mAbs and polyclonal antibodies.** Three-week-old female BALB/c mice were purchased from SLC, Japan. The mice (n=5) were housed under pathogen-free conditions, in accordance with the NIH Guide for Care and Use of Laboratory Animals, and the Animal Care and Use Committee of Hokkaido University also approved this experiment. Purified bacteria were injected intraperitoneally three times at 10 day intervals with the antigen mixed with Freund’s complete adjuvant (BD Diagnostics). Ten days after the last injection, mice were intravenously injected with the bacteria. Three days later, the spleen of mice (n=2) producing P. acanthamoebae antibodies with a high titre was removed for cell fusion of the spleen and mouse myeloma cells (P3-X63-Ag8-U1), which were kindly provided by Dr S. Kobayashi, Hokkaido University, Japan. We finally established hybridoma clones specific to either P. acanthamoebae or amoeba on solid medium using a ClonaCell-HY kit (StemCell) according to the manufacturer’s protocol. The isotype and subclass of mAbs were determined using an IsoStrip Mouse Monoclonal Antibody Isotyping kit (Roche Applied Science) according to the manufacturer’s instructions. Culture supernatants of the hybridomas were used for the experiments as described below. mAb titres were confirmed via assessment of the reactivity of diluted mAbs with P. acanthamoebae-infected amoebae fixed with cold 70 % (v/v) ethanol on a 96-well microtitre plate. Also, all mice sera were collected from all immunized mice, pooled and used as polyclonal antibodies to assess bacterial attachment of inhibition to amoebae (see below).

**Reactivity of mAbs against bacteria and amoebae.** Amoebae [5 × 10^5] cells per well of a 24-well plate with (for immunofluorescence to determine localization of molecules recognized by mAbs) or without glass coverslips (12 mm diameter, Sigma) (for preparation of antigens on dot blots, Western blots and zymographs); 5 × 10^4 cells per well of a 96-well plate (for immunofluorescence screening of mAbs) were infected with or without P. acanthamoebae at an m.o.i. of 10 by centrifugation at 700 g for 60 min. After centrifugation, cultures were placed into fresh PYG medium and incubated for up to 3 days at 30 °C within a normal atmosphere. Amoebae were gently rinsed with Page’s modified Neff’s amoeba saline (PAS) (Fukumoto et al., 2010) and then fixed with cold 70 % (v/v) ethanol. Fixed amoebae were then used for immunofluorescence microscopy (see below). Amoebae in cultures without coverslips were gently scraped from the plate, collected by centrifugation and then used as antigens for dot blots, Western blots and zymographs.

**Dot blotting.** Freeze–thawed antigens were deposited on a nitrocellulose membrane at different concentrations and then thoroughly air-dried. Membranes were further blocked with 3 % skimmed milk dissolved in PBS with 0.05 % (v/v) Tween 20 (PBS-T) for 30 min at room temperature. Following blocking, mAbs (10-fold diluted in culture supernatant) were incubated with the membrane for 60 min at room temperature, washed with PBS-T, and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG+IgM (1: 500 dilution) (Wako Pure Chemical) for 60 min. The membrane was washed, and then developed with 3,3’-diaminobenzidine tetrahydrochloride (DAB) (Dojin).

**Western blotting.** Freeze–thawed antigens were boiled for 5 min in a reducing sample buffer that included 2-mercaptoethanol, before approximately 10 µg of the protein was loaded and separated on a 10 % (w/v) SDS-polyacrylamide gel (40 mA, 90 min). Separated proteins were subsequently transferred to a PVDF membrane by semi-dry electroblotting. Membranes were blocked with 3 % (w/v) skimmed milk in PBS and then incubated with mAbs (10-fold diluted in culture supernatant), both for 1 h at room temperature. HRP-conjugated goat anti-mouse IgG+IgM (1: 500 dilution) was used as a secondary antibody for 1 h at room temperature, before the reaction was visualized with an HRP/ECL kit (Invitrogen).

**Immunofluorescence.** Amoebae grown either directly on a 96-well plate or on a coverslip of a 24-well plate were washed in cold PBS containing 5 % (w/v) BSA, and then fixed in cold 70 % (v/v) ethanol. Fixed cells were incubated with mAbs (10-fold diluted in culture supernatant) for 1 h at room temperature, and then reacted with a secondary antibody, FITC-labelled anti-mouse IgG+IgM antibody (Jackson ImmunoResearch), in the presence or absence of DAPI. Staining traits were then determined using either a conventional fluorescence microscope (Nikon) (for screening) or an FSX100 Bio imaging Navigator fluorescence microscope equipped with
deconvolution (Olympus) (for determining localization of molecules recognized by mAbs).

**Assessment of inhibition by mAbs of the attachment of bacteria to amoebae.** For the inhibition assay, amoebae [5 × 10^4 cells per well (96-well plate)] were incubated for 60 min at 30°C, allowing attachment to the bottom, and then cultured with the bacteria at an m.o.i. of 10 or 20 in the presence or absence of each of the mAbs (50 μl of fivefold-diluted culture supernatant) in a 150 μl mixture adjusted with PAS for 1 h at 4°C. In some experiments, either serine protease (PMSF, Sigma) or metalloprotease inhibitor (1,10-phenanthroline) (Sigma) was added to the cultures at a final concentration of 1 mM; these working concentrations did not have any harmful effects on amoebal viability. After washing with PAS, the amoeba were fixed with cold 70% (v/v) ethanol and then stained with *P. acanthamoeba*-specific polyclonal antibodies followed by FITC-labelled anti-mouse IgG (Sigma). After washing, numbers of amoebae with a FITC signal indicating bacterial attachment were estimated under a conventional fluorescence microscope.

**Procedure for quantification of infective progeny.** The number of infective progenies of *P. acanthamoeba* was determined by the amoeba-infectious units (AIU) assay, using co-culture with amoebae, as previously described (Matsuo *et al.*, 2008). Finally, the number of bacterial infectious progenies in a culture was defined as the AIU value.

**Assessment of proteolytic activity.** Freeze–thawed amoebal lysates (equivalent to 2 × 10^6 amoebae) were incubated for 60 min at 4°C with or without either each of the mAbs (5 μl of a fivefold dilution of the culture supernatant) or protease inhibitors (final concentration 1 mM) in a 50 μl mixture adjusted with PAS. The lysate (20 μl of each mixture) was loaded onto 10% (w/v) acrylamide gels containing 0.1% (w/v) gelatin as a substrate (PAGE-gelatin). After electrophoresis (40 mA, 90 min), the enzymes were renatured by rinsing the gels in Tris-buffered saline (TBS; 20 mM Tris/HCl, pH 7.5, 150 mM NaCl) containing 2.5% (v/v) Triton X-100 for 1 h to remove SDS, and then incubated at 37°C overnight in TBS. Gels were stained with 0.25% (w/v) Coomassie brilliant blue in 50% (v/v) ethanol and 15% (v/v) acetic acid. After treatment with decolouring solution containing 5% (v/v) methanol and 7% (v/v) acetic acid, gelatin proteolysis was detected as broad colourless bands on an otherwise blue gel.

**Statistical analysis.** Statistical software (StatView, version J4.5, Abacus Concept) was used for all statistical analysis. Comparisons between the AIU values or the inhibition rates were assessed by an unpaired t test. A P value < 0.05 was considered significant.

**RESULTS**

**Obtaining mAbs and assessment of their effects on *P. acanthamoeba* attachment to amoebae**

Hybridomas of 954 clones were successfully selected from the solid medium and used to assess reactivity to either *P. acanthamoeba* or amoebae. Dot blotting, Western blotting...
and immunofluorescence were further used to select 13 hybridoma clones that produced specific mAbs against molecules from either *P. acanthamoebae* (five clones) or amoebae (eight clones) (Table S1). Western blotting analysis revealed mAbs (except mAb79) within the amoebal lysate as smear-like bands regardless of the bacterial infection, suggesting that the target molecule was lipid or carbohydrate. Because of the lack of a Western blot reaction, mAb311 appeared to recognize a conformational epitope of an amoebal molecule. Since the reactivity of mAb79 was limited to denatured antigens on the Western blot, this mAb was omitted from our subsequent analysis.

**Inhibition of the attachment of *P. acanthamoebae* to amoebae by obtained mAbs**

We firstly assessed whether the obtained mAbs could inhibit the attachment of *P. acanthamoebae* to amoebae using an inhibition assay, and found that four mAbs (mAb38, mAb300, mAb311, mAb562) dramatically inhibited bacterial attachment to amoebae (Fig. 1a, b). In particular, a maximal inhibition of 20% (mAb311) was obtained compared with the medium alone (control). Furthermore, using an AIU assay we determined whether this attachment inhibition could influence the increase in the number of infectious progenies in amoebae. Addition of mAbs that had an inhibitory effect to the infected amoebal cultures inhibited bacterial growth to a statistically significant extent (Fig. 1c). Inhibitory mAbs clearly reacted with the amoebae, but not the bacteria, suggesting that following successful infection, *P. acanthamoebae* could potentially attach to several amoebal molecules involved in attachment or development.

**mAbs with inhibitory effects recognize either the amoebal surface or released molecules**

Staining patterns among the mAbs used were unique, and all mAbs exhibited different levels of reactivity to the released molecules. In particular, mAb300 reactivity to released molecules was characterized by a very strong staining pattern (Fig. 2, mAb300). In contrast, while the target molecule localized to both the surface membrane and cytoplasm, release of the molecule recognized by mAb311 was minimal (Fig. 2, mAb311). Since mAb311 recognized a molecule on the amoebal surface (Fig. 3, indicated by the dashed square for mAb311), the molecule recognized by mAb311 may be a candidate amoebal receptor for *P. acanthamoebae* attachment following successful infection.

In addition, expression of all the molecules on the infected amoebal cultures drastically decreased depending on cultivation time and bacterial maturation (Fig. 3, images at 72 h p.i.), suggesting that expression had been modified due to the infection. Although mAb562 also reacted with a released molecule, the degree of release was much weaker than that for mAb38 and mAb300 (Fig. 3, mAb562 at 18–48 h p.i.). Furthermore, a difference in co-localization with the bacteria was also observed; while the molecules recognized by mA38, mAb300 and mAb562 co-localized with the bacteria present either inside or outside the amoebae, co-localization of the molecule recognized by mAb311 with the bacteria inside amoebae was limited (Fig. 3b). Taken together, these results indicated that concurrent bacterial attachment to amoebae was required for several amoebal released molecules and an amoebal surface molecule, suggesting that *P. acanthamoebae* may co-opt the amoebal secretion system to enhance infection.

![Fig. 2. Representative fluorescence images of mAbs reacting with uninfected amoebae.](image-url) Amoebae grown on a coverslip for 24–48 h were used for this experiment. Fixed cells were stained with each of the mAbs and then incubated with secondary FITC-labelled antibody (green) in the presence of DAPI (blue). FITC, single staining; merged with DAPI, double staining with mAbs and DAPI. The dashed square indicates an enlarged area in the right-hand panel (arrow). Magnification, ×100.
**P. acanthamoebae** attachment to amoebae required for serine protease activity is independent of the molecules recognized by mAbs

Since it is well known that amoebae secrete a large amount of serine protease likely to be involved in amoebal pathogenesis (Anger & Lally, 2008; Dudley et al., 2008; Moon et al., 2008), the molecules recognized by the inhibitory mAbs may be proteases. To confirm this, we assessed the effect of protease inhibitors on bacterial attachment to amoebae. Contrary to our expectation, the inhibition of protease with either PMSF or 1,10-phenanthroline did not produce any synergistic effect of the mAbs on bacterial attachment (Fig. 4a), although PMSF itself significantly inhibited bacterial attachment in the absence of the mAbs. We also found no synergistic effect among mAbs on bacterial attachment (Fig. 4b). Thus, our results indicated that **P. acanthamoebae** attachment to amoebae was concurrently, yet independently, required for serine protease activity and the release of the molecules recognized by the mAbs. This suggested that the attachment process was complex and exhibited a ‘domino-like’ effect. Zymograph analysis also revealed that the obtained mAbs did not have any inhibitory effect on amoebal protease activity, suggesting that the epitopes recognized by the mAbs may not be associated either with the active sites or with other parts of the amoebal proteases (Fig. 4c).

**DISCUSSION**

Among the mAbs that we obtained, four specific to amoebae were found to significantly inhibit bacterial attachment. These results indicate that multiple molecules derived from the host amoebae are likely to be required for bacterial attachment, as indicated by the different staining patterns among the amoebal cultures. This inhibition also significantly influenced bacterial growth within the amoebae, suggesting that bacterial attachment to amoebae is likely to be directly associated with successful bacterial replication. In addition, because our experiment was conducted at 4 °C, the influence of phagocytosis was minimal (Hodinka & Wyrick, 1986). Thus, our findings indicate that the attachment of **P. acanthamoebae** to amoebae is dependent on specific ligand–receptor interactions.

Expression levels of the target molecules recognized by mAb38 and mAb300 dramatically decreased during the infection, suggesting that the presence of **P. acanthamoebae** in amoebae can modify the expression of these target molecules. Pathogenic chlamydiae have been reported to degrade several cellular matrixes, actin or intermediate...
Fig. 4. Synergistic effect of the mAbs and protease inhibitors on the inhibition of *P. acanthamoebae* attachment to amoebae. (a) Synergistic effects between each of the mAbs and protease inhibitors. Amoebae were cultured with the bacteria at an m.o.i. of 10 in the presence or absence of each of the mAbs (fivefold-diluted culture supernatant) with or without either PMSF or 1,10-phenanthroline (final concentration 1 mM) for 1 h at 4 °C. Data are expressed as the mean (percentage) and SD. Asterisks indicate *P*<0.05 versus the value of the medium control. (b) Synergistic effects among the mAbs in the presence or absence of protein inhibitors. Amoebae were incubated with bacteria at an m.o.i. of 20 (see above). Data are expressed as the mean (percentage) and SD. NS, Not statistically significant. MAb38a, mixture of mAb300 with mAb38; mAb311b, mixture of mAb562 with mAb311; mAb38c, mixture of 300mAb, 311mAb and mAb562mAb with mAb38. MedE, commercial medium for hybridoma growth supplied by StemCell. Asterisks indicate *P*<0.05 versus the mean value among four mAbs without any protease inhibitor. All experiments were performed at least three times independently. (c) Representative zymograph image.
bacteria within the amoebae, suggesting that these molecules may be also required for the normal developmental cycle of bacteria, including maturation and secondary infection. More interestingly, as well as the molecules recognized by the mAbs, bacterial attachment to the amoebae was independently required for serine protease activity because the mAbs did not produce a synergistic effect. Since amoebae (Acanthamoeba) mainly secrete serine protease, which is important in amoebal pathogenesis (Anger & Lally, 2008; Dudley et al., 2008; Moon et al., 2008), the bacteria may be easily targeted by this protease during infection. Taken together, we propose an attachment model of P. acanthamoebae to amoebae following successful infection that uses both amoebal molecules and serine protease activity in a ‘domino-like’ manner (Fig. 5).

Attachment mechanisms of the pathogenic chlamydiae related to P. acanthamoebae have been extensively studied using epithelial cells, such as HeLa, HEp-2 and McCoy cells (Abromaitis & Stephens, 2009; Chen & Stephens, 1997; Kim et al., 2011; Rasmussen-Lathrop et al., 2000; Taraktchoglou et al., 2001; Wuppermann et al., 2001; Zhang & Stephens, 1992). Data from these experiments indicate that heparin, which is the most common glycosaminoglycan, attaches not only to the bacteria during attachment to host cells but also to those released from the host cells that possess a secondary infectious ability (Chen & Stephens, 1997; Rasmussen-Lathrop et al., 2000; Taraktchoglou et al., 2001; Wuppermann et al., 2001; Zhang & Stephens, 1992). A recent study has also revealed that the attachment and entry of C. trachomatis require the host protein disulfide isomerase (Abromaitis & Stephens, 2009). Furthermore, the novel finding has been reported that fibroblast growth factor 2 is necessary to enhance the binding of C. trachomatis to host cells in a heparin/glycan-dependent manner (Kim et al., 2011). These findings suggest that the attachment of pathogenic chlamydiae via the use of several molecules derived from host cells is a complex process. P. acanthamoebae diverged from the ancestral chlamydiae more than 700 million years ago, and evolved to live in unicellular amoebae, thereby forgoing the process of genome reduction to overcome changes in harsh natural environments (Horn, 2008; Horn et al., 2004). However, the multi-molecular mechanism associated with P. acanthamoebae attachment to amoebae is likely to share some similarities with that found in pathogenic chlamydiae, and may therefore allow us to better understand the complicated pathogenic chlamydial attachment process.

In conclusion, using mAbs we have successfully demonstrated that concurrent P. acanthamoebae attachment to amoebae is required for the expression of several amoebal released molecules and serine protease activity in a ‘domino-like’ manner, implying that the underlying P. acanthamoebae–host amoeba interaction is complex. Furthermore, although the molecules recognized by the mAbs are still unknown, this information could provide us with new insights into the complicated attachment and entry mechanisms that underlie the interactions between intracellular
parasites and their host cells, especially those involved in chlamydial pathogenesis.

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