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

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SUMMARY

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 a crucial step in bacterial pathogenesis, yet remains undetermined. Hence, we established monoclonal antibodies (mAbs) specific to either *P. acanthamoebae* or amoebae in an attempt to elucidate the involved attachment mechanism. Hybridomas of 954 clones were assessed, and we found 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 in amoebae, but also those released from amoebae (except mAb311). Furthermore, serine-protease inhibitor had an inhibitory effect on the bacterial attachment to amoebae, although none of the mAbs had any synergetic effect on the attachment inhibition by the protease inhibitor. Taken together, we concluded that *P. acanthoamebae* attachment to amoebae is concurrently 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 as 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 *et al*., 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 range in natural environments, such as rivers and in soil (Greub, 2009; Horn *et al*., 2004). These species can grow and survive dependently within the free-living amoeba *Acanthamoeba*, which is the most abundant genus of amoebae (Khan, 2006).

Parachlamydia acanthamoebae and *Simkania negevensis* have been 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 supporting

the pathogenic role of *P. acanthamoebae* in humans. Several studies have reported that parachlamydial DNA was detected by polymerase chain reaction 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 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 relating to host cell attachment, which is a critical step for successful infection, still remain unknown.

We therefore aimed to establish cultured *P. acanthamoebae* and amoeba-specific mouse monoclonal antibodies (mAbs) in an attempt to analyse bacterial attachment to host amoebae. We here show that the bacterial attachment to amoebae was concurrently 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^{TM}) was purchased from the American Type Culture Collection (ATCC) (Manassas, VA). 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* Bn_9 (VR-1476) was also purchased from the ATCC, and propagated in the amoeba culture system according to methods described previously (Matsuo *et al*., 2008). Bacteria were further purified by density-gradient-ultra centrifugation with percoll (Sigma, St. Louis, MO) (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).

Establishment of mAbs and polyclonal antibodies. Three-week-old female BALB/c mice were purchased from SLC (Hamamatsu, Shizuoka, 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 immunized intraperitoneally three times at 10 day intervals with the antigen mixed with Freund's complete adjuvant (BD Diagnostics, Franklin Lakes, NJ). Ten days after the last injection, mice were intravenously injected with the bacteria. Three days later, the spleen of mice (*n*=2) producing *P. acanthamoeba* antibodies with a high titer was respectively 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 amoebae on solid medium using a ClonaCell-HY kit (StemCell,

Vancouver, Canada) according to the manufacturer's protocol. Isotype and subclass of mAbs were determined using an IsoStrip Mouse Monoclonal Antibody Isotyping Kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. Culture supernatants of the hybridomas were used for the experiments as described below. MAb titers 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 microtiter plate. Also, all mice sera were collected from immunized all 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 well^{-1} of a 24-well plate with (for immunofluorescence to determine localization of molecules recognized by mAbs) or without glass-cover slips (12mm in diameter)(Sigma) (for preparation of antigens on dot blot, western blot and zymograph); 5×10^4 cells well^{-1} of a 96-well plate (for immunofluorescence screening of mAbs)] were infected with or without *P. acanthamoebae* at a multiplicity of infection (MOI) of 10 by centrifugation at 700 × g for 60 min. After centrifugation, cultures were placed into fresh PYG medium and incubated for up to three 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 cover slips were gently scraped from the plate, collected by centrifugation and then used as antigens for dot blot, western blot and zymograph.

i) Dot blot. Freeze-thawed antigens were deposited on a nitrocellulose membrane at different concentrations and then thoroughly air-dried. Membranes were further blocked with 3% skim milk dissolved in phosphate-buffered saline with 0.05% (v/v) Tween 20 (PBS-T) for 30 min at room temperature. Following blocking, mAbs (10-fold diluted 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 + M (1:500 dilution) (Wako Pure Chemical, Osaka, Japan) for 60 min. The membrane was washed, and then developed with DAB (Dojin, Kumamoto, Japan).

ii) Western blot. 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) sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (40mA, 90 min). Separated proteins were subsequently transferred to a polyvinylidene difluoride membrane by semi-dry electroblotting. Membranes were blocked with 3% (w/v) skim milk in PBS and then incubated with mAbs (10-fold diluted culture supernatant), both for 1 h at room temperature. HRP-conjugated goat anti-mouse IgG + M (1:500 dilution) was used as a secondary antibody for 1 h at room temperature, before the reaction was visualized with an enhanced HRP-ECL color kit (Invitrogen, Grand Island, NY).

iii) Immunofluorescence. Amoebae grown either directly on a 96-well plate or on a cover slip 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 culture supernatant) for 1 h at room temperature, and then reacted with a secondary antibody, FITC-labeled anti-mouse IgG + IgM antibody (Jackson ImmunoResearch), in the presence or absence of 4',6-diamidino-2-phenylindole (DAPI). Staining traits were then determined using either a conventional fluorescence microscope (Nikon, Tokyo, Japan) (for screening) or a Bio-imaging Navigator fluorescence microscope equipped with deconvolution (FSX100, Olympus, Tokyo Japan) (for determining localization of molecules recognized by mAbs).

Assessment of inhibition by mAbs on the attachment of bacteria to amoebae. For the inhibition assay, amoebae [5×10^4 cells $well^{-1}$ (96-well plate)] were incubated for 60 min at 30°C, allowing attachment to the bottom, and then cultured with the bacteria at MOI 10 or 20 in the presence or absence of each of the mAbs (50 μl of 5-fold diluted culture supernatant) in a mixture of 150 μl adjusted with PAS for 1 h at 4°C. In some experiments, either serine-protease [phenylmethylsulfonyl fluoride (PMSF)] (Sigma) or metaro-protease inhibitor (1,10-phenanthroline) (Sigma) was added to the cultures at a final concentration of 1mM; 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*-polyclonal antibodies following FITC-labeled anti-mouse IgG (Sigma). After washing, numbers of amoebae with a FITC signal indicating bacterial attachment were then estimated under a conventional fluorescent microscope.

Quantification procedure of infective progeny. The number of infective progenies of *P. acanthamoebae* was determined by the amoeba-infectious units (AIU) assay, using

co-culture with amoebae as previously described (Matsuo *et al*., 2008). Finally, numbers of bacterial infectious progenies in cultures was defined as AIU value.

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

Statistical analysis

Statistical software (StatView, version J4.5, Abacus Concept, Berkeley, USA) 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

Establishment of mAbs and assessment of their effect on *P. acanthamoebae*

attachment to amoebae

As a result, hybridomas of 954 clones were successfully selected from the solid medium and used for the assessment of reactivity to either *P. acanthamoebae* or amoebae. Dot blot, western blot and immunofluorescence were further used to select 13 hybridoma clones that produced specific mAbs against molecules for 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 amoebal molecule. Since the reactivity of mAb79 was limited to only denatured antigens on the western blot, the mAb was omitted from our subsequent analysis.

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

We firstly assessed if established 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 and B). In particular, the maximal inhibitory rate was achieved at 20% (mAb311) compared with the medium alone (control). Furthermore, we determined if this attachment inhibition could influence an increase in the number of infectious progenies in amoebae using an AIU assay. Addition of mAbs that had an inhibitory effect within the infected amoebal cultures statistically inhibited bacterial

growth (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 recognized either amoebal surface or released molecules

Staining patterns among the mAbs used were quite 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, see mAb300). In contrast, while the target molecule localized to both the surface membrane and cytoplasm, the released molecule recognized by mAb311 was minimal (Fig. 2, see mAb311). Since mAb311 recognized a molecule on the amoebal surface (Fig. 3, indicated by the dashed square for mA311), the molecule recognized by mAb311 may be an amoebal receptor candidate 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 pi), suggesting this had been modified due to the infection. Although mAb562 also reacted to the released molecule, the degree of release was much weaker than that for mAb38 and mAb300 (Fig. 3, mAb562 at 18-48h pi). Furthermore, a difference in co-localization with the bacteria was also observed; while the molecules recognized by

mA38, mAb300 and mAb562 co-localized to the bacteria present either inside or outside the amoebae, co-localization of the molecule recognized by mAb311 to the bacteria inside amoebae was limited (Fig. 3B). Taken together, these results indicated that the bacterial attachment to amoebae was concurrently 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.

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 always 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 performed an experiment to assess 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 synergetic 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 synergetic 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 released 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 established

mAbs did not have any inhibitory effect on amoebal protease activity, suggesting that the epitopes recognized by the mAbs may be not likely to be associated with active site on the amoebal proteases or the protease itself (Fig. 4C).

DISCUSSION

Among the mAbs that we established, 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 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 these target molecule's expressions. Pathogenic chlamydiae have been reported to degrade several cellular matrixes, actin or

intermediate fiber filaments required for inclusion formation, which is regulated via the activation of chlamydia-associated protease factor (CPAF) (Christian *et al*., 2011; Jorgensen *et al*., 2011; Zhong, 2011). Because recent genome research has demonstrated the presence of a gene homologue in *P. acanthamoebae*, the CPAF-like enzyme is a potential candidate for causing this modification (Horn, 2008). We also found that mAb311 strongly reacted with the amoebal surface, although its expression disappeared as the bacteria matured. Although the exact reason for such down-regulation is unknown, it is possible that the targeted molecule on the amoebal surface might act as a receptor for bacterial attachment, and is no longer expressed after the bacteria have gained entry to the host amoebae.

Molecules recognized by mAb38, mAb300, mAb562 co-localized on bacteria released from the amoebae after bacterial maturation. These findings indicated that after release, the bacteria are covered with the amoebal molecules, which subsequently may be required for secondary infection to occur. In addition, the molecules recognized by all mAbs studied (mAb38, mAb300, mAb311, mAb562) co-localized onto the spots stained by DAPI possibly showing the 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 using 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, or 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 indicated that heparin, which is the most common glycosaminoglycan, attaches to the bacteria not only 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). Recent study also revealed that the attachment and entry of *C. trachomatis* requires the host protein disulfide isomerase (Abromaitis & Stephens, 2009). Furthermore, it reported a novel finding in which the 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 year ago,

and evolved to live in unicellular amoebae, thereby forgoing the process of genome reduction to overcome changes of 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, we have successfully demonstrated using mAbs that *P. acanthamoebae* attachment to amoebae is concurrently 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 amoebae 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 underlying the interactions between intracellular parasites and their host cells, especially those involved in chlamydial pathogenesis.

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Figure legends

Fig. 1. Inhibition of *P. acanthamoebae* attachment to and growth in amoebae by the addition of mAbs. (A) Representative fluorescence images showing successful attachment (mAb399) and the inhibition of bacterial attachment to amoebae (mAb311). Positive control, without mAb; Negative control, without infection. Arrows indicate bacterial attachment to amoebae. (B) Change of infectious rate (attachment rate) of the bacteria to amoebae when incubated without (medium control) or with the mAbs. Data are expressed as the average (percentage) + the standard deviation (SD). The experiment was performed at least three times independently. *p<0.05 versus the value of medium control. (C) Change in the numbers of infectious progenies when incubated without (medium control) or with the mAbs. Numbers of infectious progenies were determined using an AIU assay. Data are expressed as the average (the numbers of infectious progenies) + SD. All experiments were performed at least three times independently.

Fig. 2. Representative fluorescence images of mAbs reacting with uninfected amoebae. Amoebae grown on a cover slip for 24-48 h were used for this experiment. Fixed cells were stained with each of the mAbs and then incubated with secondary FITC-labeled antibody (Green) in the presence of DAPI (Blue). FITC, single staining. Merged with DAPI, double staining with mAbs and DAPI. Dashed square indicates the enlarged area in right panel (Arrow). Magnification, × 100.

Fig. 3. Representative fluorescence images of the mAbs that reacted to the amoebae infected with *P. acanthamoebae*, and co-localized to the bacteria cluster released from the amoebae. (A) Representative fluorescence images showing co-localization of the molecules recognized by mAbs with the bacteria. *P. acanthamoebae*-infected amoebae grown on a cover slip for 24-48 h were used for this experiment. Fixed cells were stained with each of the mAbs and then incubated with secondary FITC-labeled antibody (Green) in the presence of DAPI (Blue). Magnification, × 400. Dashed squares indicate the enlarged areas in right panel. "White" was presumed to indicate co-localization of the molecules recognized by mAbs with the bacteria (White arrows). (B) Prevalence of co-localization of the molecules recognized by mAbs with the bacteria. To determine the percentage of the co-localized molecules recognized by the mAbs with the bacteria released from amoebae, at least five fields were randomly selected and the numbers of bacterial clusters showing "white spots" were counted (see above). White columns indicate uninfected amoebae. Black columns indicate infected amoebae. Data are expressed as the average + the SD. *p<0.05 versus the value of each mAb on the uninfected amoebae.

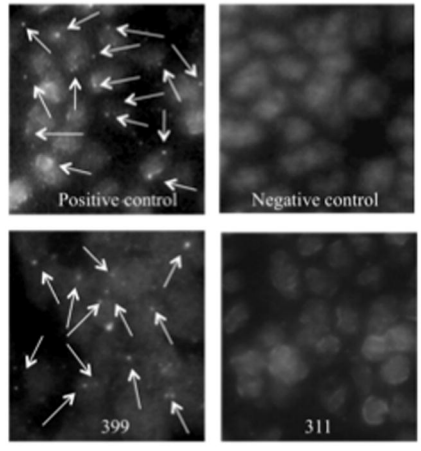
Fig. 4. Synergistic effect of the mAbs and protease inhibitors on the inhibition of *P. acanthamoebae* attachment to amoebae. (A) Synergistic effects between each of the mAbs and protease inhibitors. Amoebae were cultured with the bacteria at MOI 10 in the presence or absence of each of the mAbs (5-fold diluted culture supernatant) with or

without either PMSF or 1,10-phenanthroline (final concentration, 1mM) for 1 h at 4°C. Data are expressed as the average (percentage) + the SD. *p<0.05 versus the value of medium control. (B) Synergistic effect among the mAbs in the presence or absence of protein inhibitors. Amoebae were incubated with bacteria at MOI 20 (see above). Data are expressed as the average (percentage) + the SD. NS, not statistical significant. MAb38a, mixture of mAb300 with mAb38. MAb311b, mixture of mAb562 with mAb311. MAb38c, mixture of 300mAb, 311mAb and mAb562mAb with mAb38. *p<0.05 versus the value averaged among four mAbs without any protease inhibitor. All experiments were performed at least three times independently. (C) Representative zymograph image.

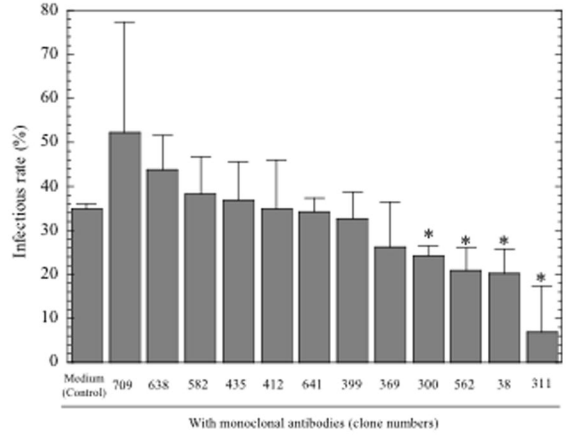
Fig. 5. Model of *P. acanthamoebae* attachment to amoebae following successful infection. Dashed square represents a requirement for serine-protease activity. Arrows indicate bacterial maturation in the amoebae. Pa, *P. acanthamoebae*. Molecule(s), each of the targets recognized by the mAbs.

Fig. 1

A


Positive control
Negative control
399
311

B


Infectious rate (%)
80
70
60
50
40
30
20
10
0
Medium (Control)
709
638
582
435
412
641
399
369
300
562
38
311
With monoclonal antibodies (clone numbers)

C

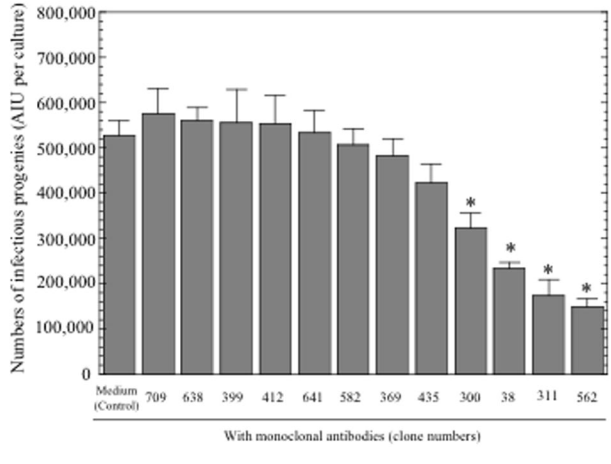

Numbers of infectious progenies (AIU per culture)
800,000
700,000
600,000
500,000
400,000
300,000
200,000
100,000
0
Medium (Control)
709
638
399
412
641
582
369
435
300
38
311
562
With monoclonal antibodies (clone numbers)

Fig. 2

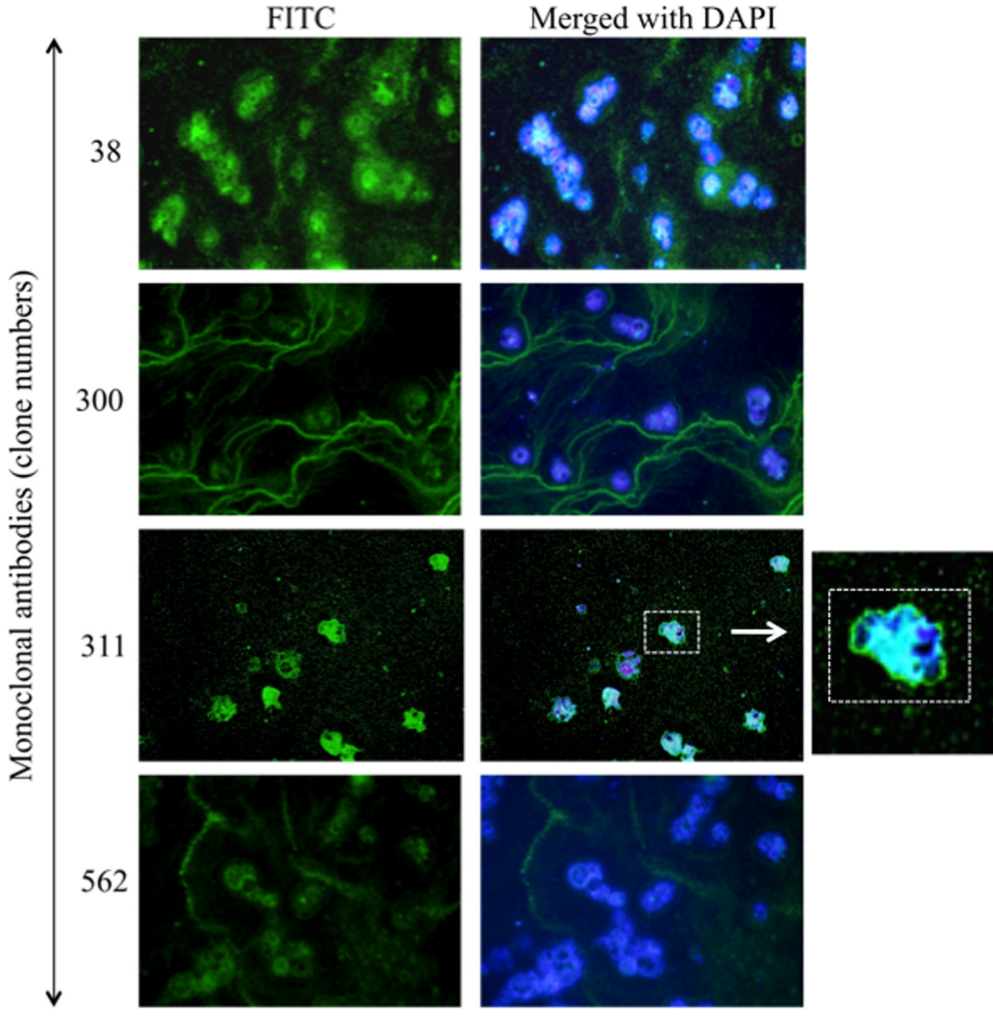

FITC
Merged with DAPI
Monoclonal antibodies (clone numbers)
38
300
311
562

Fig. 3

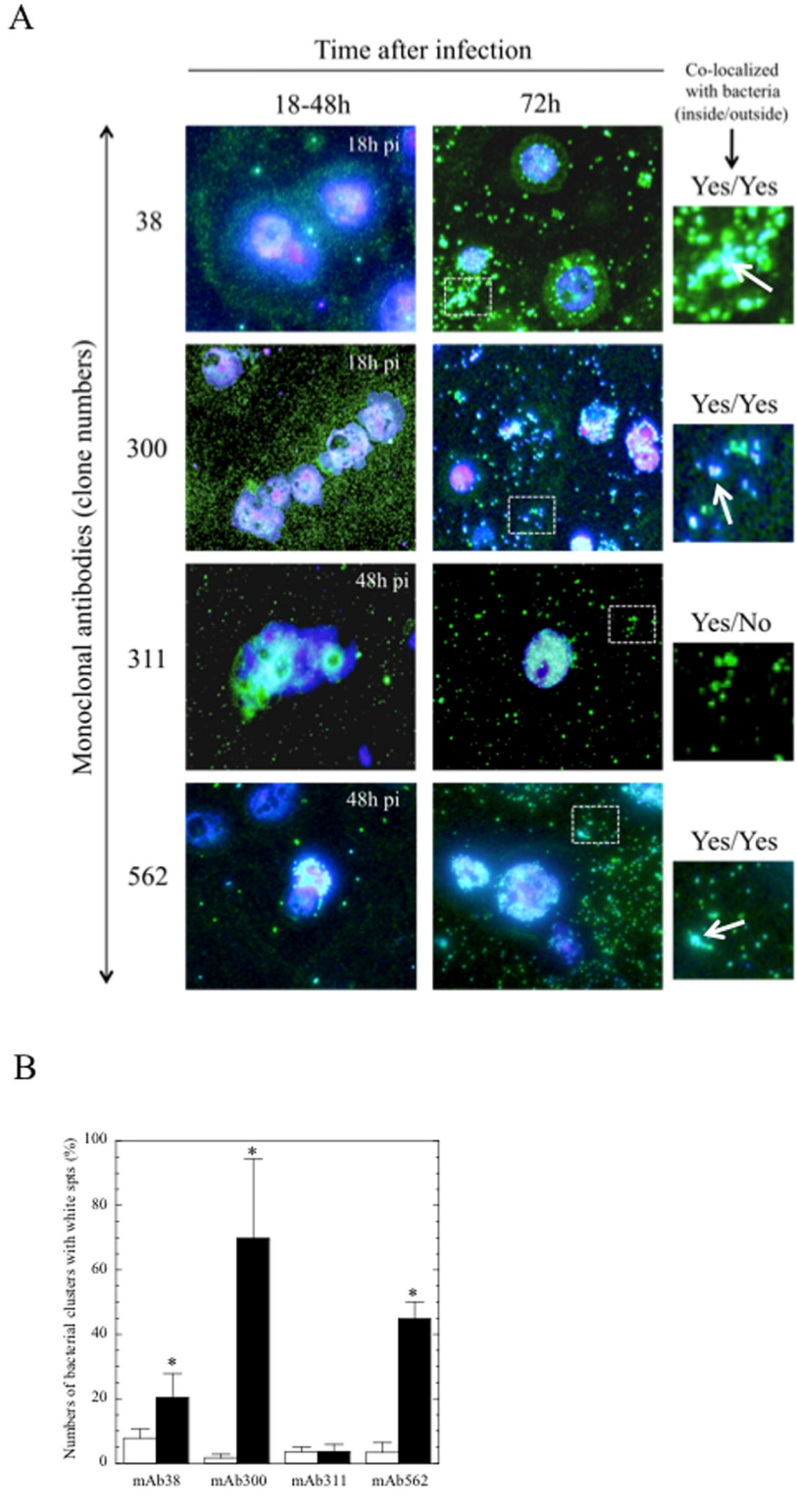

A
Time after infection
18-48h
72h
Co-localized with bacteria (inside/outside)
Monoclonal antibodies (clone numbers)
38
300
311
562
18h pi
18h pi
48h pi
48h pi
Yes/Yes
Yes/Yes
Yes/No
Yes/Yes
B
Numbers of bacterial clusters with white spts (%)
100
80
60
40
20
0
mAb38
mAb300
mAb311
mAb562

Fig. 4

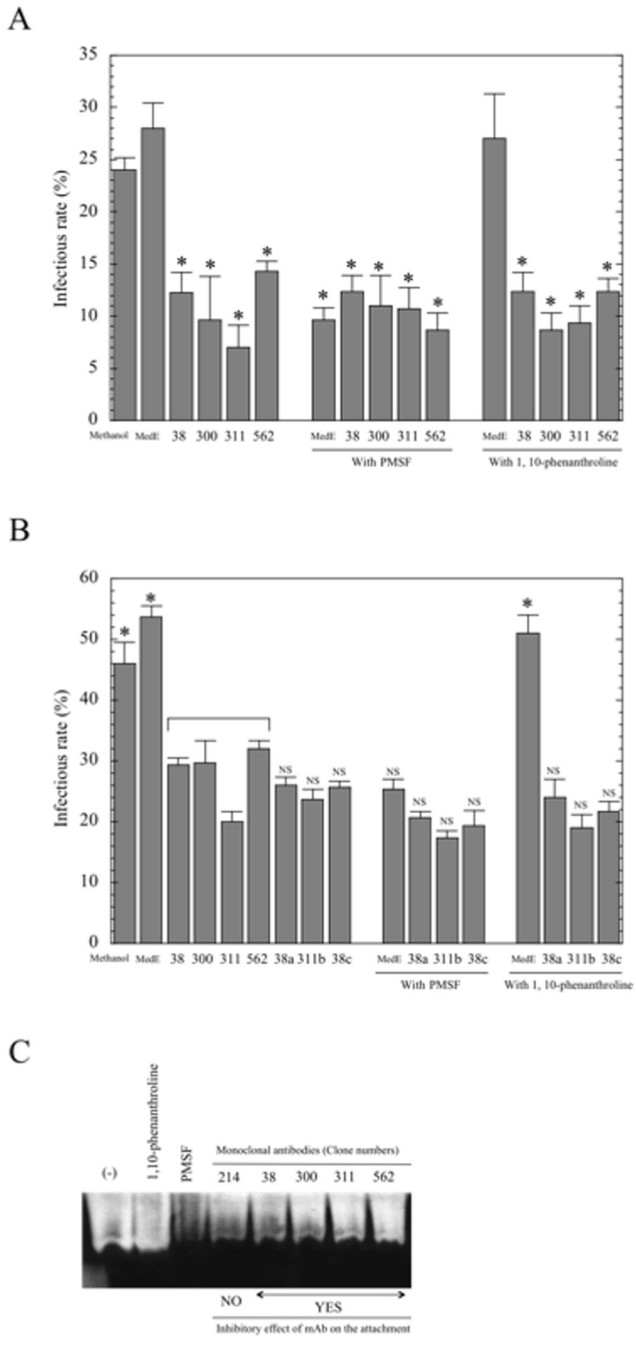
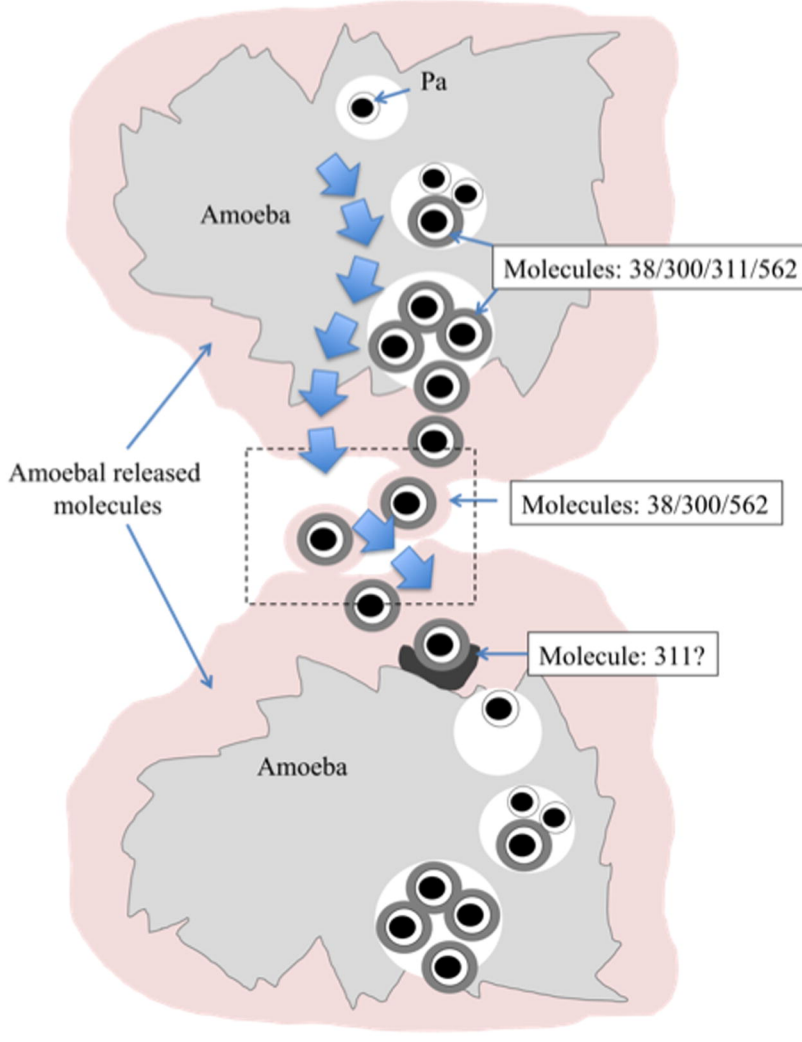

A
Infectious rate (%)
35
30
25
20
15
10
5
0
Methanol
ModE
38
300
311
562
ModE
38
300
311
562
With PMSF
ModE
38
300
311
562
With 1, 10-phenanthroline
B
Infectious rate (%)
60
50
40
30
20
10
0
Methanol
ModE
38
300
311
562
38a
311b
38c
NS
ModE
38a
311b
38c
With PMSF
ModE
38a
311b
38c
With 1, 10-phenanthroline
C
(-)
1,10-phenanthroline
PMSF
Monoclonal antibodies (Clone numbers)
214
38
300
311
562
NO
YES
Inhibitory effect of mAb on the attachment

Fig. 5


Pa
Amoeba
Molecules: 38/300/311/562
Amoebal released molecules
Molecules: 38/300/562
Molecule: 311?
Amoeba