

## Original article

# Human IgG inhibits IgA1 protease-dependent adherence of *Haemophilus influenzae* strains to human lung epithelial cells

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**Background:** IgA1 protease may enhance the bacterial infection in human beings. However, the molecular mechanism of bacterial adherence to eukaryotic cells is unclear.

**Objective:** Reveal the mechanisms of IgA1 protease-dependent and non-protease bacterial adherence to eukaryotic cells.

**Method:** Type I and type II IgA1 proteases from *iga* genes (GenBank DQ683355 for NTHi465, DQ683356 for NTHi500 and DQ683357 for Nm430) were cloned, expressed, and purified. Cellular assays for adherence of IgA1 protease-producing and -non-producing and typable and nontypable strains of *H. influenzae* to human lung carcinoma cells (A549) were carried out in the presence of human antibodies.

**Results:** Adherence of protease-producing strains and non-producing strains to human epithelial cells was significantly dependent on the enzyme activity. In addition, human IgG was an inhibitor to IgA1 protease-dependent adherence of *H. influenzae* strains to human cells. However, IgA1 antibodies were irrelevant to IgA1 protease-dependent adherence.

**Conclusion:** IgA1 protease was required for adherence of pathogenic bacteria to human epithelial cells in IgA1 protease-producing bacteria, and human IgG inhibits the adherence, but not for IgA1 protease non-producing bacteria.

**Keywords:** Cellular adherence, *iga* gene; IgA1 protease subtypes, IgG inhibition, *Neisseria meningitides*, typable and nontypable *Haemophilus influenzae* (THi and NTHi)

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IgA1 protease was first identified in *Streptococcus sanguis*, a species involved in the early stage of dental plaque [1], and was subsequently found in other pathogenic bacteria including *Neisseria meningitides* (*N. meningitides*), *Haemophilus influenzae* (*H. influenzae*), and *Streptococcus pneumoniae* (*S. pneumoniae*) [2]. The pathogenic role of the enzyme had been an experimental

problem until cellular assays were developed. IgA1 proteases had been classified into four types: serine protease, threonin protease, cysteine protease, and metalloprotease.

The serine protease is the most important and widely present enzyme. It has further been divided into three subtypes according to different cleavage sites in the hinge region of IgA1: type I proteases cleave the Pro231-Ser232 bond and type II proteases cleave the prolyl-threonyl bond at position 235-236. Both types I and II enzymes have been found in some important pathogenic bacteria [3], such as *N. meningitidis*, *N. gonorrhoeae* and *H. influenzae*. However, type III serine proteases, a relatively rare enzyme in these bacterial strains have not been

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characterized [4]. Each pathogenic bacterium produces only one type of the protease, i.e. metalloprotease in some streptococcal bacteria, cleaving human IgA1 only at the prolyl-threonyl bond at position 235-236 in the hinge region of human IgA1 molecule. Though type III serine IgA1 protease was not commonly detected in bacterial strains, some non-typable *H. influenza* (NTHi) isolates have been reported to produce the subtype enzyme [5].

Four distinguishing features in the precursor IgA1 protease are the signal peptide, the protease domain, a short linker, and  $\beta$ -domain. The protease domain is composed of approximately 1,000 residues with a special sequence of VLGDSGSPLF identified as the catalytic site of serine protease. The serine residue is believed to be one of the reactive site residues [2]. Thus, the presence of this special sequence could be a marker for serine type protease. The  $\beta$ -domain contains heterogeneous sequences in the N-terminus and a highly conserved C-terminus comprising approximately 300 residues that may be required for protein translocation [6].

IgA1 proteases are highly specific for human IgA1. It does not hydrolyze any other subclasses of human immunoglobulin. This is because the IgA1 protease cleaves the heavy chain of the antibody in the hinge region where the special primary structure is not present in IgA2 or other subclasses of immunoglobulin [7]. The cleavage results in separation of Fc and Fab fragments. The latter remains bound to cognate antigens, thus forming a mask of surface epitopes to prevent subsequent recognition by other intact antibodies [8]. Therefore, the Fc-mediated functions (e.g., immune complex elimination) are lost by such proteolytic cleavage. According to Weiser et al. [9], after degradation by IgA1 proteases, the retained Fab fragments selectively unmask the bacterial ligands used in interactions with host cells, providing means for enhanced adherence. For years after the IgA1 protease had been reported, the pathogenic role of the protease was not well understood in relation to colonization and pathogenic virulence because of difficulties in setting up experimental animal models [10]. However, quantitative analysis of IgA1 protease activity in pathogenic bacteria isolated from patients with invasive diseases suggested that the quantity of IgA1 protease activity could be related to the virulence of pathogenic bacteria. For example, levels of IgA1 protease activity in cerebrospinal fluid or blood from

meningococcal patients were found to be significantly higher than those obtained from asymptomatic carriers [11]. In addition, an investigation of NTHi-related diseases revealed that the activity of IgA1 protease in bacterial strains isolated from blood and sputum of symptomatic individuals was significantly higher than that of IgA1 protease in bacterial strains isolated from throat swabs of asymptomatic carriers [12]. These findings provided evidence to show that IgA1 protease activity could be a determinant of virulence that contributes to the pathogenic phenotype.

NTHi is a common etiologic agent responsible for otitis media and chronic sinusitis. It is also an important cause of community-acquired pneumonia, especially in children in developing countries and patients with underlying chronic lung disease [13, 14]. In previous studies, we demonstrated that NTHi has become a dominant strain responsible for the pathogenesis of the diseases in southern Taiwan after years of application of vaccines against type b and other subtypes of *H. influenza*. Thus, NTHi has become an important strain in clinical assessments.

In some swine pathogens, such as *Streptococcus suis*, IgA1 protease was also found to cleave IgA1 and cause immuno-reaction [15, 16]. However, the mechanism of action of the enzyme was not clarified. The substrate of IgA1 protease in human and other primates is an immunoglobulin predominantly located on the mucosal membrane. Therefore, it is considered an essential defense against potential harmful pathogens and opportunistic microbial flora colonizing the lower respiratory tracts. However, this defense mechanism has never been proved by experimental evidence.

Prolyl boronic acids [3] and human serum [6] have been found to inhibit proteolytic activity toward human IgA1, but the molecular mechanism and anti-pathogenic role of the inhibition remain unclear. In this paper, we investigated the mechanisms of IgA1 protease-dependent and independent bacterial adherence to eukaryotic cells, and found that IgA1 protease enhances adherence of bacteria to epithelial cells, the event of which can be inhibited by human IgG.

## Materials and methods

### *Bacterial strains and growth conditions*

Both NTHi and typable *H. influenza* (THi) strains were used in this study. Strains NTHi465 (type II enzyme), NTHi500 (type I enzyme) and NTHi558 (enzyme-negative), and THi46679 (Hic, type II

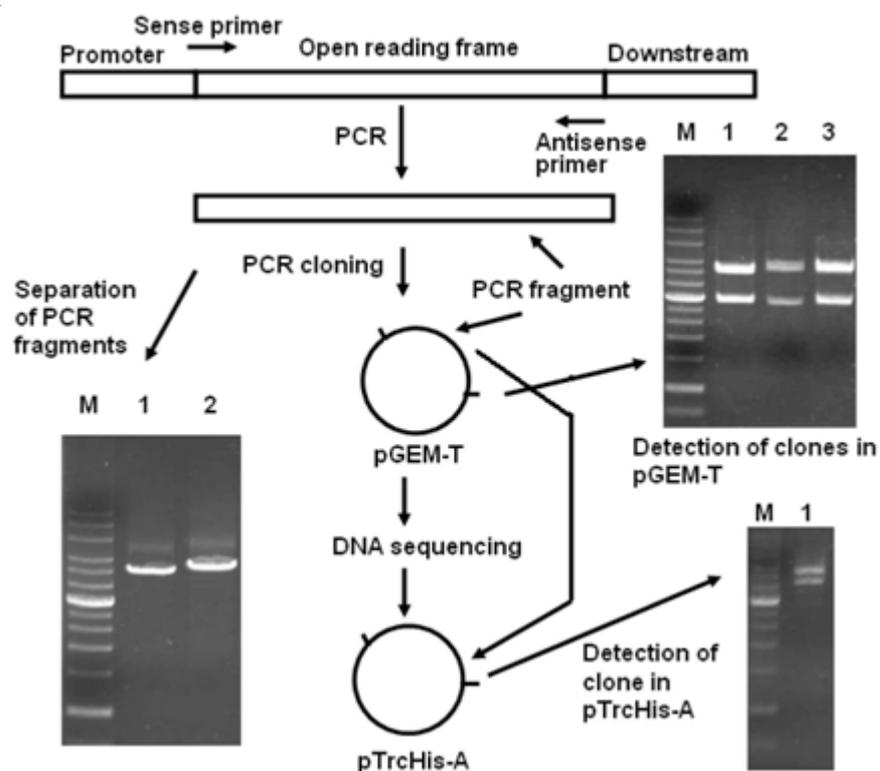
enzyme) and THi46644 (Hic, enzyme-negative) were isolated from patients with invasive diseases. Both NTHi and THi strains were grown on chocolate agar plates (BD-BBL, NJ, USA) at 37°C in an atmosphere of 5% CO<sub>2</sub> and 95% air for 18 to 20 hours. Broth cultures were prepared in brain-heart infusion broth (Difco) supplemented with X factor (hemin, 10 µg/mL, Sigma) and V factor (nicotinamide adenine dinucleotide, 2 µg/mL) in an atmosphere of 5% CO<sub>2</sub> and 95% air for 18 to 20 hours. *N. meningitidis* (Nm) strain 430 containing type II enzyme was isolated from blood from a meningitis patient, and grown in TSB broth (Tryptic Soy Broth, Difco) at 37°C for 20 hours.

**Preparation of genomic DNA, PCR, PCR cloning and DNA sequencing**

Incubation of bacterial culture supernatants with human IgA1 followed by detection of the immunoglobulin fragments could show the presence or absence of active IgA1 protease, our isolated strains. To purify genomic DNA, NTHi and Nm strains were grown as described above. Their genomic DNA

was purified with a Genomaker-kit (Invitrogen) according to the manufacturer's instructions. Dried DNA dissolved in 50 µL of sterilized TE buffer was used as template in polymerase chain reactions (PCR).

PCR primers (sense 5' CGC GGA TCC TTA AAA CGG TAA AAC CTT ATG 3' and antisense 5' CGC GAA TTC TTT TGC TAT GAA TTT AGT ATT 3') [1, 17] were designed to amplify the Nm430 *iga* gene, and primers (sense 5' CGC CTC GAG ATG CTA AAT AAA AAA TTC AA ACT C 3' and antisense 5' CGC AAG CTT CGG GCT TAT TAA AAA CTAAAA CTT AG 3') [18, 19] were designed to amplify the NTHi500 and NTHi465 *iga* genes. PCR reactions (25 cycles) were performed in a programmable thermocycler (ThermoHybaid). Then, the products were checked by agarose gel electrophoresis. Appropriate DNA fragments, which had been purified using Band-prep kit, were cloned into pGEM-T for DNA sequencing. Then, the DNA fragment was transferred to pTrcHisA for expression, as shown in Fig. 1.



**Fig. 1** Schematic diagram of PCR amplification of *iga* genes, PCR cloning, and gene expression. The left gel shows the PCR-amplified *iga* gene fragments from genomic DNA templates of NTHi465 and Nm430 strains. The upper right gel shows the restriction fragments from pGEM-T vector and cloned *iga* genes, NTHi465 (lane 1), NTHi500 (lane 2) and Nm430 (lane 3). The bottom right gel shows the separation of restriction fragments of NTHi465 from expression vector pTrcHis-A (lane 1).

### **Expression, purification and test of subtypes of recombinant IgA1 protease**

LB (3L) containing 50 µg/mL of ampicillin was inoculated with 30 mL of stock BL21 (DE3) cells containing appropriate plasmid. Incubation was carried out for another six hours before harvesting by centrifugation at 5,000g for 20 minutes. Ammonium sulfate was added to the supernatant to 70% saturation with gentle stirring at 4°C to precipitate crude recombinant protein. Then, this was dissolved with sterilized ddH<sub>2</sub>O and dialyzed against 50mM Tris-HCl (pH 7.5) before applying to a DE52 anion-exchange column (Whatman). The enzyme was eluted from the column in the same buffer. The purity was checked using SDS-PAGE. Protein concentration was calculated using the following equation:

$$(\text{mg/mL}) = 1.45 \text{ OD}_{280} - 0.74 \text{ OD}_{260}$$

Recombinant protease (0.1 µg) was incubated with 4 µg of either human IgA1-λ (Calbiochem) in 50 mM Tris-HCl (pH 7.5) or human IgG purified from protein A-Sepharose. The reaction was stopped with SDS-PAGE buffer, and the reaction products were separated by SDS-PAGE. Polypeptide bands were revealed by staining with Coomassie blue.

### **Assays for bacterial adherence to human epithelial cells**

Cellular assay for bacterial adherence to human epithelial cells in the presence of recombinant IgA1 protease was modified from a protocol for *Hap* protein [20]. A549 human lung carcinoma cells (ATCC CCL-185) were seeded in 24-well plates at a density of 4x10<sup>4</sup> cells/well. Then, they were washed in PBS (pH 7.2) and maintained in culture medium (Ham's F12K with 2mM L-glutamine/1.5g/L sodium bicarbonate/10% CCS, GIBCO, Langley, Oklahoma, USA). Clinical isolates of NTHi500, 558 and THi46679 (Hic) and THi46644 (Hic) were grown in supplemented brain-heart infusion broth to mid-logarithmic phase (OD<sub>620</sub>=0.3-0.4) to determine their colony-forming unit (CFU).

Bacterial adherence to human epithelial cell monolayer was investigated using adherence assays. Briefly, bacterial culture (7x10<sup>5</sup>-2x10<sup>6</sup> CFU) was inoculated onto a viable epithelial cell monolayer, to which purified IgA1 protease had been added. In another set of assays, either human IgA1-λ or IgG was added at 1.138 µg/µL with or without addition of

IgA1 protease. The plates were then centrifuged at 1,200g for five minutes to facilitate the contact between the bacteria and the epithelial cells. After incubation for one hour at 37°C in 5% CO<sub>2</sub>, the plate was gently shaken and rinsed twice with PBS to remove nonadherent organisms. Cell-associated bacteria were lifted off by treatment with 0.05% trypsin and 0.02% EDTA. Serial dilutions of the inoculum or adherent bacteria were quantified by plating on chocolate agar. Adherence ratio was calculated by dividing the number of adherent CFU per well by the number of inoculated CFU. Relative adherence rate was calculated assuming a control adherence of 100%.

### **Statistic analysis**

At least five independent assays for bacterial adherence were conducted. Data were analyzed using Student *t*-test. Although results were plotted against inoculum control to show the significance, each treatment (assays with addition of IgA1 protease) was also analyzed against all other treatments (assay with IgA1 protease plus IgA1).

## **Results**

### **The α- and β-protein domains exhibited different conserved regions**

PCR fragments for *iga* genes were 5kbp from NTHi465 and NTHi500 and 5.5kbp from Nm430 templates (**Fig. 1**) (GenBank access number DQ683355 for NTHi465, DQ683356 for NTHi500, and DQ683357 for Nm430).

Amino acid sequential alignments [21, 22] showed that NTHi proteases contained three autoproteolytic sites at C-terminus of α-protein, namely Pro1025-Ser1026, Pro1044-Ala1045 and Pro1049-Ser1050 (NTHi465 number from signal peptide), but different in Nm430 protease, as shown in **Fig. 2**. Notably, there was a 53-residual peptide inserted between Pro1046 and Ala1047 in NTHi500 protease. The alignments demonstrated that a conserved domain (Cys814-Pro1025), the β-protein, was presumably cleaved free from the precursor protease.

An α-protein domain (**Fig. 2**) in NTHi protease begins from Ser1020 to Pro1318 (Ser988 to Pro1379 in Nm amino acid sequence), with highly variable compositions of identical amino acid sequence. Even the two NTHi proteases had only 45.8% identical sequences in the region. In addition, the length of this region was also variable, with about 390 residues in

Nm and NTHi500, but only 299 residues in NTHi465 protease. Moreover, although the cleavage site was present within the deduced N-terminal end of the  $\alpha$ -protein domain (Pro1024-Ser1025) in Nm protease, the corresponding site was not present in NTHi protease.

Another notable sequence (the surface domain in Fig. 2) was present in all species. It was located between the  $\alpha$ -protein and  $\beta$ -core, and was covalently linked to the membrane-embedded  $\beta$ -core. Alignments of Nm430 protease with NTHi465 and NTHi500 enzymes showed that it was 13.2% identical (32.7% similarity) between Nm430 and NTHi465/NTHi500, whereas up to 75.7% identical sequences were present in the two NTHi proteases even though they belong to different subtypes of serine protease.

#### Protease subtypes in relation to primary sequences

IgA1 serine protease subtypes have been found in a number of organisms [5, 23], but the relationship between sequential specificity and the cleavage site is unknown. Sequence alignments of NTHi465 (type II), Nm430 (type II), and NTHi500 (type I) proteases showed that the most significant variable region in protease domain was located from Ser216 to Asp240, but it is unlikely the determinant for subtypes. This is because the same subtype from NTHi465 and Nm430 exhibited variation in the region. The determinant residues for subtype enzymes could be located around

the reactive site residues (His100, Asp164, and Ser298 (His75, Asp139, and Ser273 in mature protease)). Some residues in type II enzymes (Tyr140, Ala185, Asn196, Gly215, Gly239, Lys259, Asn272, and Ser278 in NTHi465 and Nm430) have been substituted by alternative amino acids in type I enzymes.

#### Recombinant IgA1 protease expression, purification, and detection of enzymic activity

The *iga* genes from NTHi500, NTHi465 and Nm430 were cloned into pTrcHis-A (Invitrogen), respectively for expression, but the poly-histidine tag was not used. The recombinant protease domain should be autotransported outside of the host cell. Figure 3 shows SDS-PAGE of purified recombinant IgA1 protease, cleaved fragments of human IgA1- $\lambda$  and IgG. Purification from the cultured supernatants showed that about 95% homogeneity could be reached in a single chromatography as detected by SDS-PAGE (Fig. 3A), which showed that the autoproteolytic cleavage was processed correctly. *In vitro* incubation of human IgA1- $\lambda$  with recombinant protease showed that all the subtypes were truly reproduced (Fig. 3B, left). Moreover, we tested the specificity of the recombinant proteases with protein A-Sepharose-purified human IgG, and the results showed that the proteases were highly specific to human IgA1 (Fig. 3B, right).

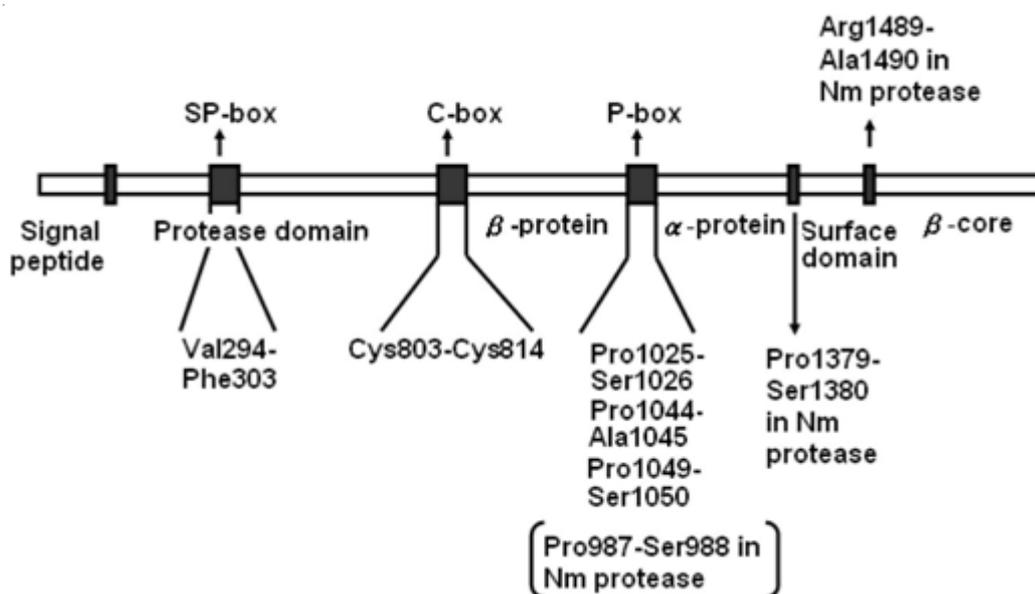
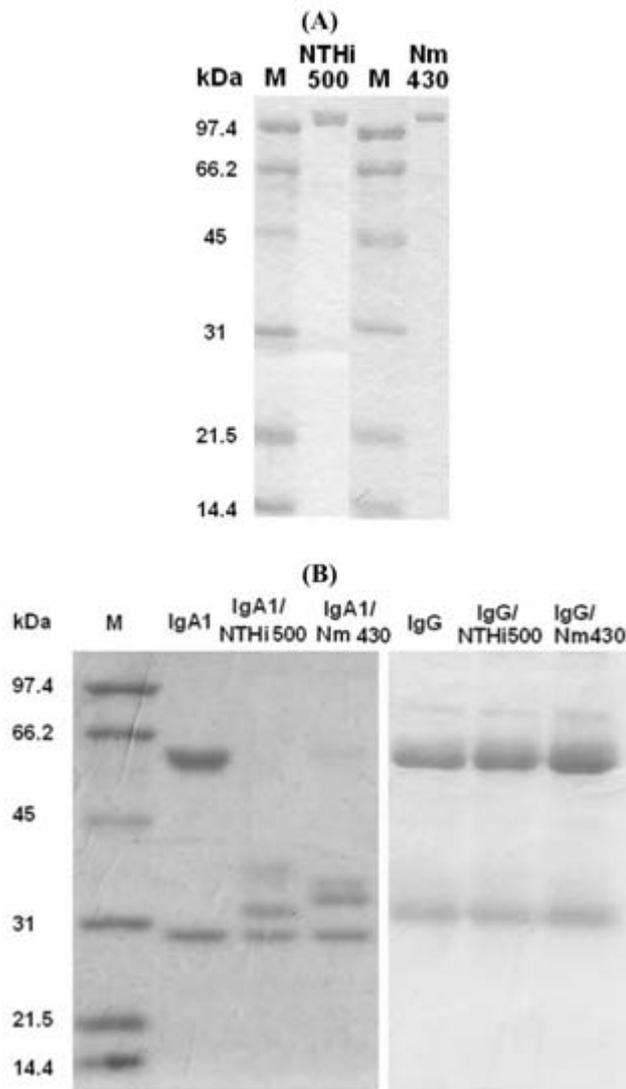


Fig. 2 Schematic diagram of special features of IgA1 protease. The amino acid numbers were given according to the precursor protease of NTHi465.



**Fig. 3** SDS-PAGE of purified recombinant IgA1 protease, cleaved fragments of human IgA1- $\lambda$  and IgG. **(A)** SDS-PAGE of purified IgA1 protease. **(B)** SDS-PAGE of cleaved IgA1- $\lambda$  by recombinant NTHi500 and Nm430 proteases (left) and SDS-PAGE of IgG incubated with recombinant NTHi500 and Nm430, respectively (right).

***Recombinant IgA1 protease enhances the adherence of NTHi cells to human epithelial cells***

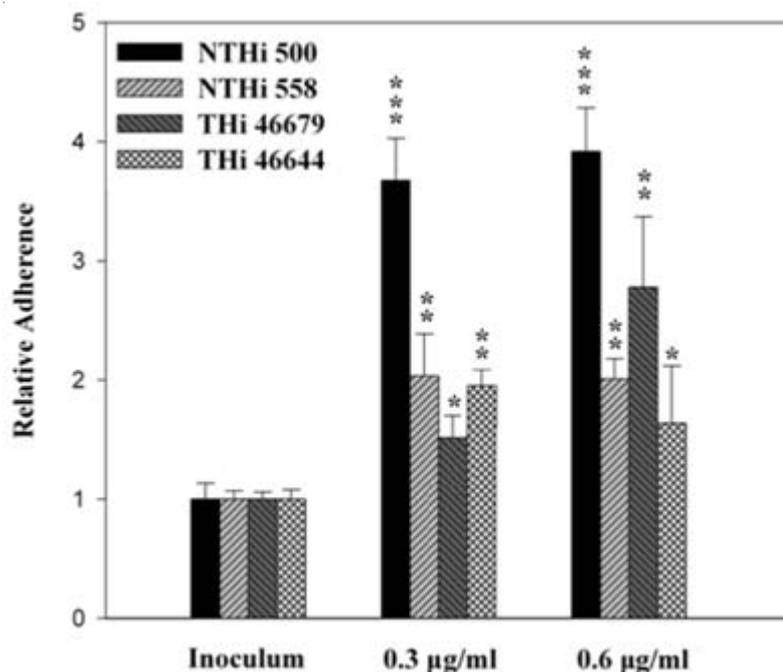
Four *H. influenzae* strains (protease-producing NTHi500 and THi46679, protease-negative NTHi558

and THi46644) were used in the cellular adherence assays with the recombinant proteases. **Figure 4** shows cellular assays for bacterial adherence to human epithelial cells in the presence of recombinant IgA1 protease. Relative adherence of each strain in the absence of recombinant IgA1 protease was set to 100% (**x**), and that of each strain in the presence of IgA1 protease (**y**) was a ratio of **y/x**. The results showed that all of the strains were significantly

dependent on IgA1 protease to adhere to human epithelial cells. Interestingly, under the stimulation of 0.3-0.6  $\mu\text{g/ml}$  of its own recombinant IgA1 protease, more NTHi500 cells adhered on the human cells than cells in the control inoculum ( $p < 0.005$ ). Adherence of the two IgA1 protease-negative strains, NTHi558 and THi46644, was also dependant on the protease.

***Human IgG inhibits IgA1 protease-dependent adherence of H. influenzae strains to human cells***

To mimic the pathogenic environment of bacterial infection, IgA1- $\lambda$  and IgG were mixed respectively with recombinant proteases, and then added to the cell culture along with the *H. influenzae* strains. First,

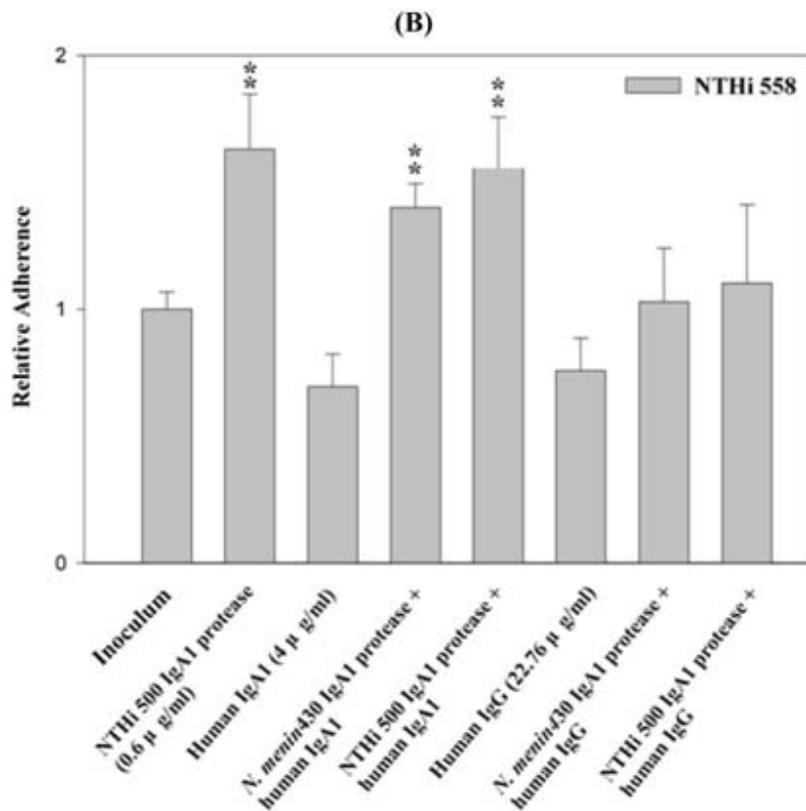
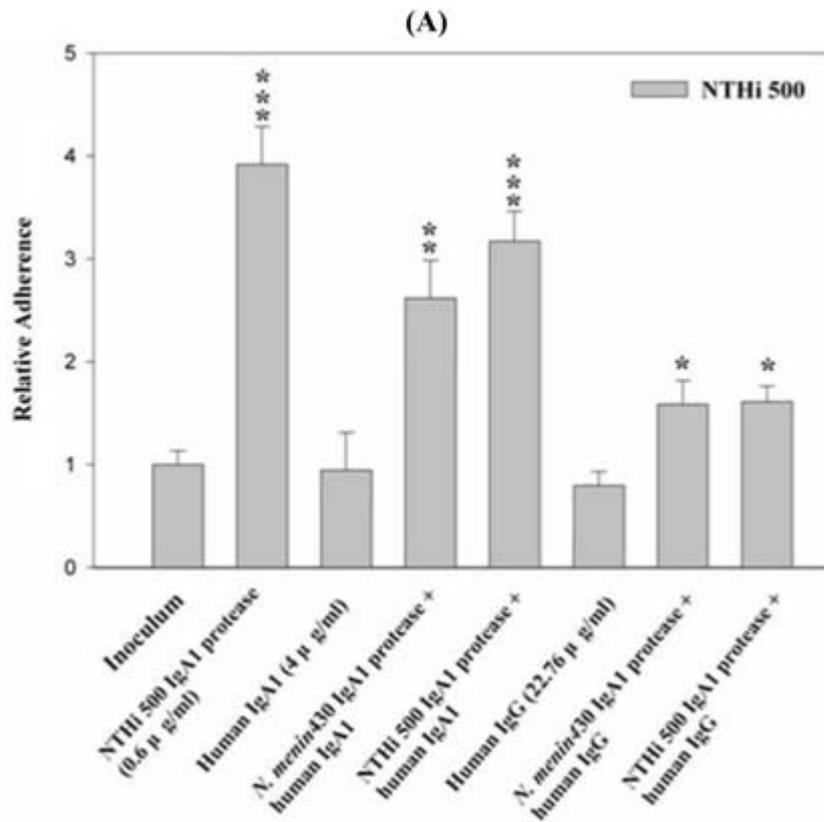


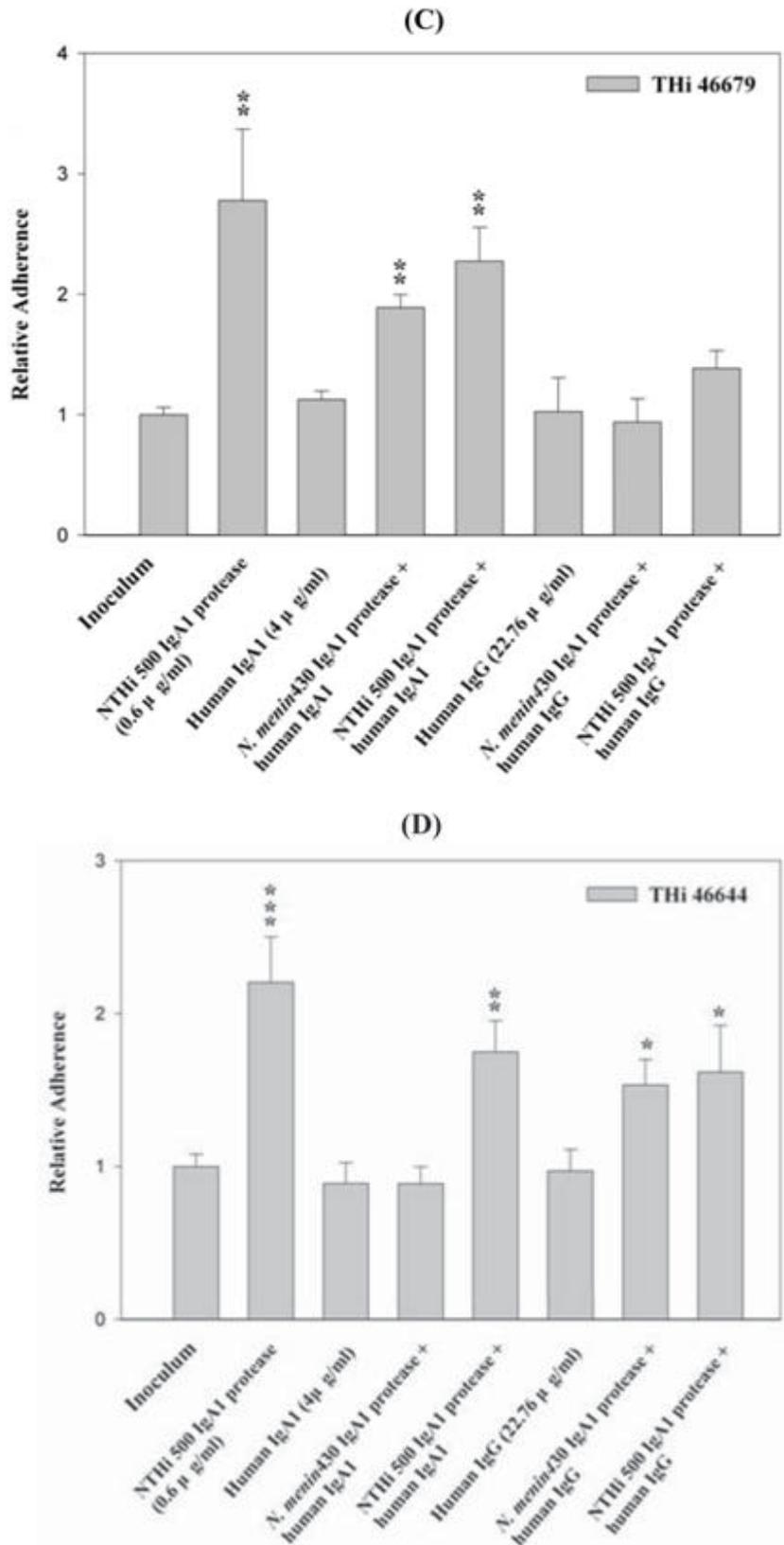
**Fig. 4** Cellular assays for bacterial adherence to human epithelial cells in the presence of recombinant IgA1 protease. *H. influenzae* cells of NTHi500 (protease-producing), NTHi558 (protease-nonproducing), THi46679 (protease-producing) and THi46644 (protease-nonproducing) were used in the assays, which were carried out in absence (inoculum control) or presence (0.3 µg/mL and 0.6 µg/mL) of recombinant IgA1 protease. Star symbols stand for significant differences in comparison with the inoculum control (\*stands for significant difference,  $p < 0.05$ ; \*\* for very significant difference,  $p < 0.01$ ; \*\*\* for extremely significant difference,  $p < 0.005$ ).

we tested the effect of IgA1 and IgG on adherence of NTHi to the epithelial carcinoma cells. We found that NTHi500 IgA1 protease (0.6 µg/mL) alone and the protease mixed with human IgA1-λ (4 µg/mL) had a significant effect on induction of bacterial adherence ( $p < 0.005$ ); however, no significant difference was observed between NTHi500 protease alone and NTHi500 protease with human IgA1-λ on bacterial adherence to epithelial cells (**Fig. 5A**). Relative adherence of NTHi500 in the presence of 0.6 µg/mL of Nm430 IgA1 protease increased significantly in comparison with the control, but human IgG (4µg/ml) reduced the adherence in the presence of either Nm or NTHi500 protease. Similarly, Nm IgA1 protease significantly promoted bacterial adherence to epithelial cells. The same set of experimental data showed that human IgG alone did not seem to have any inductive effect on the adherence of the two NTHi strains to human cells (**Fig. 5**).

We tested the level of adherence of THi strains to epithelial cells in the presence of IgA1 protease alone, IgA1-λ alone, IgG alone, IgA1 protease with IgA1-λ or IgA1 protease with IgG. As shown in **Fig. 5C**, NTHi500 IgA1 protease significantly

promoted the adherence of the THi46679 strain to the cell surface ( $p < 0.01$ ) in comparison with the inoculum control; however, the presence of IgA1-λ alone did not seem to enhance or decrease the strain's adherence in comparison with the control. Interestingly, human IgG inhibited the role of IgA1 protease in all of the assays. The effect of NTHi500 protease on NTHi500 strain's adherence was significantly attenuated by IgG (**Fig. 5A**). This result was similar to that obtained in the assays with THi46644 (**Fig. 5C**) with the exception of Nm430 IgA1 protease in the presence of human IgG (column 7 of **Fig. 5D**). The molecular mechanism of action of Nm430 IgA1 protease with either human IgA1 or IgG, in this particular case IgA1, protease-non-producing strain is not clear. However, the effect of NTHi500 protease on the adherence of NTHi558 was brought down from a very significant level ( $p < 0.005$ ) to non-significant level ( $p > 0.05$ ) in comparison with the control (**Fig. 5B**) in another IgA1 protease-non-producing strain, NTHi558. However, most assay results were consistent with and similar to other strains when assayed with THi46679 (**Fig. 5D**).





**Fig. 5** Cellular assays for bacterial adherence to human epithelial cells in the presence of recombinant IgA1 protease with or without IgA1 and IgG. (A) Assays with NTHi500 strain. (B) Assays with NTHi558 strain. (C) Assays with THi46679 strain, and (D) assays with THi46644 strain.

## Discussion

Since bacterial adherence to eukaryotic cells is the first step for infection, it causes much attention in infectious diseases. The surface domain of neisserial IgA1 protease has been shown to play a role in induction of bacterial adherence to human epithelial cells [24], suggesting that the protease is required for the first step of infection, specially for IgA1 protease-producing bacteria. However, the comparison of IgA1 protease-producing and non-producing bacteria in the presence of human IgG has not been reported.

NTHi has become a dominant strain of *H. influenzae* due to years of vaccine application against THi strains (Hib and Hic) and other pathogenic organisms. However, pathogenesis of NTHi infection is not well understood. Although the typable genome of *H. influenzae* has been reported [25], pulse-field agarose gel electrophoresis (PFGE) has demonstrated that there is great genomic heterogeneity among THi strains. We attempted to isolate *iga* genes from three subtypes, but the type III enzyme was so rare that we failed to find the subtype-producing strain in 129 NTHi isolates from patients with invasive diseases.

Sequence analysis showed that the specificity of subtype enzymes might be located within the protease domain. Furthermore, a conservative region in the precursor IgA1 protease is notably the  $\beta$ -core at the C-terminus, which contains highly conserved amino acid sequences with other autotransporter proteins (Hap and Hia/Hsf) [26, 27]. This is probably because both the protease domain and the  $\beta$ -core have highly specific functions, and any gross conformational change may greatly alter the functions of the two domains.

The  $\alpha$ -protein is a highly variable domain in IgA1 proteases from all sources, but its function remains unclear. There are three proteolytic sites (P-box) in NTHi proteases and one proteolytic site in the Nm protease at the N-terminal ends of the domain. A proteolytic site is also present at the C-terminus of  $\alpha$ -protein in Nm proteases (see **Fig. 2**). Therefore, it is presumably cleaved and released free. The surface-exposed domain is a small region composed of only 107 amino acids and remains covalently linked with the membrane-embedded  $\beta$ -core. Amino acid sequential similarity is 32.7% (13.2% identical) between NTHi465 and Nm430 proteases, but over 75.7% identical sequences are present between the two NTHi genes. The variation of the surface domain has been noticed in earlier studies [28, 29], but the

pathogenic role of variation remains unknown. We observed another conservative domain,  $\beta$ -protein, (**Fig. 2**), but its role is still unclear.

All subtypes of IgA1 protease were reproduced after expression and purification from *E. coli* strain BL21 (DE3), as shown in **Fig. 3A**. They remained highly active after purification. Inhibition of IgA1 protease activity has been studied with prolyl boronic acids [3] and human serum [4], but the mechanism of action and effect on bacterial adherence are unknown. St Geme et al. [30] reported finding a high-molecular-weight protein of NTHi that promoted attachment of bacteria to human epithelial cells. Further studies of that high-molecular-weight protein revealed that it could be a protein related to IgA1 protease [27, 29]. In addition, Nm type II IgA1 protease promotes bacterial survival within epithelial cells [20]. NTHi has been found to be associated with many respiratory-related diseases [31]. Thus, we tested NTHi and THi strains in an *in vitro* adherence assay with human lung carcinoma (epithelium) cells (A549). IgA1 protease increased the adherence rates of both IgA1 protease-producing NTHi/THi strains and IgA1 protease-negative strains to lung carcinoma cells.

The present study experimentally showed that IgA1 protease played a very significant role in promoting adherence of NTHi/THi strains to epithelial cells, as shown in **Fig. 4**. We found that the adherence rate was related to the concentration of IgA1 protease, because all the IgA1 protease-producing strains have higher relative adherence rates (2.8-3.9) than IgA1 protease-nonproducing strains (2.25-2.65) in the presence of the same amount of recombinant gA1 protease (0.6  $\mu\text{g}/\text{mL}$ ). In addition, the level of activity of IgA1 protease was associated with infection and invasion. In testing the role of IgA1 protease with or without human immunoglobulins (IgA1 and IgG), we found that IgA1- $\lambda$  alone, IgG alone, or a mix of any one immunoglobulin with IgA1 protease did not seem to enhance bacterial adherence to human cells. However, different responses between protease-producing strain NTHi500 and protease negative strain NTHi558 to the stimulation were statistically demonstrated (see **Fig. 5A** and **5B**). For example, when the concentration of IgA1 protease reached 0.6  $\mu\text{g}/\text{mL}$ , the enhancement was extremely significant ( $p < 0.005$ ) in the assays with NTHi500, but only very significant with NTHi588. Furthermore, the presence of human IgG had a negative effect in both strains, inhibiting the bacterial adherence from an extremely

significant level ( $p < 0.005$ ) to a significant level ( $p < 0.05$ ) with NTHi500, but to the same level as the inoculum control with NTHi588, suggesting IgA1 protease-producing and negative strains are dependent on IgA1 protease to infect host cells.

Although recombinant IgA1 protease is unable to hydrolyze human IgG, as shown in **Fig. 3B** (right), the subclass immunoglobulin significantly inhibited IgA1 protease-dependent bacterial adherence. The adherence of NTHi500 was brought down from an extremely significant level ( $p < 0.005$ , **Fig. 5A**) to a significant level ( $p < 0.05$ ), whereas THi46679's adherence dropped from a very significant level ( $p < 0.01$ ) to an insignificant level (**Fig. 5C**). Similar results with IgA1 protease negative strains (NTHi558 and THi46644) could be repeatedly reproduced (**Fig. 5B** and **5D**). These results strongly suggest that human IgG may be a native inhibitor of IgA1 protease-dependent adherence. The adherence results also suggest that there must be atomic interactions between IgA1 protease and human IgG, although IgA1 protease cannot hydrolyze the covalent structure of human IgG.

In conclusion, IgA1 protease enhanced bacterial adherence to human epithelial cells. IgA1 protease-producing *H. influenzae* strains (NTHi500/THi46679) depended significantly on IgA1 protease to adhere to human epithelial cells, and could be inhibited by human IgG. However, for IgA1 protease negative strains (NTHi558/THi46644), bacterial adherence did not seem to depend absolutely on IgA1 protease to adhere to human epithelial cells. The inhibition of IgA1 protease-dependent adherence in the presence of human IgG might be caused by molecular interactions between the two proteins.

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