Relationship of IL-8 production and the CagA status in AGS cells infected with *Helicobacter pylori* exposed to low-pH and activating transcription factor 3 (ATF3)

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Abstract

We examined the kinetics of IL-8 production and CagA status in AGS infected with *Helicobacter pylori*, 26695 (Western CagA-type), HPK5 (Eastern CagA-type) and isogenic *cagA*-disrupted mutants, exposed to different pH (pH6 and pH3). IL-8 was produced in the early and late phases after infection in CagA-dependent and -independent manners, respectively, irrespective of CagA-type. The wild-type exposed to low-pH tremendously reduced IL-8 level at early phase, but restored with urea, suggesting that low-pH exerted the kinetics of *H. pylori*-induced IL-8 production in CagA-dependent manner and urea was necessary for effective induction. CagA and phosphorylated CagA increased time-dependently after infection. Phosphorylated CagA from 26695, but not HPK5, rapidly peaked, consistent with the kinetics of IL-8 induction and appearance of hummingbird phenotype. ATF3 transcripts peaked late phase by wild-type, however, induced in two peaks early and late phases by *cagA*-disrupted mutants, indicating that different CagA-type proteins altered ATF3 induction in the infected cells.

Key words: kinetics of IL-8 production and CagA status, different CagA-type *H. pylori* strains (Western CagA-specific sequence (WSS) and Eastern CagA-specific sequence (ESS)), low-pH condition, activating transcription factor 3 (ATF3),

Introduction

Helicobacter pylori, a Gram-negative bacterium preferentially colonizes in the human stomach, modulates the mucosal immune system and causes a variety of gastrointestinal diseases (Kikuchi, 2002; Stoicov et al., 2004). *H. pylori* is grouped in two families, type I and II (Censini et al., 1996; Xiang et al., 1995). Type I, not II, harbors the multigene 40-kb *cag* pathogenicity island (*cag*PAI) possessing bacterial virulence and uses a type IV secretions system (TFSS) encoded by *cag*PAI to deliver the virulence factors such as CagA into the target cells (Rohde et al., 2003; Fischer et al., 2001).

During bacterium-epithelial cell interaction, *H. pylori* injects CagA directly into the attached cells. The translocated CagA subsequently undergoes tyrosine phosphorylation in the host cells by the Src family tyrosine kinases (SFK). The major tyrosine-phosphorylated CagA binds specifically to SHP-2 tyrosine phosphatase and deregulates phosphatase activity involved in carcinogenesis (Bentires-Alj et al., 2004; Hatakeyama and Higashi, 2005; Azuma et al., 2004; Higashi et al., 2002a; Souza et al., 2002; Neel et al., 2003). The minor tyrosine-phosphorylated CagA binds to C-terminal Src kinase (Csk) and inhibits SFK activity. Once tyrosine-phosphorylated CagA· SHP-2 triggers a signal cascade, which results in cell scattering and elongation (hummingbird), the recruitment of transcription factors and the release of cytokines including interleukin (IL)-8. Cell scattering is also induced by nonphosphorylated CagA with Grb-2 (Stein et al., 2002; Odenbreit et al. 2000; Backert et al., 2000; Covacci and Rappuoli 2003; Tsutsumi et al., 2003). Several studies reported *H. pylori* infection-induced

IL-8 release through multiple signaling pathways including VirD4-CagA-dependent and -independent manners (Fisher et al., 2001; Naito et al, 2006; Nozawa et al., 2004; Selbach et al., 2002; Kim et al., 2006).

Five-amino-acid motif EPIYA (Glu-Pro-Ile-Tyr-Ala) of CagA was subclassified into EPIYA-A, -B, -C and -D based on the sequences surrounding EPIYA motifs. In Europe, North America, Africa and Australia, the 'A-B-C'-type (Western CagA-specific sequence (WSS)) is prevalent, whereas that in East Asia is the 'A-B-D'-type (Eastern CagA-specific sequence (ESS)). The ESS-type CagA exhibits stronger SHP-2 binding, greater morphogenetic activity and severe inflammation than WSS-type CagA because EPIYA-D matches the high-affinity binding sequence for the SH2 domains of SHP-2, while, EPIYA-C differs the binding sequence at the pY-5 position. These results from the experiments using CagA transfection of epithelial cells. However, bacterium-cell interaction is indispensable to inject CagA into target cells, suggesting that other bacterial factors contribute to the alteration of molecular behavior in the infected cells.

H. pylori colonizes, adapts to low-pH and persistently infects in the stomach. There are controversial reports that *H. pylori cagA* is repressed (Merrell et al., 2003; Karita et al., 1996) or induced (Allan et al., 2001) by low-pH. Expression of *H. pylori* CagA varied with growth phase and pH exposure in parallel with the level of transcription variation within bacterium. However, the situation of CagA such as translocated CagA and its phosphorylation in target cells infected with *H. pylori* exposed to low-pH is little known.

For the first time microarray analysis showed that activating transcription factor 3 (ATF3) was induced at late phase (48 h) after *H. pylori* infection (Sepulveda et al., 2002). ATF3 is a member of the ATF/CREB subfamily of the larger basic region/leucine zipper transcription factor family. Normally expressed at low level, ATF3 is rapidly induced in response to diverse stress conditions or cell growth (Pan et al., 2005; Allan et al., 2001). ATF3 activates some target genes but represses others, depending on the promoter and cellular contexts (Hai et al., 1999). ATF3 can homodimerize, but it preferentially heterodimerizes with c-Jun, JunB, JunD, ATF2 and growth arrest/DNA damage C/EBP homologous protein to facilitate DNA binding to activating protein 1 or ATF/CRE consensus sites (Allan et al., 2001). However, it is unclear how ATF3 induction is regulated.

In this study, we employed four *H. pylori* strains, 26695 (WSS-type CagA), HPK5 (ESS-type CagA) and these *cagA*-disrupted mutants, to investigate the CagA status including its phosphorylation and physiological activities involved in morphological features affected by *H. pylori* in different pH conditions. The kinetics of *H. pylori*-induced IL-8 was in CagA-dependent and -independent manners, irrespective of CagA-type. However, different efficiency of IL-8 induction was observed between different CagA-type strains. Furthermore, we first demonstrated the relationship between CagA and ATF3 induction in the infected cells.

Materials and methods

Cell lines and *Helicobacter pylori* strains AGS cells (a human gastric adenocarcinoma epithelial cell line, ATCC CRL1739c) utilized were cultivated in RPMI 1640+7 medium including L-Glutamine, $NaHCO_3$, kanamycin (60 μg/ml) and streptomycin (20 μg/ml) (Nikken bio medical, Japan) supplemented 10% (vol/vol) fetal bovine serum (Biochrom, Ltd) at 37℃ in an atmosphere containing 5% CO_2. Two wild-type *H. pylori* strains; HPK5 (ESS-type CagA) (Takeuchi et al., 1998) and 26695 (WSS-type CagA), and each isogenic *cagA*-disrupted mutant strains; HPK5CA and 26695CA were used in this study. The each isogenic *cagA*-disrupted mutant possessing insertion of kanamycin cassette in the *cagA* locus was constructed by homologonous recombination with pcagA-plasmid gifted, as previously described (Takeuchi et al., 1998). These mutant strains were confirmed by PCR with specific primers (cagA-F1, 5′-TGCATGCAGGAGAAACAATGACTAACG-3′ and cagAR, 5’-TGCTTAATTAAGATTTTTGGAAACC-3’) and western blotting. All strains were grown on Brucella broth (Becton Dickinson) solidified with 1.4% agar (Wako Pure Chemical Industries) including vancomycin (10 μg/ml) supplemented with 10% horse serum (Invitrogen) at 37℃ under microaerobic conditions. Bacteria grown well were cultivated in 10ml of Brucella broth medium (pH 6) (Brucella-broth) supplemented with 10% horse serum in 10 ml of 100-ml conical flasks as previously described (Takeuchi et al., 2006). The bacterial growth was measured by determine optical density at 600nm (OD_{600}) with spectrophotometer (Biochrom Ltd), and colony forming units (CFU) were determined for bacterial viability.

Co-culture of AGS cells with *H. pylori* strains AGS cells (0.5×10^6) cultured in RPMI 1640+7 medium in six-well culture plates (Sumitomo bakelite) for two days were washed two times with phosphate-buffered saline (PBS) and then co-cultured with or without the strains at a multiplicity of infection (MOI) of 150 in RPMI 1640 medium (Invitrogen) without serum and antibiotics for three days at 37℃ under microaerobic conditions. The supernatants and cells were collected at appropriate time after co-culture such as 3, 6, 12, 24 and 48 hours and were utilized for measurement of IL-8 and western blotting, respectively. For synchronized infection, the plate was centrifuged by PlateSpin (Kubota) for 5 minutes at 600 ×g after adding the bacteria to AGS cells.

Preparation of whole-cell extracts At appropriate time-points (3, 6, 12, 24 and 48 hours), AGS cells were washed vigorously with PBS, incubated in PRIA buffer containing 50 mM Tris-HCl (pH 7.4), 1% Noidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM Ethylene Glycol Bis (β-aminoethyl ether)- N,N,N',N'-tetraacetic Acid, 1 mM NaF, 100 μM sodium orthovanadate, 100 μM Phenylmethylsulfonyl fluoride and a commercially available protease inhibitor mixture tablet (Roche Molecular biochemical) for 15 minutes and then harvested by scraping, followed by centrifugation (10,000 rpm) for 10 minutes to remove debris. The supernatants were added in 2× lysis buffer containing 50 mM Tris (pH 6.8), 10 % (wt/vol) sodium dodecyl sulfate (SDS), 12% (vol/vol) 2-mercaptoethanol, 0.1% (vol/vol) bromophenol blue (BPB) and 20% (wt/vol) glycerol and then boiled for 5 min for western blot.

Western blot analysis Equivalent amounts of total cell extracts (200 μg) were fractioned into SDS-polyacrylamid gel electrophoresis (5% SDS-PAGE) and electrotansferred onto nitrocellulose membranes (Millipore Corp.) by the apparatus (Marysol). The blots were blocked with 4% (wt/vol) dried skim milk (Nacalai tesque) in Tris-buffered saline with Tween 20 (TBST) at room temperature and incubated with PY99 antibody (1:1000, Santa Cruz) to detect phosphorylated tyrosine protein. The membrane was incubated with ECL anti-mouse IgG peroxidase-linked species-specific antibody (1/1000, Amersham Biosciences) and developed using the ECL Plus Western Blotting Detection Reagents (Amersham Biosciences). The membrane was soaked in buffer containing 0.1 M 2-mercaptoethanol, 2 % (wt/vol) SDS, 0.0625 M Tris (pH 6.7) for 30 minutes at 50℃ and washed 2 times with TBST for stripping. The blots were blocked with skim milk, reprobed with anti-CagA antibody (1:1000, Austral Biologicals) and then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:1000, Jackson Immunoresearch).

Immunoprecipitation One mg of the protein extracts was incubated for 1hour at 4℃ with either polyclonal rabbit anti-*H. pylori* CagA or monoclonal PY99 antibodies and then incubated with a 20 μl aliquot of Protein G Plus-agarose beads (Santa Cruz) at 4℃ for overnight. The beads were washed three times with lysis buffer and proteins were eluted by boiling for 10 minutes in 2× electrophoresis sample buffer (50 mM Tris, 10% SDS, 12% 2-mercaptoethanol, 20% glycine and 1% BPB). The immunoprecipitated proteins were subjected to western bolt analysis.

Measurement of IL-8 secretion from AGS cells The culture supernatant was centrifuged at 7,000 rpm for 10 minutes to eliminate unattached bacteria and was measured by IL-8 enzyme-linked immunosorbent assay (ELISA) (BioSource International) according to the manufacturer's instructions. All samples were measured in triplicate in at least three independent experiments.

Morphological examination AGS cells (1×10^5) were grown in six-well plates containing RPMI 1640+7 medium supplemented with 10% fetal bovine serum for 2 days to reach monolayers at approximately 30% cell confluence. The cells were washed with PBS and four *H. pylori* strains (26695, 26695CA, HPK5 and HPK5CA) suspended in RPMI 1640 medium without serum and antibiotics were added to AGS cells at a MOI of 150. Morphological changes of these cells were microscopically observed for 2 days to evaluate the appearance of elongation phenotype (hummingbird). The hummingbird is characterized by the production of thin needle-like cell protrusions. Smaller protrusions that were also occasionally seen in the uninfected control cells were not counted.

Acid exposure Aliquot of Bacteria grown well in Brucella-broth (pH 6) was incubated in Brucella-broth (pH 3) adjusted by hydrochloric acid supplemented with 10% horse serum for 15 minutes at 37℃ under microaerobic conditions and utilized for co-culture with AGS cell at a MOI of 150. In this acid exposure experiment, we used Brucella-broth (pH 3) without urea (pH3-U-) and with urea (5 mM) (pH3-U+). The viability of bacteria incubated in all conditions for 15 minutes was determined by CFU.

Sequence analysis The genomic DNA of HPK5 extracted by a QIAamp®DNA Mini Kit (Qiagen) was subjected to polymerase chain reaction (PCR) to amplify full sequence of *cagA* using primers, cagA-F1 and cagAR. PCR was performed following conditions: pre-heat at 96°C for 2 min, followed by 40 cycles of 96°C for 30 s, 52°C for 30 s and 72°C for 3.5 min. The amplicon (approximately 3.5-kb) was cloned into the pGEM-T-Easy vector (Promega) to construct pCAH5ZN. A variety of deletion mutants based on the pCAH5ZN was taken by Erase-a-Base® system kit (Promega) and was subjected to sequence analysis (Bio Matrix Research, Japan).

RT-PCR for ATF3 To seek whether the translocated CagA affects on transcriptional level of ATF3, total RNA extracted from AGS cells co-cultured with strains using TRIzol reagent (Invitrogen) according to the manufacture's instruction was subjected to RT-PCR. After DNase treatment (Promega), reverse transcription was performed using oligo$(dT)_{12\text{-}18}$ primer (Invitrogen) and PowerScript™ Reverse transcriptase (Clontech) with methods of supplier. The primers are as follows: ATF3 sense primer, 5′-GCTGTCACCACGTGCAGTATCTCA-3′ and ATF3 antisense primer, 5′-CTGTTCCTCCTCTTGCTGACAAGC-3′. The mRNA level for ribosomal protein *L7a* was measured at the same time to normalize and to serve as the internal control. The sequences of *L7a* sense and antisense primers were 5′-TTTGGCATTGGACAGGACATCC-3′ and 5′ -AGCGGGGCATTTCACAAAG-3′, respectively. PCR condition for ATF3 was as follows: pre-heat at 95°C for 1 min, followed by

30 cycles of 94°C for 30 s, 57°C for 20 s and 72°C for 60 s. The condition of PCR for *L7a* was same except the cycle was 32 (Pan et al., 2005).

Results

IL-8 production from AGS To determine whether *H. pylori* infection affects the kinetics of IL-8 production in AGS cells, we monitored the level of IL-8 release for 48 hours (Fig. 1A). The levels of IL-8 induced by wild-type strains (26695 and HPK5) increased steadily with time extending and reached the highest level. However, the kinetics of IL-8 production induced by 26695 was faster and greater compared to that by HPK5; the highest levels were at approximately 12 h and 48 h in 26695 and HPK5, respectively.

The isogenic *cagA*-disrupted mutants (26695CA and HPK5CA) showed a lower level of IL-8 production than the corresponding parent strains over all. For early phase (until 12 h), the mutants showed less than 50% of IL-8 level induced by the corresponding parent strains. In particular, a significant suppression of IL-8 level induced was still observed at 24 h by 26695CA. At 48 h (late phase), there was no significant difference in IL-8 production induced by wild-type and *cagA* mutant strains. To eliminate the possibility that the bacterial motility influences such difference in the IL-8 production level at early phase due to bacterial moving and attaching to AGS, synchronized infection was carried out to propel the bacterium to attach AGS quickly. The results were same as those with non-synchronized infection (data not shown).

The effect of low acid condition (pH 3) on IL-8 production Simultaneously the bacteria exposed to either pH3-U- or pH3-U+ were subjected to co-culture for the measurement of IL-8 release from AGS (Fig. 1B and C). The levels of IL-8 release induced by 26695 and 26695CA exposed to pH3-U- were dramatically reduced at 6 h and 24 h, respectively, whereas, HPK5 and

HPK5CA exposed to the same medium showed significantly lower level of IL-8 production over all (Fig. 1B) compared to those in pH 6 (Fig. 1A). The kinetics of IL-8 production induced by bacteria exposed to pH3-U- was completely recovered when the bacteria were exposed to pH3-U+ (Fig. 1C). These data are consistent with those in pH 6, except for HPK5CA which the IL-8 level was not restored with urea.

Identification of CagA and tyrpsine-phosphorylated CagA proteins in AGS

The status of CagA proteins in AGS co-cultured with *H. pylori* strains was examined by western blotting with immunoprecipitation (Fig. 2). CagA and phosphorylated CagA proteins derived from wild-type strains gradually increased during infection, but neither CagA nor phosphorylated CagA proteins was observed in mutants (Fig. 2A). CagA and phosphorylated CagA proteins derived from HPK5 reached the highest level at 12 h and 24 h, respectively. However, both CagA proteins from 26695 rapidly reached the highest at 6 h and kept the peak level until 24 h. We performed synchronized infection to determine whether bacterial motility effects on the status of CagA proteins in early phase after infection, which revealed no difference on the CagA status (data not shown).

The different kinetics of IL-8 production was observed in bacteria exposed to low-pH. Next, we analyzed the CagA and phosphorylated CagA proteins in AGS co-cultured with *H. pylori* incubated in acid conditions (Fig. 2B) to determine the effect of acid condition (pH 3) on CagA status. In bacteria exposed to low-pH mediums (pH3-U- and pH3-U+), CagA in AGS infected with 26695 gradually increased time-dependently as in normal condition (pH 6).

However, the time reached to the peak level in pH3-U- and pH3-U+ was 24 h and 12 h, respectively, indicating that the appearance of CagA protein shifted later. The CagA was dramatically reduced in early phase (3 h and 6 h) in pH3-U-, but recovered with urea (pH3-U+). In addition, the phosphorylated CagA which was not detected at early phase in pH3-U- became present in pH3-U+. Regarding the CagA status in AGS infected with HPK5 exposed to low-pH, CagA was little over all in pH3-U-, but restored gradually time-dependently in pH3-U+. The phosphorylated CagA did not appear over all in pH3-U-, whereas these became weakly present at late phase (24 h and 48 h) in pH3-U+. These data were distinct from the results obtained from 26695. Low-pH condition used showed no effect on the viability of wild-type strains (data not shown).

Amino acid sequence of HPK5 CagA We sequenced HPK5 *cagA* and then compared the amino acid sequence of 26695 CagA. The aliment of amino residues demonstrated that three EPIYA motifs, critical for the tyrosine phosphorylation of CagA (Stein et al., 2002), existed in C-terminal half of HPK5 CagA, resulting in ESS-type. 26695 CagA possessing two EPIYA and one EPIYT motifs is WSS-type. Approximately 10 amino acid sequences following third EPIYA at position of 972, involved in binding of phosphorylated CagA to SHP-2 molecule via SH-2 domain (Backert et al., 2001; Azuma et al., 2004; Higashi et al., 2002a), differed between HPK5 and 26695 strains (Table 1). Three putative tyrosine phosphorylation motifs (TPMs) and four cAMP-dependent phosphorylation motifs (CPMs) (Segal et al., 1999) were compared in both aliments of CagA. The TPM motif at Y-122 and Y-899 were active and the motif near the

C-terminus was inactive in both strains. Three out of four CPM motifs were active at K-223, K-454 and K-904 in 26695, whereas two active CPM motifs were observed at K-223 and K-923 in HPK5.

Morphological characteristics Interaction of host cells with *H. pylori* induced a hepatocyte growth factor-like response in AGS (hummingbird), which is characterized by spreading and elongated growth of the cell (Segal et al., 1999). We microscopically observed the appearances of such phenotypes with four strains for 2 days. Only 26695 induced the hummingbird phenotype time-dependently, but not in other three strains over all (data not shown). The AGS cells infected with 26695 showed the hummingbird at 6 h, irrespective of synchronized infection. Furthermore, we examined the morphological features of AGS cells infected with 26695 exposed to low-pH (pH 3) (Table 2). The hummingbird was observed at 6 h and 24 h with 26695 incubated in pH 6 and pH3-U-, respectively, indicating that the appearance of the phenotype was dramatically delayed. Using 26695 incubated in pH3-U+, the delayed time improved, but not completely compared to that in pH 6.

RT-PCR for ATF 3 We investigated the effects of *H. pylori* infection on the induction of ATF3 transcripts by RT-PCR (Fig. 3). ATF3 was definitely increased time-dependently in wild-type strains with different level and peaked at late phase (48 h), consistent with previous results (Sepulveda et al., 2002), despite ATF3 is an early response gene. Interestingly, the highest level was observed temporally at 6 h and 12 h only in *cagA*-disrupted mutants, HPK5CA and 26695CA, respectively. The experiments was performed two times and showed same

results. The ratio of ATF3 normalized with house keeping gene *L7a*, and was calculated and confirmed by NIH imager ver. 1.60.

Discussion

Pro-inflammatory chemokine IL-8 production depends on the presence of *cag*PAI in *in vitro* (Censini et al., 1996; Smita et al. 1998) and *vivo* studies (Peek et al., 1995; Yamaoka et al., 1996). In contrast, CagA was not always the responsible factor for IL-8 production (Fisher et al., 2001; Selbach et al., 2002). Our results demonstrated that *cagA* mutants significantly decreased IL-8 level in the early phase after infection, which indicated that CagA was involved in IL-8 production in the early phase, but not in late phase, leading to phase variation of IL-8 production in CagA-dependent and -independent manners during *H. pylori* infection. In addition, IL-8 level reduced tremendously in the early phase of the wild-type strains exposed to low-pH was restored with urea. These data suggest that the acid pH exerts the kinetics of *H. pylori*-induced IL-8 production in CagA-dependent manner and urea was necessary for effective induction of IL-8. The suppression of IL-8 level induced by HPK5 exposed to low-pH was observed until the late phase, which was distinct from 26695. Thus, the acid condition might have had more long-term effect on IL-8 secretion induced by HPK5 than 26695 due to the CagA sequence and/or individual properties in response to low-pH including gene regulation/expression (Merrell et al., 2003; Bury-Mone et al., 2004).

Three EPIYA(T) motifs of CagA might be necessary to enhance IL-8 release in AGS transfected with different *cagA* fragments, WSS- and ESS-CagA types (Kim et al., 2006; Azuma et al., 2004; Higashi et al., 2002b). However, the amount of IL-8 secreted over all in their study was not so high. We showed that CagA was involved in IL-8 induction at early phase

irrespective of CagA-specific sequences. However, the levels of IL-8 production induced by wild-type strains considerable differed at early phase. These suggest that not only CagA-specific sequences but strain-specific factors affect the enhancement of IL-8 induction as an early response to *H. pylori* infection.

CagA and phosphorylated CagA proteins increased time-dependently in AGS infected with wild-type strains. In particular early phase, phosphorylated CagA derived from 26695 rapidly peaked before 6 h, but those from HPK5 was delayed (24 h), consistent with the kinetics of IL-8 induction. Regarding 26695 exposed to low-pH, both proteins were little at 6 h and phosphorylated CagA was still little even CagA was completely recovered with urea. However, both proteins from HPK5 exposed to low-pH showed neither phosphorylated CagA nor the kinetics of CagA increased time-dependently. The CagA, but not phosphorylated CagA, was restored with urea. These results indicate that *cagA* expression of HPK5 is more defective than 26695 by low-pH exposure and raised the possibility that the acid condition influences the efficiency of translocation of CagA into the infected cell. Microarray analyses of low pH-responsive genes revealed that *cagA* expression increased within 26695 (Bury-Mone et al., 2004; Allan E. et al., 2001., Ang et al., 2001), however were strongly repressed within G27 (Merrell et al., 2003), suggesting that there are differences in *cagA* expression that are intricately linked to the strains examined. Interestingly, our studies demonstrated that phosphorylated CagA derived from wild-type strains exposed to low-pH were little or not detected even in the presence of huge CagA detected. These suggest that the molecular processing of CagA to enter

the translocation channel including possible secretion chaperone-like protein may be defective within bacterium exposed to low-pH. Indeed, the involvement of both N- and C-terminal regions of CagA including 20 amino residues in CagA translocation via TFSS was shown, suggesting that such terminal signal sequences would be also required for recruitment to the secretion apparatus and/or for translocation across the bacterial outer membrane (Hohlfeld et al., 2006). The ESS-type CagA might be more sensitive to low-pH in term of the structural and functional features of the molecular chaperone of CagA within bacterium; however, we should investigate more to elucidate these problems.

The hummingbird appeared at 6 h by 26695 (WSS-type), but not 26695CA, supporting the morphological features associated with phosphorylated CagA (Segal et al., 1999; Higashi et al., 2002a). The appearance of the hummingbird induced by 26695 exposed to low-pH was delayed and somewhat was restored with urea, consistent with the detected kinetics of phosphorylated CagA. Based on binding affinities of different CagA-type to SHP-2, ESS-type CagA could induce effectively the change in hummingbird phenotype than WSS-type CagA (Higashi et al., 2002a). However, the phenotype induced by HPK5 (ESS-type) was absent in our study, suggesting that this CagA interaction may be independent of tyrosine phosphorylation (Mimuro et al., 2002) and/or dependent on signal cascade by strain *per se*.

ATF3 was up-regulated at 48 h after infection with WSS-type strain (ATCC 43504) for the first time (25). However, ATF3, an early response gene, is rapidly induced as “stress response” by many extracellular signals and agents known to induce cell death or the JNK/SAPK signaling

pathway (Weir et al., 1994; Drysdale et la., 1996; Yu et al., 1996), which is involved in cellular growth, tissue remodeling, cytoskeletal reorganization and inflammation (Hai et al., 1999). We also demonstrated that ATF3 transcript peaked at late phase irrespective of CagA-type. It should be noted that ATF3 was induced in two peaks early and late phases only by *cagA*-disrupted mutants, indicating that both CagA-type proteins altered the induction of ATF3 in the infected cells. In contrast to previous reports (Higashi et al., 2002a; Tsutsumi et al., 2003; Naito et al., 2006), AGS infected with 26695 (WSS-type), but not HPK5 (ESS-type), showed the hummingbird in our study. The differences on the kinetics of ATF3 induction and/or strain-specific factors might contribute the morphological features via CagA-ATF3 signal cascade in the complicated *H. pylori*-related molecular pathways. These findings provide new insights to understand the extra molecular signaling pathway in CagA-dependent manner.

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Figure legends

Figure 1. *H. pylori*-induced IL-8 production. AGS cells were infected with *H. pylori* added at a MOI of 150. Culture supernatant was collected at indicated time-point and measured by ELISA.

A: *H. pylori* exposed to pH 6 B: *H. pylori* exposed to pH3-U- C: *H. pylori* exposed to pH3-U+

 :without infection  : 26695  : 26695CA (26695 *cagA*-disrupted mutant)

 : HPK5  :HPK5CA (HPK5 *cagA*-disrupted mutant)

Figure 2. Western blotting analysis of AGS cells infected with *H. pylori* strains 26695, HPK5, 26695CA (26695 *cagA*-disrupted mutant) and HPK5CA (HPK5 *cagA*-disrupted mutant).

A: *H. pylori* exposed to pH 6 B: *H. pylori* exposed to either pH3-U- or pH3-U+

Figure 3. RT-PCR for ATF3 mRNA. Total RNA was extracted form AGS cells infected with wild-type or these isogenic *cagA*-disrupted mutant strains. The amplicons (70-bp) of both ATF3 and ribosomal protein *L7a* were detected on 2% SDS-polyacrylamide gel.

Table 1. EPIYA motifs, tyrosine phosphorylation motifs (TPMs) and cAMP- dependent phosphorylation motifs (CPMs) in CagA amino acid sequences

Strain							
26695[a]	KNST**EPIYA**	VASPE**EPIYT**		SASP**EPIYA**TIDDLGGPFPLKRHD			
HPK5[a]	KNSA**EPIYA**	ATSPE**EPIYA**		SASP**EPIYA**TIDFDEANQAGFPLR			
location[c]	899	918		972			
	TPM at	CPM at	CPM at	TPM at	CPM at	CPM at	TPM at
location[c]	Y-122	K-223	K-454	Y-899	K-904	K-923	Y-1031
26695[b]	QKFGDQR<u>Y</u>	K<u>K</u>QS	K<u>K</u>DT	KNSTEPI<u>Y</u>	K<u>K</u>KT	KKVN	DKLKDSTKK
HPK5[b]	RKFGDQR<u>Y</u>	K<u>K</u>QS	KKDP	KNSAEPI<u>Y</u>	KKKA	K<u>K</u>VS	DELKDSTKK

[a] The EPIYA(T) motifs are boldface. The different amino acid is shaded.

[b] Active TPM = [RK]-X(2,3)-[D,E]-(2,3)-[Y]; active CPM = [RK](2)-X-[ST]. Y-899, K-904 and K-923 are in the variable region. Amino acid is designated by standard one-letter code. The active amino acid is underlined.

[c] The number denotes location to amino acid residue in 26695 sequence.

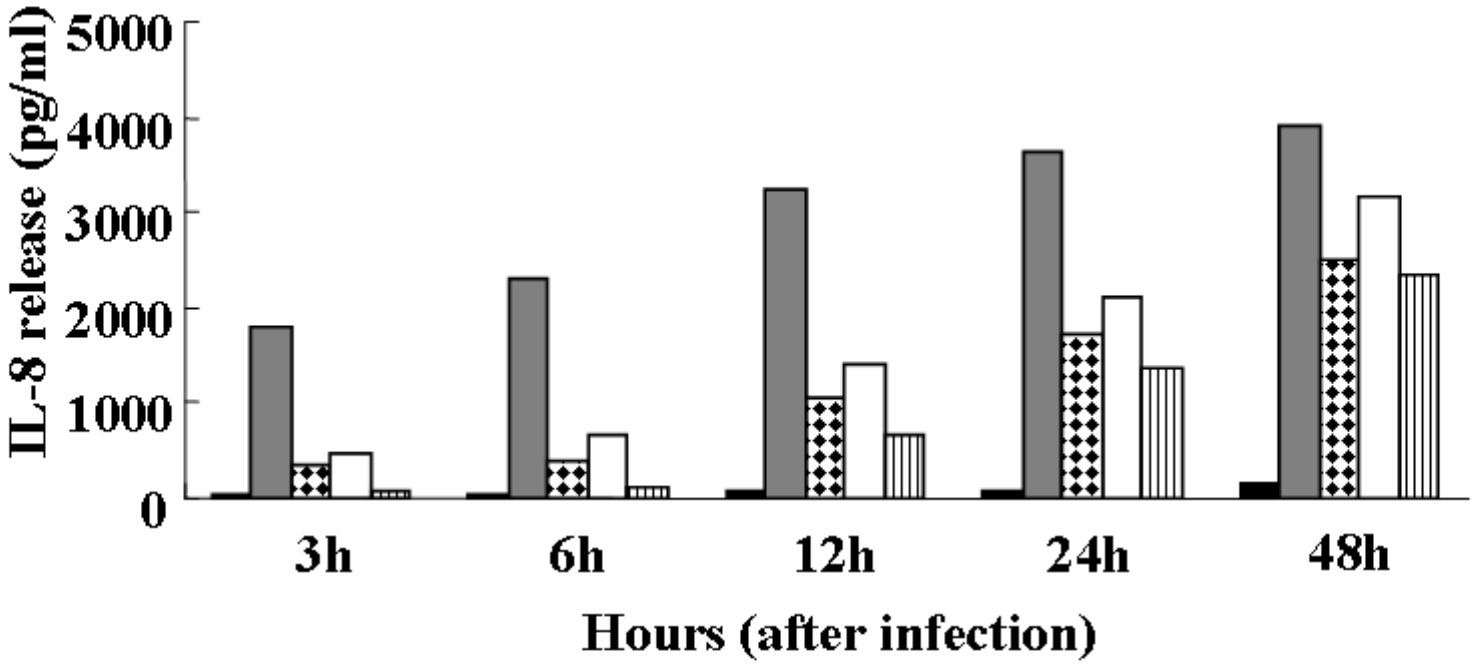
Table 2. The morphological changes (hummingbird phenotype) of AGS cells infected with 26695 exposed to different pH (3 and 6)

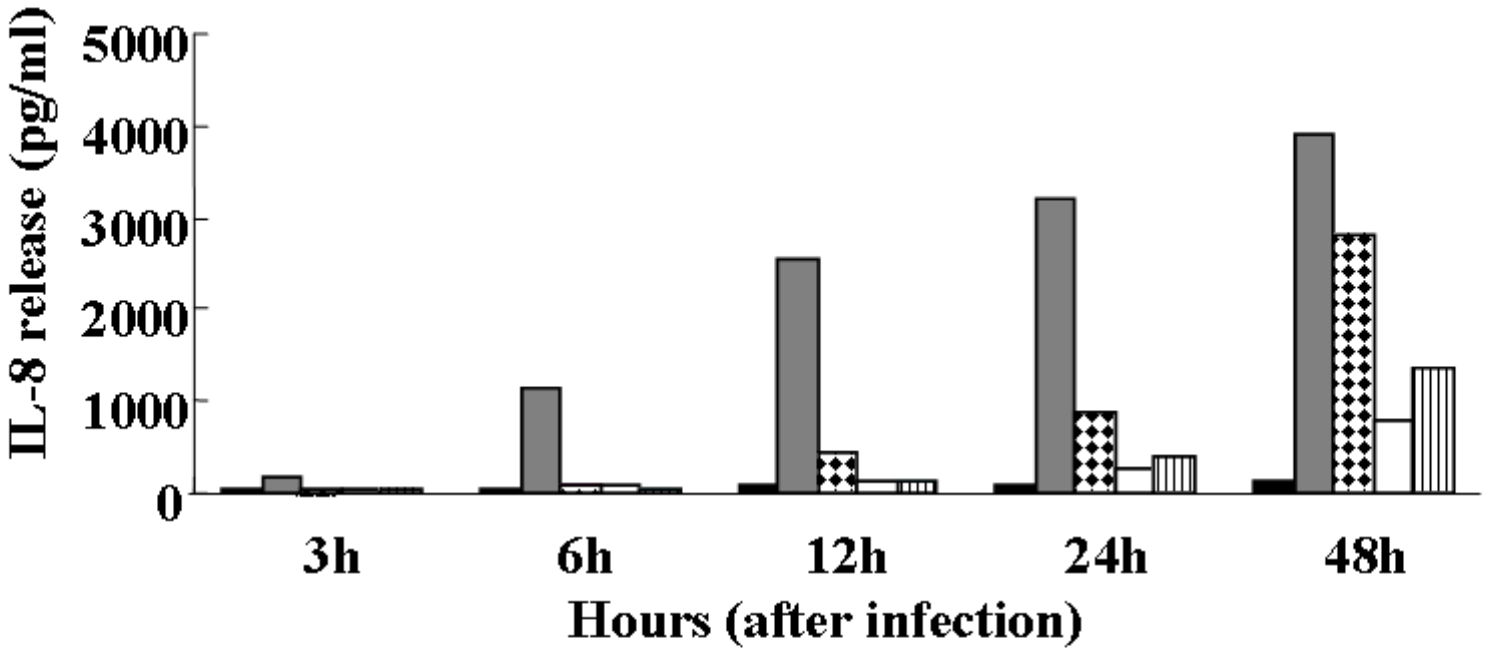
	3h	6h	12h	24h	48h
pH6	-[a]	±[b]	+[c]	+	+
pH3-U-	-	-	-	±	+
pH3-U+	-	-	±	+	+

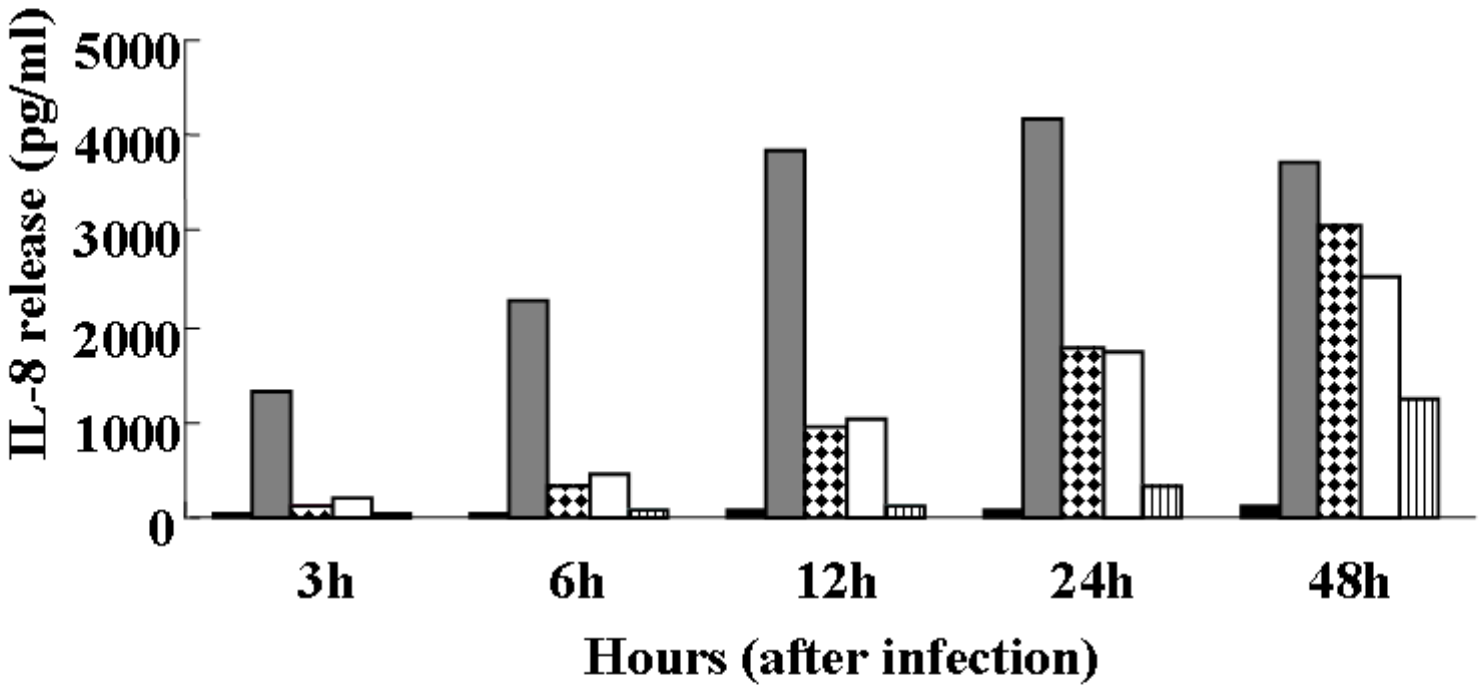
[a] -: the number of hummingbird phenotype is <1%

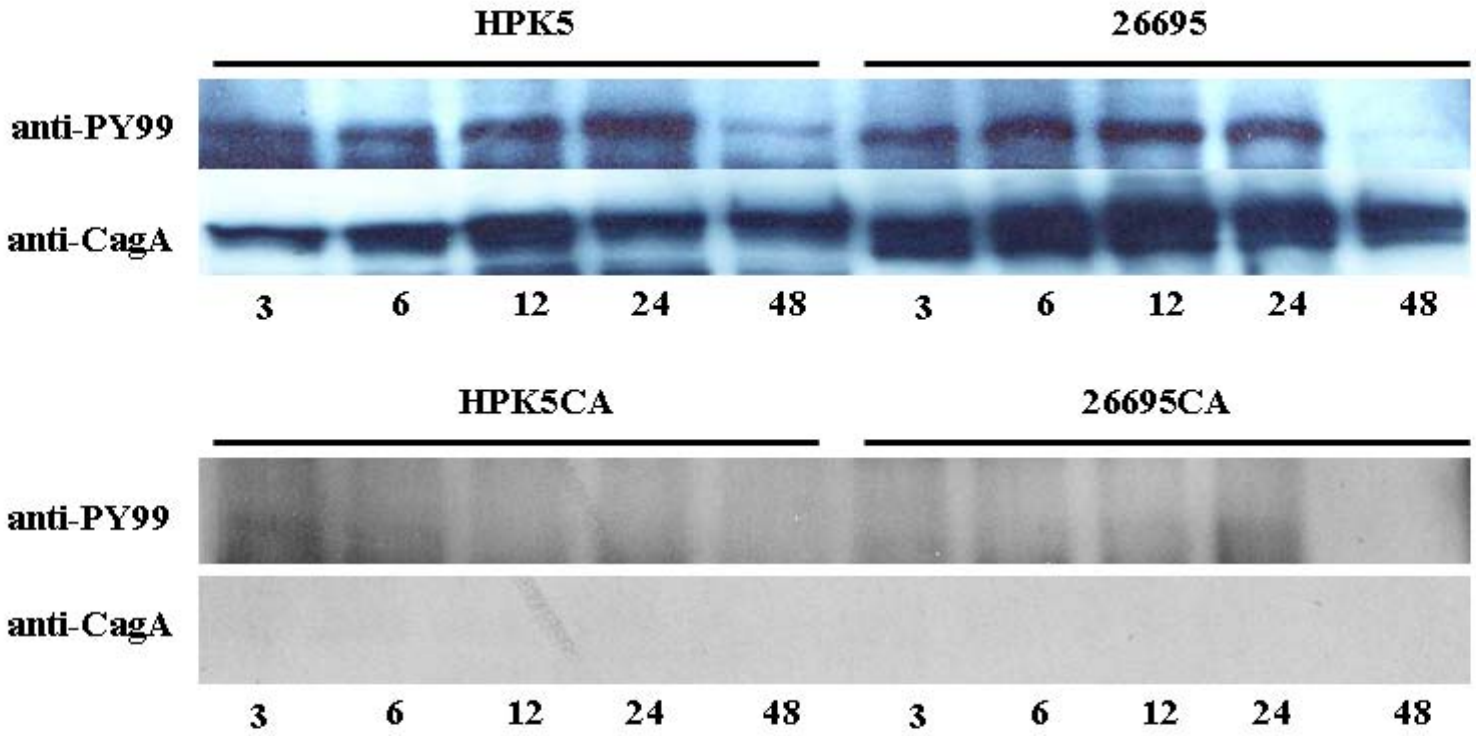
[b] ±: the number of hummingbird phenotype is 1~5%

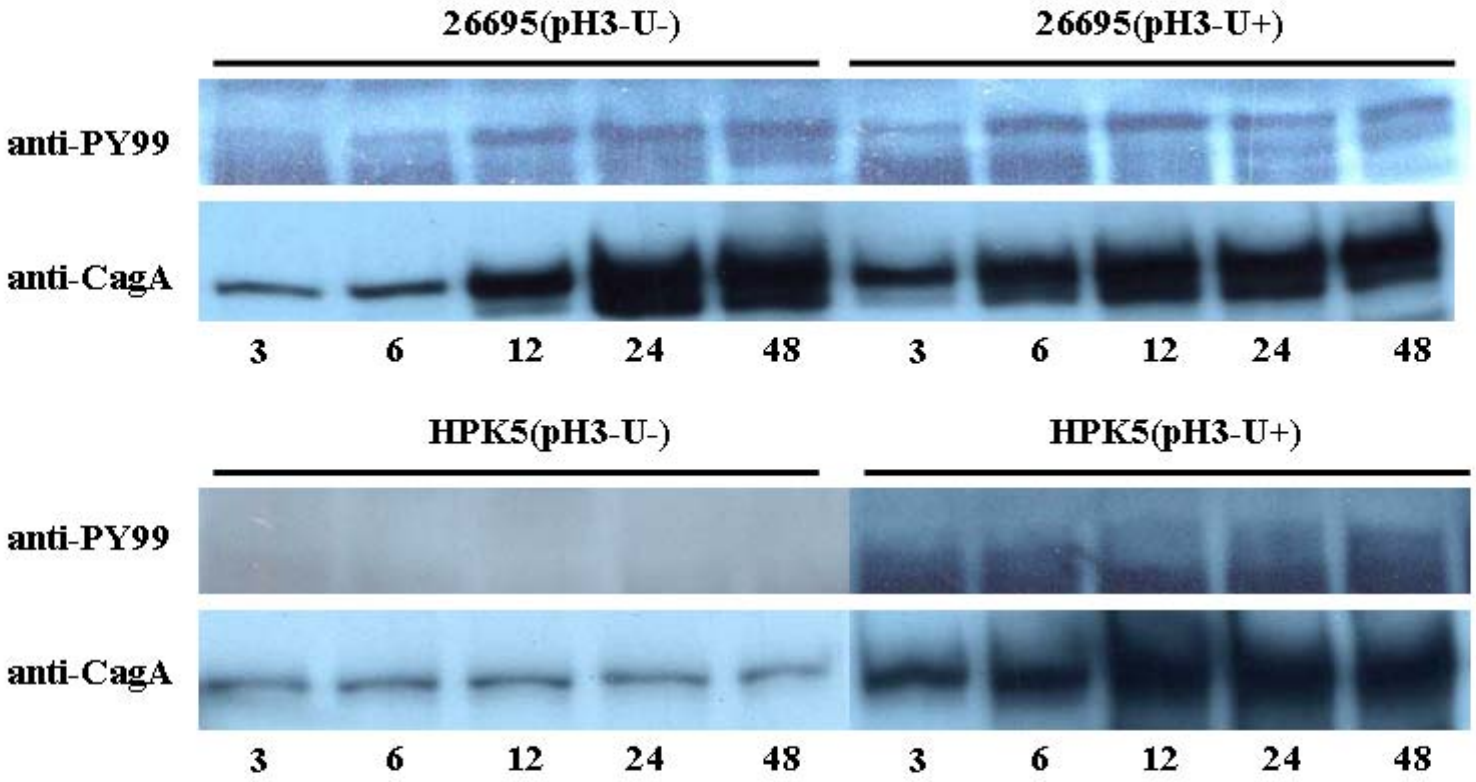
[c] +: the number of hummingbird phenotype is >5%

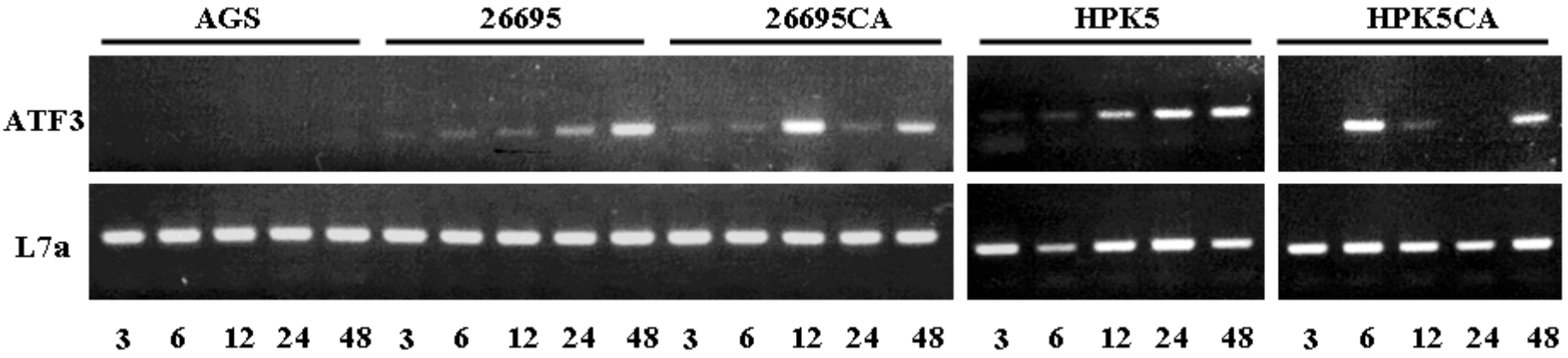

IL-8 release (pg/ml)
5000
4000
3000
2000
1000
0
3h
6h
12h
24h
48h
Hours (after infection)


IL-8 release (pg/ml)
5000
4000
3000
2000
1000
0
3h
6h
12h
24h
48h
Hours (after infection)


IL-8 release (pg/ml)
5000
4000
3000
2000
1000
0
3h
6h
12h
24h
48h
Hours (after infection)


HPK5
26695
anti-PY99
anti-CagA
3
6
12
24
48
3
6
12
24
48
HPK5CA
26695CA
anti-PY99
anti-CagA
3
6
12
24
48
3
6
12
24
48


26695(pH3-U-)
26695(pH3-U+)
anti-PY99
anti-CagA
3
6
12
24
48
3
6
12
24
48
HPK5(pH3-U-)
HPK5(pH3-U+)
anti-PY99
anti-CagA
3
6
12
24
48
3
6
12
24
48


AGS
26695
26695CA
HPK5
HPK5CA
ATF3
L7a
3 6 12 24 48 3 6 12 24 48 3 6 12 24 48 3 6 12 24 48 3 6 12 24 48