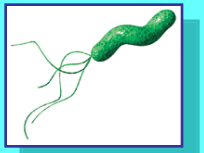

Induction of IL-8 production by *Helicobacter pylori* strains with different *cagA* genotype and *oipA* functional status.

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ABSTRACT

IL-8 is a potent neutrophil chemotactic and activating proinflammatory cytokine, thought to be related to the mucosal infiltration with neutrophils and mononuclear cells characteristic of the *Helicobacter pylori*-related gastritis, and suggested to have a role in *H.pylori*-associated gastroduodenal diseases. Its induction by *H.pylori* strains is increased if a functional *cag* pathogenicity island (PAI) is present in the *H.pylori* strains or if their outer inflammatory protein (*oipA*) gene is in the functional on-status. The *cagA* positivity, expression of the presence of the *cag* PAI, and the *oipA* on-status are characters usually considered to be well correlated with each other.

During a recent study on the *Helicobacter pylori* virulence genotypes circulating in Western Sicily, Italy, we isolated some strains in which the presence of *cagA* was associated with the presence of *babA2* and *vacA*s1 and *vacA*m1 alleles, but not with the *oipA* on-status, evaluated by DNA sequencing, on the basis of the number of the CT dinucleotide repeats in the 5' region of the gene.

Mainly to obtain evidence of the phenotypic functional status of their *oip*A genes, the Sicilian strains were cocultured with AGS gastric cancer cells and the IL-8 production was assayed by ELISA.

Results are discussed with respect to the hypothesis that mutations in the promoter region of the *oipA* gene can prevent the switch effect of the CT repeats on the expression of a functional open reading frame.

INTRODUCTION

The ability to stimulate interleukin-8 (IL-8) production in gastric cell cultures is largely considered by several Authors as indicative of, and responsible for, the *H.pylori* capacity to induce gastric inflammation characterized by mucosal infiltration with neutrophils and mononuclear cells [1].
This capacity is closely related to the progression of *H.pylori* infection "in vivo" and its detection can be useful for the evaluation of *H.pylori* virulence.
Among the microbial factors involved in IL-8 secretion, a main importance has been attributed to the genes of the *cag* pathogenicity island (PAI), whose presence, revealed by the cytotoxin-associated gene (*cagA*), is associated with a more severe clinical outcome, and whose encoded proteins enhance the bacterial virulence not only by altering protein tyrosine phosphorylation but also by increasing host cell cytokine production. A higher IL-8 secretion is usually induced by *cagA*$^+$ than by *cagA*$^-$ *H.pylori* strains, although, *in vivo*, some *cagA*$^-$ infections can be associated with severe gastric cellular infiltration and, *in vitro*, significant levels of IL-8 can be produced by some *cagA*$^-$ strains in cell lines as MKN45, AGS and KATO III [1].
Because of the last observations, the presence has been suggested of virulence factors other than *cag* PAI involved in IL-8 production and, because *in vitro* experiments indicate that IL-8 is produced from the epithelial cells only after viable *H.pylori* are attached to the cells, the possibility was evaluated that some bacterial outer membrane proteins (OMPs) are also responsible for a pro-inflammatory activity. In fact, a *H.pylori* OMP, specifically the OipA (outer inflammatory protein-A), has been proposed to be an important virulence factor associated with enhanced IL-8 secretion and increased inflammation both *in vitro* and *in vivo* [1] [2] [3].
The functional status of OipA is regulated by the slipped-strand repair mechanism based on the number of the CT dinucleotide repeats in the 5' region of the *oipA* gene (switch on = functional and switch off = non-functional) and, currently, the presence of a functional OipA protein can be predicted by the PCR-based sequencing of the signal region of the gene. Isolates that contain the *cag* PAI typically also have *oipA* with functional status "on" [1] [2] [4].
As the strong correlation of *oipA* frame status with *cagA* status can make difficult to establish the relationship of *oipA* status to pathogenicity, interesting observations could be obtained by assays performed on the rare isolates not exhibiting such type of relationship.
Here results are shown of IL-8 titration tests carried out on AGS gastric cancer cell line supernatants after infection with 32 *H.pylori* clinical strains recently isolated in Palermo, Sicily, in a study in which it was observed that the presence of cagA was not always associated with OipA on-status.

MATERIALS AND METHODS

H.pylori strains

Thirtytwo *H.pylori* strains were examined, originally isolated from biopsy samples of 30 patients who had undergone endoscopy for gastric symptoms (13 with inactive chronic gastritis CG, 7 with active chronic gastritis CGA, 9 with active chronic gastritis and gastric or duodenal peptic ulcer CGA+PU and only 1 with gastric cancer GC).
The isolated strains, stored to -80°C, were seeded on Columbia agar medium (Oxoid, Basingstoke, Hampshire, England) added with 7% horse blood and 0.4 % of selective supplement. The plates were incubated at 37°C under microaerobic conditions (CampyGen Oxoid) for 3 days.
H.pylori bacteria had been previously identified on the basis of characteristic colony morphology, appearance on Gram staining, rapid urea hydrolysis, positive catalase, oxidase production and the API Campy Kit (bioMérieux) [5].

Extraction of bacterial DNA

Genomic DNA was extracted from bacterial suspensions in sterile distilled water (200 µl) boiled for 10 min, and centrifuged for 5 min at 14,000 x g. Aliquots of 50 ng of the genomic DNA were used for PCRs.

Genotyping of *H.pylori*

DNA from each strain was used for amplify: *ureaseA*, *cagA*, *vacA*, *babA2* and *oipA*, using the specific primers [6], [7], [8], [9].
PCR was carried out in a 100 µl mixture containing two to four µl of each DNA solution, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM MgCl2, 200 µM dNTPs, 200 nM of each primer, and 2.5 U of Ampli Taq Gold polymerase (Applied Biosystems). The amplification was performed in a Perkin-Elmer ThermoCycler 2400 under the following conditions: 10 min at 95°C, followed by 40 cycles at 94°C for 45 sec, 56°C for 45 sec, and 72°C for 45 sec, and a final extension at 72°C for 5 min. PCR products were analyzed after electrophoresis on a 2% agarose gel.
To determine the expression status of *oipA* gene, a PCR-sequencing-based methodology was applied to detect the correct number of CT-dinucleotide repeats [4] [8].

Cell colture and *H.pylori* coinfection

AGS human gastric epithelia cells were grown into 24 well-plates ($1x10^5$ cells/ml) in Ham's F12 mediun supplemented with 10% fetal bovine serum (FBS), at 37°C in a 5% CO_2 atmosphere for 48h.
Next, the medium was aspirated from wells, serum-free F12 medium was added, and the plates were incubated for an additional 24h.
At the same time *H.pylori* isolates were grown in Columbia agar medium for 48h and reseeded on new plates for more 24h.
For IL-8 stimulation, AGS cells were cocultured with *H.pylori* suspensions at 0.9 MacFarland torbidity (Densimat BioMerieux).
After cocoltivation for 6h and 24h, the cell culture supernatant was collected, centrifuged for 5 min at 14,000 x g to remove particulates and stored at -80°C until IL-8 production assay [10], [11].

IL-8 assay

The amount of IL-8 in each sample was measured by an enzyme-linked immunosorbent assay using the Quantikine-Human CXCL8/IL-8 kit (R&D Systems) following the manufacturer's instructions.

Tab.1 Relationship between gastric disease and *H.pylori cagA vacA* and *babA2* genotypes in 30 Sicilian patients included in the study.

Gastric disease*	No of patients	*H. pylori* Genotypes
CG	13	8 *vacAs2m2cagA-* 2 *vacAs1m2cagA-* 1 *vacAs1m2cagA+* 2 *vacAs1m1cagA+*
CGA	7	5 *vacAs1m1cagA+* 1 *vacAs1m1cagA-* 1 *vacAs1m2cagA+*
GCA+PU	9	5 *vacAs1m1cagA+* 2 *vacAs1m2cagA+* 1 *vacAs2m2cagA-* (antrum) } ** 1 *vacAs1m2cagA+* (corpus) } ** 1 *vacAs1s2m1m2cagA+*
GC	1	1 *vacAs1m2cagA+*

* CG = inactive chronic gastritis
CGA = active chronic gastritis
CGA+PU = active chronic gastritis and gastric or duodenal peptic ulcer
GC = gastric cancer

** two different strains from a singol patients

Tab.2: Variations in the signal-sequence coding region of the *oipA* gene and *cagA* status in 32 *H.pylori* strains from Sicily, Italy

Strain reference	Sequence of the signal peptide coding region		No. of CT repeats	*oipA* status	*cagA* status
D13	ATGAAAAAAGCCCTCTTACTAACTCTCTCTCTCT.....	CGTT	6	on	-
8	ATGAAAAAAGCTCTCTTACTAACTCTCTCTCTCT.....	CGTT	6	on	-
D40	ATGAAAAAAGCTCTCTTACTAACTCTCTCTCTCT.....	CGTT	6	on	-
3DA	ATGAAAAAAACTCTTTTA.....CTCTCTCTCTCT.....	CGTT [a]	6	off	-
V28C	ATGAAAAAAACTCTTTTA.....CTCTCTCTCTCTCTCTCT.	CGTT [a]	9	off	-
V28A	ATGAAAAAAACTCTTTTA.....CTCTCTCTCTCTCT.....	CGTT [a]	7	off	-
V17	ATGAAAAAAGCCCTCTTA.....CTCTCTCTCTTTCTCT.....	CGTT [b]	5+2	on	-
V08	ATGAAAAAAGCTCTTTTA.....CTCTCTCTCTCTCTCT.....	CGTT [c]	8	on	-
D56	ATGAAAAAAACTCTTTTA.....CTCTCTCTCTCTCTCT.....	CGTT [c]	8	on	-
401	ATGAAAAAAGCTCTCTCTCTAACTCTCTCTCT.....	CGTT	5	off	-
E01	ATGAAAAAAACTCTCTTACTAACTCTCTCTCTCTCT.....	CGTT	8	off	-
5	ATGAAAAAAGCTCTCTTA.....CTCTCTCTCTCTCTCT.....	CGTT [c]	8	on	+
D41	ATGAAAAAAGCTCTCTTACTAACTCTCTCTCTCT.....	CGTT	6	on	+
202	ATGAAAAAAGCTCTCTTACTAACTCTCTCTCTCT.....	CGTT	6	on	+
D14	ATGAAAAAAGCCCTCTTACTAACTCTCTCTCTCT.....	CGTT	6	on	+
D44	ATGAAAAAAGCTCTCTTACTAACTCTCTCTCTCT.....	CGTT	6	on	+
D51	ATGAAAAAAGCCCTCTTACTAACTCTCTCTCTCT.....	CGCT	6	on	+
201	ATGAAAAAAGCTCTCTTACTAACTCTCTCTCTCTCTCTCT.	CGTT	9	on	+
V18	ATGAAAAAAGCTCTCTTA.....CTCTCTCTCTCTCTCT.....	CGTT [c]	8	on	+
102	ATGAAAAAAGCTCTTTTA.....CTCTCTCTCTTTCTCT.....	CGTT [b]	5+2	on	+
V03	ATGAAAAAAGCCCTCTTA.....CTCTCTCTCTTTCTCT.....	CGTT [b]	5+2	on	-
V14A	ATGAAAAAAGCCCTCTTACTAACTCTCTCTCT.....	TGTT	5	off	-
V14C	ATGAAAAAAGCCCTCTTACTAACTCTCTCTCTCT.....	CGTT	6	on	+
D55	ATGAAAAAAACTCTTTTA.....CTCTCTCTCTCT.....	CGTT [a]	6	off	+
D30	ATGAAAAAAGCTCTCTTACTAACTCTCTTTTTCT	CGTT [d]	3+1	on	+
D39	ATGAAAAAAGTTCTATTA.....CTCTCTCTCTCTCTCT.....	CGTT [c]	8	on	+
203	ATGAAAAAAGCCCTCTTA.....CTCTCTCTCTTTCTCT.....	CGTT [b]	5+2	on	+
206	ATGAAAAAAGCCCTCTTA.....CTCTCTCTCTCTCTCT	CGTT [c]	8	on	+
303	ATGAAAAAAGTTCTATTA.....CTCTCTCTCTCTCTCT.....	CGTT [c]	8	on	+
105	ATGAAAAAAGCTCTTTTA.....CTCTCTCTCTTTCTCT.....	CGTT [b]	5+2	on	+
103	ATGAAAAAAGTTCTATTA.....CTCTCTCTCTCTCT.....	CGTT [a]	7	off	+
K	ATGAAAAAAGCCCTCTTACTAACTCTCTCTCTCT.....	CGTT	6	on	+

[a] = ORF with six, seven or nine repeats is out of frame due to deletion of CTAA sequence

[b] = ORF with seven CT repeats and one TT insertion is in frame due to deletion of CTAA sequence

[c] = ORF with eight CT repeats is in frame due to deletion of CTAA sequence

[d] = ORF with four CT repeats and two TT insertion without deletion of CTAA sequence is in frame

Analises based on R. de Jonge [1] and T. Ando [2]

Tab.3: IL-8 stimulation by 32 *H.pylori* strains characterized by different genotypes and *oipA* status

No. of patient	Strain reference	Genotypes	*oipA* status	IL-8 assay	
				6h pg/ml	24h pg/ml
1	D13	*vacAs2m2babA2+cagA-*	on	114,9	369,1
2	8	*vacAs2m2babA2-cagA-*	on	60,6	140,6
3	D40	*vacAs1m2babA2+cagA-*	on	37,8	121,7
4	3DA	*vacAs2m2babA2-cagA-*	off	107,6	247,4
5	V28 C	*vacAs2m2babA2-cagA-*	off	120,1	344,8
	V28 A	*vacAs2m2babA2-cagA-*	off	35,7	121,5
6	V17	*vacAs2m2babA2-cagA-*	on	ND	80,8
7	V08	*vacAs2m2babA2-cagA-*	on	ND	107,8
8	D56	*vacAs2m2babA2-cagA-*	on	33,7	374,8
9	401	*vacAs1m2babA2-cagA-*	off	41,9	386,1
10	E01	*vacAs2m2babA2-cagA-*	off	ND	549,0
11	5	*vacAs1m1babA2+cagA+*	on	1158,2	1388,5
12	D41	*vacAs1m1babA2+cagA+*	on	523,7	1164,5
13	202	*vacAs1m2babA-cagA+*	on	742,9	1115,7
14	D14	*vacAs1m1babA2+cagA+*	on	1011,0	1867,9
15	D44	*vacAs1m1babA2+cagA+*	on	597,6	2326,1
16	D51	*vacAs1m1babA2+cagA+*	on	360,7	951,4
17	201	*vacAs1m2babA2+cagA+*	on	1566,2	2543,1
18	V18	*vacAs1m1babA2-cagA+*	on	325,8	545,6
19	102	*vacAs1m1babA2+cagA+*	on	476,8	788,6
20	V03	*vacAs1m1babA+cagA-*	on	108,9	343,2
21	V14 A	*vacAs2m2babA2-cagA-*	off	88,4	279,5
	V14 C	*vacAs1m2babA2+cagA+*	on	789,5	1540,4
22	D55	*vacAs1m1babA2+cagA+*	off	1020,9	1427,8
23	D30	*vacAs1m1babA2+cagA+*	on	256,2	308,7
24	D39	*vacAs1m1babA2+cagA+*	on	ND	2115,6
25	203	*vacAs1m2babA2+cagA+*	on	3832,3	1787,1
26	206	*vacAs1m2babA2+cagA+*	on	442,7	593,5
27	303	*vacAs1m1babA2+cagA+*	on	1451,1	1237,3
28	105	*vacAs1m1babA2+cagA+*	on	603,1	1506,4
29	103	*vacAs1s2m1m2babA2-cagA+*	off	970,3	2004,8
30	K	*vacAs1m2babA2-cagA+*	on	1028,0	885,9

ND= not determined

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