A Role for the Vacuolating Cytotoxin, VacA, in Colonization and Helicobacter pylori–Induced Metaplasia in the Stomach

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Carriage of Helicobacter pylori strains producing more active (s1/i1) forms of VacA is strongly associated with gastric adenocarcinoma. To our knowledge, we are the first to determine effects of different polymorphic forms of VacA on inflammation and metaplasia in the mouse stomach. Bacteria producing the less active s2/i2 form of VacA colonized mice more efficiently than mutants null for VacA or producing more active forms of it, providing the first evidence of a positive role for the minimally active s2/i2 toxin. Strains producing more active toxin forms induced more severe and extensive metaplasia and inflammation in the mouse stomach than strains producing weakly active (s2/i2) toxin. We also examined the association in humans, controlling for cagPAI status. In human gastric biopsy specimens, the vacA i1 allele was strongly associated with precancerous intestinal metaplasia, with almost complete absence of intestinal metaplasia in subjects infected with i2-type strains, even in a vacA s1, cagA+ background.

Keywords. gastric cancer; pathogenesis; SPEM; Helicobacter pylori; virulence; colonization.

Helicobacter pylori is a gram-negative microaerophilic bacterium that infects about half the world’s population and persists lifelong if untreated. Colonization of the human gastric mucosa with H. pylori stimulates a chronic inflammatory response with an associated lifetime risk of 10%–20% for peptic ulceration [1] and approximately 1% for gastric adenocarcinoma [2]. The risk of disease in an infected individual is determined by a combination of host factors, including genetics polymorphisms [3], environmental factors such as smoking [4], and, critically, the virulence of the infecting strain. H. pylori produces multiple virulence factors, including cytotoxin-associated gene A (CagA) and the vacuolating cytotoxin (VacA), each of which is associated with a marked increase in the risk of disease development (reviewed by Kusters et al [5]).

VacA, a pore-forming toxin, was named for the vacuoles it induces in gastric epithelial cells [6] but has many other effects, including apoptosis induction in gastric epithelial cells [7] and inhibition of proliferation and IL-2 secretion by T cells [8]. CagA is translocated into gastric epithelial cells by a type IV secretion apparatus encoded within the cag pathogenicity island (cagPAI), where it induces morphological changes and proinflammatory cytokine secretion (reviewed by Noto and Peek [9]).

The cagPAI may be present or absent in a given H. pylori strain. The vacA gene is present in all strains but is polymorphic, having 2 alternative allelic variants at its signal (s1/s2), intermediate (i1/i2), and middle (m1/m2) regions [10, 11]. The s2 forms of VacA form pores slowly and inefficiently and induce minimal vacuolation, if any [12]. The s1 forms are more active, especially if they also have the i1 intermediate region [11]. The midregion type affects binding to some cell lines [10, 13]. Association studies in humans examining the importance of VacA in disease are hindered by a linkage...
between cagA and vacA genotypes [14]; most vacA s1 strains isolated are also cagA+, and most vacA s2 strains are cagA−. We reported elsewhere that the vacA i1 allele is the best independent predictor of gastric adenocarcinoma risk in humans, based on a multivariate analysis including vacA s, i, and m types and cagA [11]. Associations of i1-type vacA with peptic ulceration, gastric atrophy, and carcinoma have since been confirmed by us and others [15–17]. To date, however, there is no direct experimental evidence that these associations are causal, in part because animal models for studying pathogenesis are poor. Few H. pylori strains will infect mice, and the widely used SS1 strain [18] lacks cagPAI functionality [19, 20] and expresses nontoxigenic s2/i2 VacA. An alternative cagPAI+ derivative (PMSS1) [21] is also VacA s2/i2. Although many H. pylori strains produce VacA and CagA in humans, both are absent from other Helicobacter species naturally infecting animals (eg, Helicobacter felis). VacA and CagA have profound effects on mouse gastric epithelial cells, but murine T cells are resistant to VacA-mediated inhibition [22].

Despite these limitations, several studies have examined the role of s2/i2 VacA during infection in animal models. Early studies reported little or no difference in colonization or disease between wild-type and vacA null strains [23, 24], but Salama et al [25] showed that carriage of vacA conferred a competitive advantage over vacA null mutants and that presence of vacA facilitated colonization of mice with lower inoculating doses. More recently, Oertli et al [26] reported that vacA null mutants colonized mice at reduced levels while inducing stronger T-helper 1 and T-helper 17 responses. In nature, vacA null H. pylori strains are very rarely found—the gene is maintained in bacterial populations—but it is unclear why the nonvacuolating s2/i2 form is maintained so widely or what role it might play during infection.

We aimed to characterize the role of different vacA polymorphic forms in H. pylori pathogenesis. Uniquely, we determined effects of VacA polymorphisms on colonization, inflammation, and precancerous changes in mice. We also showed that the vacA i1 allele is associated with an increased risk of intestinal metaplasia in the gastric mucosa of infected human patients.

**MATERIALS AND METHODS**

**H. pylori Strains**

The strains used in this study were SS1s1i1, SS1s1i2, SS1s2i2, and SS1null. All strains were isogenic and constructed on the SS1 background, with vacA genotypes as indicated. All strains were minimally passaged. Mutants were generated by natural transformation of wild-type SS1 with plasmid pJR100 [11], containing the s1/i1-encoding region of vacA from strain 60190, to make SS1s1i1 or with plasmid pCTB2cat [10] to make SS1s1i2 and SS1s2i2. After allelic exchange by homologous recombination and chloramphenicol resistance marker rescue, the vacA allele in each transformant was sequenced to confirm its genotype. The SS1s2i2 strain expresses the same VacA type as wild-type SS1, in similar quantities (Figure 1) but also contains the chloramphenicol resistance cassette present in the other mutants. SS1null was produced by inserting a kanamycin resistance cassette into the vacA coding sequence using plasmid pCTB8::Km [27]. Because strains expressing s2/i1 VacA are almost never found in human infections, an SS1s2i1 strain was not constructed for this study.

**Vacuolation Assay**

RK13 cells (American Type Culture Collection CCL-37) were maintained at 37°C and 5% carbon dioxide in Ham F12 medium supplemented with 2 mmol/L L-glutamine and 10% fetal bovine serum (Sigma-Aldrich). Bacterial water extracts were prepared as described elsewhere [10] and applied at 75 µg/mL to 1 × 10⁶ cells per well in 96-well plates in the presence of 10 mmol/L ammonium chloride. After 4 hours, the extent of vacuolation was quantified with light microscopy and expressed as the mean proportion of vacuolated cells per field (≥100 cells assessed per experimental condition, 4 fields per condition, and duplicate independent experiments).

**Western Blotting**

The protein concentration of each bacterial water extract was determined by means of bicinechonic acid assay (Thermo Scientific) and adjusted to 750 µg/mL. Next, 7.5 µg of total protein per lane was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the relative VacA content of each extract was determined by Western blotting. All samples were tested on the same gel and blot. VacA was labeled by using a 1:1 mixture of rabbit polyclonal serum raised in house against VacA p33 and p55 subunits, diluted 1:10 000, and then horseradish peroxidase–conjugated goat anti-rabbit secondary antibody (Sigma), also at 1:10 000 dilution. Equal protein loading in each lane was confirmed by Coomassie staining of duplicate gels.

**Mouse Infection Study Design**

All animal procedures were carried out under Home Office project license 40/3399, according to UK Home Office and institutional guidelines and with prior approval from the University of Nottingham Animal Welfare and Ethical Review Body. Groups of 6- to 8-week-old female C57BL/6J mice (Charles River UK) were housed in individually ventilated cages and allowed water and food ad libitum. Mice were infected with doses of 10⁸ bacteria in 100 µL of Brucella broth (Difco Laboratories) by oral gavage on 3 alternate days. The viability of each inoculum was confirmed by quantitative culture after dosing [28], and no significant differences in viability were seen between the strains tested.

**Quantitative Culture From Mouse Stomachs and Histology**

After 1, 3, or 6 weeks, mice were euthanized. Stomachs were carefully halved longitudinally along the greater and lesser
curvatures and rinsed in sterile phosphate-buffered saline. Half of each stomach was manually disrupted on ice in 750 µL of Iso-
Sensitest (Oxoid, Basingstoke, UK) broth/15% glycerol, serially
diluted, and plated on Galaxo Selective Supplement A plates
(blood agar base 2 (Oxoid) plates supplemented with 10% defi-
brinated horse blood, 10 µg/mL vancomycin, 20 µg/mL bacitra-
cin, 4 µg/mL amphotericin B, 2.5 IU/mL polymyxin B sulfate
and 1.07 µg/mL nalidixic acid). The other half of the stomach
was formalin fixed, paraffin embedded and sectioned, then
stained with hematoxylin-eosin or periodic acid–Schiff/Alcian
blue. The hematoxylin-eosin–stained sections were scored for
severity of inflammation and spasmolytic polypeptide-express-
ing metaplasia (SPEM) by the same qualified histopathologist
(P. V. K.) in a blinded manner. Sections were assigned scores
of 0 (absent), 1 (mild), 2 (moderate), or 3 (severe). Because in-
flammation and SPEM were patchy in the mouse gastric muco-
sa after 1–6 weeks of infection, the length of each was measured
and normalized to the total length of corpus examined, to quan-
tify the extent of inflammation and SPEM for each mouse.

Human Studies
We studied patients attending the Queen’s Medical Centre, Not-
tington, United Kingdom, between 2002 and 2012 for routine
upper gastrointestinal endoscopy, usually for investigation of
dyspepsia. Written informed consent was obtained from all pa-
tients, with approval of the Nottingham Research Ethics Com-
mittee 2 (08/H0408/195). Gastric biopsy specimens were
collected into Iso-Sensitest broth/15% glycerol for culture and

Figure 1. Expression levels and activities of VacA from SS1 mutant series. A, Water extracts (7.5 µg total protein per lane) were Western blotted for VacA
to confirm the presence or absence of toxin expression and relative quantities of toxin production. WT, wild type. B, RK13 cells were incubated for 4 hours in
the presence of 75 µg/mL of the water extracts shown in A. The extent of vacuolation was quantified with light microscopy; vacuolating activity is presented
as the mean (± standard deviation) proportion of vacuolated cells per field of view from ≥4 fields. C, Representative microscopic images of vacuolation.
further biopsy specimens were formalin fixed for histological examination. We examined an unselected group of patients for whom histological data were available and _H. pylori_ successfully cultured. Samples from patients who had taken nonsteroidal anti-inflammatory drugs or antibiotics in the 2 weeks before endoscopy were excluded from the study. The population was 52% male with an age range of 17–82 years (median, 55 years).

A qualified histopathologist (A. A. M. Z.), who was blinded to other data, used the updated Sydney scoring system [29] to score colonization density, inflammation, activity, atrophy, and intestinal metaplasia in biopsy sections. The _H. pylori_ strains cultured from biopsy specimens were typed for _vacA_ by polymerase chain reaction (PCR), according to established methods [11, 30, 31]. PCR typing for presence or absence of the _cagA_ gene was carried out using primers A22F (5'-TTCATGGGGCTTGGTATG) and A23R (5'-ATAATCTTTGAGAGTTAGCTC) [32] alongside reference strains 60190 ( _cagA_ +) and Tx30a ( _cagA_ -). Specimens that were negative for _cagA_ at PCR were confirmed serologically with use of a CagA immunoglobulin G enzyme-linked immunosorbent assay kit (Genesis Diagnostics).

Statistical Analysis
GraphPad Prism software (version 5; GraphPad) was used for all statistical analyses. The tests used are indicated in the figure legends.

RESULTS

Production and Characterization of Isogenic _vacA_ Mutants in the SS1 Strain Background
We engineered wild-type SS1 (SS1WT, naturally _vacA_ s2/i2/m2 and unable to translocate CagA) [18, 33–35] to produce s1/i1 (SS1s1i1) or s1/i2 (SS1s1i2) VacA, using a chloramphenicol resistance cassette to recover transformants. To control for the possibility that this engineering adversely affected the strain, we also constructed a strain producing wild-type s2/i2 VacA using the same cassette (SS1s2i2). Finally, we disrupted the _vacA_ gene in SS1WT to produce SS1null mutants.

SS1WT, SS1s1i1, SS1s1i2, and SS1s2i2 had similar growth rates and adherence to AGS and RK13 cells (data not shown) and expressed similar quantities of VacA (Figure 1A). As expected, water extracts prepared from SS1s1i1 caused extensive vacuolation of RK13 cells, whereas those from SS1s1i2, SS1s2i2, and SS1null caused minimal or no vacuolation (Figure 1B and 1C).

Promotion of Gastric Colonization in Mice by Nontoxigenic s2/i2 VacA
Consistent with the findings of Salama et al [25] and Oertli et al [26], in our hands SS1null was impaired for colonization in 5 independent experiments. SS1null mutant 1 was recovered from only 1 of 19 mice 3 weeks after infection, and mutant 2 was recovered from 3 of 7 (combined data from 26 mice inoculated with SS1null are shown in Figure 2). SS1s2i2 colonized mice readily (93% of mice successfully infected), with gastric colonization densities similar to those we usually detect for SS1WT. Given that the null mutants had reduced colonization rates, indicating a role for VacA, we were surprised to find that the SS1s1i1 and SS1s1i2 strains were also impaired for colonization (Figure 2). Expression of type s1/i1 or s1/i2 VacA significantly reduced both the proportion of successful infections (24% for SS1s1i1...
and 15% for SS1\textsuperscript{s1i2}) and gastric colonization densities in successfully infected mice, compared with expression of s2/i2-type VacA (7.4-fold for SS1\textsuperscript{s1i1} \([P < .01]\) and 7.3-fold for SS1\textsuperscript{s1i2} \([P = .08]\)). This indicates that expression of nontoxigenic s2/i2 VacA confers an advantage for \textit{H. pylori} over s1/i2 or s1/i1 VacA.

Induction of Inflammation and SPEM by Expression of s1/i1-Type vs s2/i2-Type VacA in the SS1 Mouse Model

Gastric tissue sections from inoculated mice were stained with hematoxylin-eosin and scored for intensity of inflammation and SPEM. In the gastric mucosa after 3 weeks of infection, areas of inflammation and SPEM were visible and in some cases extensive (Figure 3) but were interspersed with patches of normal-appearing mucosa. Consistent with previous reports [36], SPEM in infected mice was characterized by the loss of specialized gland cells and expansion of metaplastic cells with abundant, "foamy" mucin-filled cytoplasm that stained purple/blue with periodic acid–Schiff/Alcian blue (Figure 3).

There were no correlations between colonization density and severity or extent of inflammation or SPEM. However, 3 weeks after inoculation, SPEM was significantly more extensive in mice given SS1\textsuperscript{s1i1} than in those given SS1\textsuperscript{s2i2} \((P < .05)\), and throughout the study there were trends to more severe and extensive gastric inflammation and SPEM in mice given SS1\textsuperscript{s1i1} than in those given SS1\textsuperscript{s1i2} or SS1\textsuperscript{s2i2} (Figure 4), findings further confirmed in the time course studies below.

To determine whether inflammation and SPEM would rapidly resolve after an acute phase immediately after inoculation, or progress over time, we conducted time course infection studies for SS1\textsuperscript{s1i1} and SS1\textsuperscript{s2i2}, with histological analysis 1, 3, and 6 weeks after inoculation. Inflammation and SPEM became increasingly extensive over time, and the proportion of mice in each group with moderate to severe inflammation and SPEM also increased (Figure 5). Although this experiment was to determine time courses of individual mutants, experiments were performed concurrently, so further comparisons between mutants were possible. Mice given SS1\textsuperscript{s1i1} had more severe \((P = .07)\) and extensive \((P = .08)\) SPEM than those given SS1\textsuperscript{s2i2} at 1 week after dosing, but inflammation was minimal in both groups at this early time point. SS1\textsuperscript{s1i1} and SS1\textsuperscript{s2i2} induced similar levels of inflammation and SPEM after 3 and 6 weeks, even though SS1\textsuperscript{s1i1} colonized only 1 mouse to detectable levels (SS1\textsuperscript{s2i2} colonized all mice at high levels); thus, the amount of inflammation and SPEM per viable bacterium was much higher in the SS1\textsuperscript{s1i1}-infected animals.

Figure 3. Infection with SS1\textsuperscript{s1i1} induces spasmolytic polypeptide-expressing metaplasia (SPEM) and inflammation in mouse gastric mucosa. A, Hematoxylin-eosin (HE)–stained gastric sections from a mouse 3 weeks after dosing with SS1\textsuperscript{s1i1} in Brucella broth showing inflammatory infiltrate (asterisk), loss of specialized gland cells, and marked expansion of cells with foamy cytoplasm indicative of mucinous metaplasia (arrows). B, Periodic acid–Schiff (PAS)/Alcian blue staining on gastric sections from SS1\textsuperscript{s1i1}, dosed mice after 3 weeks of infection confirmed the replacement of specialized gland cells with mucin-containing cells (blue-staining acid mucin and purple-staining neutral mucin), consistent with SPEM [36]. C, Sections from a mouse dosed with Brucella broth only. D, SPEM was absent 3 weeks after dosing with Brucella broth. Panels A and B are representative of SPEM and inflammation scores of 3 (severe); panels C and D representative of inflammation scores of 0 (absent). Bar represents 200 µm for all panels.

vacA i Region Type and Risk of Intestinal Metaplasia in Humans

Given the impact of \textit{vacA} polymorphisms on gastric histopathology in mice and the strong association between carriage of i1-type strains and gastric adenocarcinoma in human populations, we went on to examine associations of \textit{vacA} type with intestinal metaplasia in \textit{H. pylori}–infected patients.

\textit{H. pylori} was successfully isolated by culture from gastric biopsy specimens taken from 85 patients. Of these, 62% of strains (n = 53) were \textit{vacA} s1/i1 type, 27% (n = 23) were s1/i2, and 11% (n = 9) were s2/i2. Consistent with previous reports, no s2/i1-type strains were isolated from this cohort. Because carriage of s2-type strains was rare, we were unable to compare s1 and
s2 effects in this study but could directly compare i1 and i2 effects in the s1 background.

There were no differences in levels of inflammation, activity, atrophy, or colonization density between patients infected with vacA i1-type or i2-type strains (data not shown), but a marked difference was seen in levels of intestinal metaplasia (Figure 6). Intestinal metaplasia was detected in only 1 of 32 patients (3%) infected with an i2-type strain, with only mild severity, but mild, moderate, or severe intestinal metaplasia were seen in 15 of 53 patients (28%) infected with an i1-type strain ($P < .01$; Figure 6A–C).

Because vacA i1-type strains also tend to be vacA s1 type and cagA positive, we first excluded vacA s2-type strains (Figure 6D) and then vacA s1-type, cagA− strains (Figure 6E) from our analysis. The association of intestinal metaplasia with vacA i1 type remained when s2-type strains were excluded (28% of patients with s1/i1 strains had intestinal metaplasia vs 4% with s1/i2 strains; $P < .05$). When all cagA− strains were also excluded, 26% of patients (11 of 43) with i1 strains had intestinal metaplasia, compared with only 8% (1 of 13) with i2 strains. There was also a trend toward an association between the vacA i1 allele and intestinal metaplasia in patients infected with vacA s1, cagA− strains ($P = .10$; Figure 6F), although consistent with other reports, such strains were uncommon in our patient cohort.

**DISCUSSION**

Although several studies using vacA null mutant strains in animal models have aimed to clarify the contribution of the VacA toxin to pathogenesis, findings have been mixed [23–26]. The consensus now emerging is that VacA does confer a colonization advantage in mice, but in humans nearly all strains are vacA positive, and the critical determinant of disease risk is the type of toxin expressed, rather than its presence or absence. We used strains with VacA types found to occur naturally in human infection but on an isogenic background to remove all other strain differences. We selected a functionally cagPAI-null background to remove any confounding or masking effects, because CagA is known to cause inflammation in animal models [21, 37].

Consistent with findings in other SS1-mouse studies, we found that our SS1s2i2 strain colonized C57BL/6J mice readily. Our SS1null mutants had reduced colonization rates, consistent with the impaired colonization reported elsewhere for vacA-null
SS1 [25] and PMSS1 [26]. We also found that strains producing s1/i1 and s1/i2 VacA had reduced colonization success rates and densities. This is the first evidence, to our knowledge, of a positive role for the less-active s2/i2 form of VacA compared with the s1/i2 or s1/i1 forms.

Many human studies have shown associations between strains with active forms of VacA and disease, and the best of these have controlled for other virulence factors, including cag-PAI status. However, doubts about the direct importance of VacA in disease have remained, in part because animal model experiments have proved difficult and the effects of VacA described in these models have largely been confined to colonization differences. By introducing isogenic hybrid forms of VacA in a mouse-colonizing H. pylori strain with a cag-null background, we have demonstrated independent effects of VacA on induction of inflammation and metaplasia, showing that the s1/i1 type of VacA is the most pathogenic, consistent with known disease associations in humans.

Mice in the studies presented in Figures 4 and 5 were from the same supplier, housed in the same facility, and treated identically but were from multiple batches, dosed on separate occasions. Although colonization densities in infected mice were consistent within each group across 4–5 independent experiments per group (Figure 2), levels of inflammation and SPEM were quite variable. We suggest that batch variation, perhaps in non-H. pylori gastric flora, may contribute to the histological parameters measured. However, SS1s1i1 consistently induced more severe and extensive inflammation and SPEM than SS1s2i2, despite colonizing poorly. It is possible that induction of inflammation and damage by the strains producing more active VacA contributed to the reduced colonization densities achieved by these strains. However, we did not see any significant correlation between colonization density and gastric pathology in this study.

H. pylori is not a natural colonizer of mice, and there are immunological and physiological differences between mice and humans. Thus, it is important to show relevance in a human population. The results in our UK population were more dramatic than we expected, given the relatively modest differences seen in mice: intestinal metaplasia was found in many patients...
infected with \(\text{vacA} s1/i1\) strains, regardless of \(cag\) status, but in only 1 patient who did not have an \(s1/i1\) strain isolated, and in that patient the findings were grade 1 only (mild). This raises several interesting issues. First, because \(s2/i1\) strains are rarely found naturally, it might only be necessary to type the i region to identify metaplasia-associated strains. Second, it is not surprising that many \(s1/i1\) strains were not associated with metaplasia; we regard these as metaplasia-inducing strains, but many other factors, including age, length of infection, environmental cofactors, and host genetics, are likely to determine whether an individual will develop metaplasia or will have developed it by the time of the study. Furthermore, metaplasia is a patchy phenomenon in the human stomach, and single-pinch biopsy specimens may not detect it reliably.

There are some interesting differences between our findings in mice and humans. Only in mice were there associations between \(\text{vacA}\) type and inflammation. This may be because \(cag\) is the dominant driver of inflammation in humans, and in our mice, to avoid confounding effects, we used a \(cag\)-null strain background. However, in humans we saw a strong association between \(\text{vacA} i1\) strains and metaplasia despite seeing no difference in levels of gastric inflammation. This may imply that VacA is driving metaplasia through a different mechanism, for example, through a direct toxic effect on the gastric epithelium. However, there are alternative explanations; for example, there may have been different levels of inflammation earlier in life, even in childhood, that are no longer evident in long-established infection. A second interesting difference is that
even quite severe metaplasia was sometimes found in mice infected with vacA s2/i2 or s1/i2 strains. In humans, only s1/i1 strains were associated with moderate or severe metaplasia.

Human and mouse *H. pylori* infections are very different, and it would be surprising if the same level of disease was observed in both. However, because non-1 strains can cause metaplasia in mice, it is important to study the association of such strains with metaplasia in humans in larger and different populations. This may be clinically important: if vacA i2 strains are rarely associated with metaplasia and cancer in human populations, there is no need to treat them to prevent cancer.

**Notes**

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