Harnessing of urease activity of *Helicobacter pylori* to induce self-destruction of the bacterium

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Abstract

Eradication of *Helicobacter pylori* with currently available antibacterial agents is unsatisfactory due to the risk of side-effects and the emergence of resistance. The organism rapidly dies in vitro in the presence of urea at pH 6. When incubated in citrate buffer (pH 6) plus urea (10 mM) the five minute survival was 26% compared with 96% without urea and the survival progressively decreased with increasing urea concentrations, being only 9% in 50 mM urea. The bactericidal effect depended on pH as the organism survived in citrate buffer (pH 7) plus urea (50 mM). The death of the organism at pH 6 in the presence of urea was prevented by the addition of the competitive urease inhibitor hydroxyurea.

These findings indicate that destruction of the organism is mediated by its exceptionally high urease activity. Harnessing this enzyme to induce self-destruction could provide a new approach to eradicating this common infection.

About 50% of the world's adult population are infected with *Helicobacter pylori* in their gastric antral mucosa,¹ and there is now strong evidence that the organism is the single most important acquired factor in the pathogenesis of duodenal ulcers. More than 95% of patients with duodenal ulcers have the infection and eradicating it reduces the annual relapse rate from 84% to 21%.² Unfortunately, the benefits of this new approach to ulcer treatment cannot be realised as no safe and effective antibacterial regimen has been discovered that is suitable for widespread eradication of the organism in such patients.

We present the results of experiments which show an entirely new approach to killing *H. pylori*. It entails harnessing the organism's exceptionally high urease activity to induce self-destruction. The phenomenon was noted by chance during in vitro studies of the role of the organism's urease activity in allowing the bacterium to survive in gastric acid. We found that *H. pylori* died rapidly when exposed to citrate buffer at pH 6 in the presence of 50 mM urea. This was confirmed using nine fresh clinical isolates obtained by culture of endoscopic antral biopsy specimens from patients with duodenal ulcers. The

following experiments were performed to investigate this observation.

Methods

The basic experimental design consisted of measuring the survival of *H. pylori* at a range of pH and the effect on this of varying the urea concentration of the media and adding the urease inhibitor hydroxyurea. In each experiment 1 ml of a 72 hour broth culture suspension (brain-heart-infusion broth + 0.25% yeast extract + 10% horse serum) of *H. pylori* was added to 9 ml of 0.2 M citrate buffers or to isomolar unbuffered saline and incubated at 37°C. After five minutes aliquots were removed for viable bacterial counts and where appropriate for measurement of ammonium concentrations. The pH was monitored using a combined glass electrode (Radiometer GK2802C).

Using the above protocol the following studies were performed:

- The survival of the organism was compared in citrate buffer (pH6) and isomolar unbuffered saline both with and without 10 mM urea.
- The effect of varying the starting concentration of urea on survival in pH6 was assessed.
- The effect of adding the competitive urease inhibitor hydroxyurea on the survival of *H. pylori* at pH 6 in the presence of 10 mM urea was examined.
- The effect of using citrate buffer (pH7) compared with pH6 on the survival in 50 mM urea was examined.

Survival was established using the method of Miles and Misra.³ Using a microtitre modification, serial 10-fold dilutions of 20 µl volumes were made in saline. Viable colony counts were performed on horse blood agar plates after 6 days' incubation at 37°C in a microaerophilic atmosphere (BBL CampyPak gas-generating system). Survival of the culture at a known time is expressed as a percentage of the starting inoculum calculated from the dilution of the viable colony count in the initial broth suspension. The aliquots removed for biochemical analysis were immediately filtered (Gelman Sciences, Acrodisc 0.2 µm) and stored at -20°C. The ammonium concentration was measured after 1 in 15 dilution in 0.2 M phosphate buffer, pH 7.4, by an enzymatic method using the reductive amination of keto glutarate catalysed by glutamate dehydrogenase (Sigma Chemicals, UK) adapted for the Cobas Bio centrifugal analyser (Roche, Welwyn Garden City, England).

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Figure 1 Survival of *H. pylori* in citrate buffer, pH 6, with different urea concentrations.

The experiments reported were performed using the National Collection of Tissue Cultures 11637 strain of *H. pylori*. Statistical analysis was performed using the Mann-Whitney U Test.

Results

The starting inoculum was determined separately for each experiment and was consistently 10^{5-6} cfu/ml. The pH of the incubating solution remained constant in each of the experiments using citrate buffer. In the saline experiments with the urea the pH at the start ranged from pH 6.26–6.60 and at five minutes had risen to pH 7.04–7.50.

(a) EFFECT OF PRESENCE OF UREA ON SURVIVAL AT pH 6

The median five minute survival in citrate buffer pH 6 was considerably reduced when urea (10 mM) was present in the incubation solution (26%, range 0–87 with urea, *v* 96%, range 28–179 without urea, $p < 0.001$). Similar results were obtained when fresh clinical isolates of *H. pylori* were tested, confirming that this is not purely a feature of the NCTC 11637 strain.

Median five minute survival in isomolar saline was not affected by the addition of 10 mM urea (81% without urea compared with 77% with urea). The saline experiments were allowed to run for up to two and a half hours with no difference in survival.

(b) EFFECT OF INCREASING UREA CONCENTRATION ON SURVIVAL AT pH 6

The mean five minute survival at pH6 progres-

Figure 2 Effect of increasing concentrations of hydroxyurea on survival of *H. pylori* in citrate buffer, pH 6, plus 10 mM urea.

Figure 3 Survival at pH 6 versus pH 7 in presence of urea. For comparison, survival in isomolar saline with and without urea is shown.

sively decreased with increasing concentration of urea from 121% (range 69–148) in the absence of urea to only 9% (range 0–22), with 50 mM urea (fig 1).

(c) EFFECT OF ADDITION OF HYDROXYUREA ON SURVIVAL AT pH 6 IN PRESENCE OF UREA

The addition of hydroxyurea inhibited the killing effect of urea at pH 6. This protective effect increased with increasing concentration of hydroxyurea (fig 2).

The median five minute ammonium concentration at pH 6 in the presence of 10 mM urea was 2.5 mmol/l (range 1.9–7.65) compared with 1.13 mmol/l (range 0.8–1.3) in the blank incubation. When 10 mM hydroxyurea was added the median five minute ammonium concentration was reduced to 1.29 mmol/l (range 1.03–1.53) ($p < 0.001$), indicating effective inhibition of urease enzyme activity.

(d) COMPARISON OF SURVIVAL AT pH 6 VERSUS pH 7 IN PRESENCE OF UREA

When *H. pylori* was added to citrate buffer at pH 7 plus urea (50 mM) its mean five minute survival was 60% (range 31–112%) and was similar to that observed in unbuffered saline with or without 50 mM urea. The survival in buffer pH 6 plus urea (50 mM), however, was only 11% (range 2–22%) ($p < 0.001$), indicating that the killing effect is pH dependent (fig 3).

Discussion

We have shown that rapid death of *H. pylori* can be induced in vitro by changing the pH and urea concentration of its environment. Inhibition of the organism's urea activity with hydroxyurea prevents this happening. This rapid death of *H. pylori* without the use of conventional antibacterial agents indicates suicidal destruction of the bacterium mediated by its urease activity. The use of isomolar saline shows that the effect is not merely a result of the changed osmolality of the environment.

H. pylori is remarkable because of its high urease enzyme activity by which it converts urea to ammonia and carbon dioxide.⁴⁻⁶ This property is used in several tests for diagnosing the presence of the infection in man, and it has been suggested that the alkalinising effect of

the ammonia may protect the organism from being destroyed by the acidic gastric juice.

In vitro studies have shown that the bacterium can survive at pH 2.6 in the presence of urea but rapidly dies if no urea is available.⁷ Our experiments indicate that the organism's urease activity which aids survival at low pH can also result in rapid destruction of the bacterium at pH 6 when ample substrate is available. We presume that death is due to the overproduction of ammonia resulting in irreversible metabolic damage.

This would concur with the observation that the activity of the enzyme is not suppressed by ammonium,⁵ indicating the absence of a protective feedback control mechanism.

To date, no satisfactory medical regimen has been discovered for eradicating *H pylori*. To achieve eradication rates of 90% it is necessary to give two to four weeks triple treatment with tripotassium dicitrate-bismuthate, in combination with metronidazole, plus either amoxycillin or tetracycline.⁸ This complex combination treatment makes compliance difficult and is associated with side-effects, including potentially fatal pseudomembranous colitis.

The major problem with current treatment, however, is the tendency for the organism to develop resistance to metronidazole or tinidazole. Such resistance has been reported in 27%–84% of patients on first presentation with *H pylori* infection and is associated with previous treatment with nitroimidazoles.⁹

Furthermore, in a significant proportion of patients, metronidazole resistance develops during the course of eradication treatment.⁹ The use of the current anti-*H pylori* treatment is likely to result in the widespread development of resistant strains making eradication of this common infection even more difficult in future years. Consequently, there is a need for an alternative and more specific therapeutic approach.

Whether it will be possible to induce suicidal destruction of *H pylori* in vivo by changing the pH and urea concentration of its immediate environment remains to be seen. The fact that the organism is confined to the surface of the gastric mucosa without any tissue invasion means that it is accessible to orally administered urea. We have shown that the intragastric administration of urea in patients with *H pylori* results in a rapid increase of ammonia production, indicating that it gains ready access to the organism and its urease enzyme.¹⁰ The pH of

gastric juice can also be changed readily with acid inhibitory agents, and it should be possible to produce a pH of 6 in the region of the organism. It is interesting to note that patients with increased gastric pH due to pernicious anaemia or duodenogastric alkaline reflux following gastric surgery have a low prevalence of *H pylori* infection.^{11,12} Treatment with the powerful acid inhibitory agent omeprazole, which raises intragastric pH to near neutral values, has also been reported to clear *H pylori* infection in some patients.^{13,14} Omeprazole does not have any direct toxic or inhibitory effect on *H pylori* in vitro.¹⁵ It is also of interest that patients with uraemia have been reported to have a lower prevalence of *H pylori* infection.¹⁶

Our observations indicate a potential new approach to the eradication of this infection which could avoid the problems associated with current treatment.

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