Development of a ^{14}C -urea breath test in ferrets colonised with *Helicobacter mustelae*: effects of treatment with bismuth, antibiotics, and urease inhibitors

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Abstract

A ^{14}C -urea breath test analogous to its clinical counterpart is described for use in ferrets naturally or experimentally infected with *Helicobacter mustelae*. The test is performed within a sealed glass metabolism chamber through which air is drawn at a constant rate and expired breath collected into sodium hydroxide. Peak $^{14}\text{CO}_2$ production occurred approximately 1 hour after substrate administration. Both inter- and intra-animal responses were highly reproducible, with mean coefficients of variation less than 10%. Other than enhancing peak $^{14}\text{CO}_2$ levels very slightly, fasting had little influence on the response. In infant animals challenged with *H. mustelae*, breath test activity increased linearly with the total count of culturable bacteria isolated from the antrum. Treatment of established infections with colloidal bismuth subcitrate (DeNol) for 4 weeks resulted in clearance of all detectable bacteria but retention of some breath test activity. Subsequent regrowth of bacteria was paralleled by an increase in the breath test response. Inclusion of amoxycillin and metronidazole in the treatment regimen, however, eradicated all the bacteria and almost totally eliminated $^{14}\text{CO}_2$ production. This response parallels the clinically observed suppressive effect on *H. pylori* achieved with bismuth alone relative to the total eradication seen with triple therapy. A single oral dose of the urease inhibitor, flurofamide, inhibited over 90% of the response for at least 24 hours. Acetohydroxamic acid was less effective. These findings suggest that in the ferret *H. mustelae* model, breath test analysis can be a useful, non-invasive alternative to endoscopy for evaluation of agents affecting either growth of the organism or urease activity.

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The Gram negative, spiral bacterium, *Helicobacter pylori*, is known to be a major cause of chronic, non-specific histological gastritis¹⁻³ and is strongly associated with duodenal ulcer relapse.^{4,5} Studies on the precise role of this organism in the aetiology of peptic ulcer disease and its response to various treatments would be aided considerably by the availability of a suitable animal model. Adult ferrets naturally colonised with *H. mustelae* have been proposed as a model for *H. pylori* infection.^{6,7} In common with *H. pylori*, *H. mustelae* exists in or on the surface

mucus of the gastric antrum, is often but not always associated with gastritis,^{8,9} and produces large quantities of urease.¹⁰ In humans, the high endogenous urease production of *H. pylori* has been exploited in the development of either the ^{14}C - or ^{13}C -urea breath test which is a simple, non-invasive and reproducible procedure for diagnosis of infection.^{11,12} We describe here a ^{14}C -breath test devised in ferrets naturally or experimentally colonised with *H. mustelae*. Using this test, the effects of various treatments on the course of infection can be examined without recourse to endoscopy.

Methods

ANIMALS

Adult ferrets (approximately 1 kg in body weight and 7-12 months old) were housed individually and were maintained on a daily diet of fresh minced beef with diet C pellets and water ad libitum. All were confirmed to be naturally colonised with *H. mustelae* by gastroscopy using a Schott VSF-2 veterinary endoscope (6.3 mm probe diameter) under anaesthesia with sodium pentobarbitone (48 mg/kg ip, Sagatal, May and Baker, 60 mg/ml).

Infant ferrets (3-4 days old at the start of the experiments) were housed in litters with the mother for the duration of the studies (up to 21 days). Natural colonisation with *H. mustelae* was never seen before 3 weeks of age and the incidence of colonisation at 6 weeks was only about 50% (McColm, unpublished data).

BIOPSY SPECIMEN EVALUATION

The extent of *H. mustelae* colonisation in adult ferrets was assessed from two or, occasionally, three biopsy specimens taken as above. Antral specimens only were collected since fundic mucosa has a very low and sparsely distributed density of bacteria (McColm, unpublished data). Specimens were homogenised separately in 1 ml volumes of sterile brain heart infusion broth (BHIB, Oxoid Ltd) in 5 ml capacity, sterile, ground glass tissue homogenisers (Aimer Products, London). Serial 10 fold dilutions were made in BHIB and the numbers of organisms quantified from 0.1 ml aliquots cultured microaerophilically (Campypak Plus, BBL Microbiology Systems, Becton Dickinson) for 6-7 days at 37°C on chocolate Columbia agar (Oxoid Ltd., 10% v/v defibrinated horse blood). The agar contained vancomycin (50 µg/ml), polymyxin B

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Figure 1: Schematic diagram of breath test apparatus.

(20 µg/ml), and amphotericin B (100 µg/ml) to reduce/eliminate contaminants.

Infant ferrets were killed by Sagatal overdose, the entire stomach was removed, and two 4 mm diameter punch biopsy specimens were removed from as close to the pylorus as possible. These were then processed for *H. mustelae* as above.

Viable counts were expressed as log CFU (colony forming units) per biopsy specimen (adult ferrets) or log CFU/mm² of antral tissue (infant ferrets). Presumptive identification as *H. mustelae* was based on colonial and cellular morphology, Gram reaction, and urease activity using an unbuffered 10% (w/v) aqueous urea solution containing phenol red indicator.¹³

H. MUSTELAE CHALLENGE OF INFANT FERRETS

A rifampicin resistant spontaneous variant of *H. mustelae* (F251-rif R) was prepared by *in vitro* selection of a wild type isolate on chocolate Columbia agar containing 100 µg/ml of rifampicin. The organism was grown in 5 ml volumes of BHIB containing 10% horse serum and 1% Fildes and yeast (Oxoid) for 48 hours microaerophilically in 50 ml volume plastic

tissue culture flasks (Nunc). Approximately 10⁷ CFU (0.25 ml) were then administered orally to 3–4 day old ferrets using a 21G oral dosing needle. Punch biopsy specimens were processed and cultured as above on agar with and without 50 µg/ml rifampicin (in addition to the normal selective agents) in order to distinguish between the administered organism and any wild type colonisation.

BREATH TEST PROCEDURE

Unless stated otherwise, animals were not fasted before breath testing. Adult ferrets were dosed orally with 370 KBq of ¹⁴C-urea (2.1 GBq/mmol, Amersham) administered in 1 ml of sterile, distilled water. Individual ferrets were transferred immediately into sealed glass metabolism chambers (approximately 248 mm internal diameter by 165 mm high (Metabowl MK 111, Jencons Scientific Ltd, Fig 1)). Air was drawn through the chamber at a constant rate of 250 ml/minute and expired breath was collected over a 3 hour period into 2M NaOH contained in two glass Drechsel bottles linked in series. The NaOH solution (100 ml volume/bottle) was renewed every 30 minutes and triplicate 0.5 ml aliquots of the collected solutions mixed with 10 ml of scintillation fluid (Optiphase Safe, LKB) and counted for radioactivity on an LKB Rackbeta liquid scintillation counter. From the radioactivity profiles obtained, the total percentage of administered radioactivity recovered and the area under the time/radioactivity curve (AUC) value was calculated. In experiments investigating the effects of various treatments on the breath test response, the percentage reduction of the predose AUC (0–3 hours) was used as a measure of activity. Since early experiments indicated that less than 0.05% of the total recovered radioactivity reached the second collecting bottle (see Fig 1), samples were subsequently analysed for radioactivity only from the first bottle. Three hours was considered a suitable collection period since most of the ¹⁴CO₂ liberated as a result of *H. mustelae* urease is expired within this time and it avoided the possibility of any urease positive lower gut organisms (for example *Proteus* sp) contributing to the results.

Infant animals were breath tested similarly

Figure 2: Mean (SEM) breath test responses of 14 ferrets.

Figure 3: Repeat breath tests performed in a single ferret.

except that the ¹⁴C-urea (370 KBq) was administered orally in 0.1 ml of water, the duration of the ¹⁴CO₂ collection period was limited to 1 hour, and in order to achieve sufficient recoverable radioactivity, five animals were breath tested simultaneously in the same chamber.

Results

All adult ferrets examined by endoscopy were naturally colonised with *H. mustelae* (normally 10⁴–10⁵ CFU/biopsy). Each animal showed a positive breath test profile characterised by a rapid increase in ¹⁴CO₂ expiration rising to a maximum by approximately 1 hour after the ¹⁴C-urea administration. This was followed by a more gradual decline in ¹⁴CO₂ release over the next 2 hours. The mean responses of a group of 14 ferrets showing similar degrees of *H. mustelae* colonisation (approximately 10⁴ CFU/antral biopsy) are shown in Figure 2. The mean peak time of ¹⁴CO₂ release was 69 minutes and the AUC was 639,954 (DPM/minute). Nearly 10% of the total administered radioactivity was recovered in the expired breath over the 3 hour sampling period. The coefficients of variation (v) between the animals was 5.3%.

Repeat testing of the same animal on different occasions showed considerable uniformity of response. Results of repeat tests performed on five ferrets on a minimum of three occasions each gave coefficients of variation ranging from 1.3%–16.7% (mean (SD), 8.2 (6.5)). The results for one animal tested monthly for three months are shown in Figure 3.

To examine the effects of fasting on the response, five ferrets were breath tested before and after being denied food for 16 hours. Fasting did not seem to have a marked influence on the amount of ¹⁴CO₂ expired (Fig 4). Although the peak of radioactivity release was slightly higher and the subsequent rate of decline slightly more rapid after fasting, the differences were not significant. The ratios of the fasted: fed AUC values for the five animals ranged from 0.96 to 1.62, with a mean ratio of 1.19.

All adult ferrets tested were already colonised with *H. mustelae*, therefore infant ferrets (3–4 days old) which had not acquired the infection

Figure 4: Effects of fasting on mean breath test responses of five ferrets.

Figure 5: Relationship between ¹⁴CO₂ recovery and degree of antral colonisation in infant ferrets challenged with *Helicobacter mustelae*. (A) Effect of time after infection on breath test response and log bacterial counts: (B) Linear relationship of antral colonisation to breath test area under the time/radioactivity curve (AUC).

were used to investigate the effects of colonisation on the development of the breath test response. Over a 1 hour sampling time, uninfected baby ferrets showed no detectable ¹⁴CO₂ release. After a single oral challenge with the rifampicin resistant variant of *H. mustelae* (F251-rif R), however, a progressive increase in ¹⁴CO₂ release with time was observed. This increasing breath test response was paralleled by increasing *H. mustelae* numbers colonising the antral mucosa (Fig 5A). The relationship between the degree of antral colonisation and the breath test AUC seemed to be linear (Fig 5B).

As a further investigation of the relationship between numbers of organisms in the stomach and the breath test response, adult animals were treated for 28 days with colloidal bismuth subcitrate (DeNol; Brocades, 8 mg/kg/dose), without or with added antibiotics (metronidazole, 20 mg/kg/dose – for the first 10 days only and amoxycillin, 10 mg/kg/dose). All treatments were administered orally three times daily. The results of breath tests and endoscopies performed on a single animal in each treatment group 1 day before (day 1) and 1 day (day 28) and 12 days after treatment (day 40) are shown in Figure 6 A, B. The results indicate that the reduction in *H. mustelae* numbers/biopsy correlate with a reduction in the breath test AUC. Colloidal bismuth subcitrate alone did not suppress the organism count or the breath test response as effectively as the triple therapy regimen. Despite inability to culture *H. mustelae* from the ferrets by day 28, there was still a significant breath test response seen in the animal treated with colloidal bismuth subcitrate alone. This had increased by day 40 when significant *Helicobacter* regrowth had occurred (Fig 6A). In contrast, in the animal given triple therapy, no bacteria were isolated and negligible

Figure 6: Effect of DeNol alone or in combination with antibiotics on breath test response and *Helicobacter mustelae* numbers in adult ferrets. (A) DeNol; (B) DeNol plus metronidazole and amoxycillin. Dosing regimens were as follows: (A) DeNol: 8 mg/kg/dose for 28 days; (B) triple therapy: as above but with amoxycillin, 10 mg/kg/dose and metronidazole, 20 mg/kg/dose. All treatments given orally, thrice daily, for 28 days except metronidazole (first 10 days only).

breath test activity was observed on both days 28 and 40 (Fig 6B). Subsequent evaluation of the animals 3 months after therapy, showed complete restoration to pretreatment levels of bacterial numbers and breath test responses in the colloidal bismuth subcitrate treated ferret while no bacteria or breath test activity was seen in the animal given triple therapy (data not shown).

In addition to examination of the effects of long term therapy on the breath test, the effects of a single oral dose of colloidal bismuth subcitrate (8 mg/kg) were assessed. Administration of colloidal bismuth subcitrate 30 minutes before ¹⁴C-urea produced a marked but variable reduction in the breath test AUC compared with the predose value obtained 24 hours previously (Table I). The extent of the reduction ranged from 15% to almost 80% (mean of 47.5%). Since subsequent endoscopy showed no obvious reduction in *Helicobacter* in the antrum, this suggests the possibility of a direct inhibitory action of bismuth on the enzyme over and above any antibacterial effects.

The effects on the breath test of two urease inhibitors – fluoroamide and acetohydroxamic acid – were also evaluated (Table II; Fig 7). A single oral dose of 5 mg/kg of either compound administered 30 minutes before the ¹⁴C-urea resulted in 98% inhibition of the predose AUC. At this dose level, fluoroamide was still able to exert a marked inhibitory action on urease when given 6 or 24 hours before breath testing (90% and 53% reductions in predose AUC respectively). In contrast, acetohydroxamic acid showed minimal inhibition when given 6 hours before the ¹⁴C-urea and no activity when given 24 hours before. Increasing the dose concentration to 50 mg/kg showed that fluoroamide could achieve greater than 80% reduction of detectable urease activity for at least 48 hours, whereas acetohydroxamic acid had lost all activity by 24 hours (Table II, Fig 7).

Discussion

The urea breath test has proved to be a useful addition to the diagnostic methods available for the detection of *H. pylori* infections in patients. Both ¹⁴C-urea^{12 14 15} and urea labelled with the non-radioactive ¹³C isotope^{11 16} have been used as substrates for *H. pylori* urease, resulting in their hydrolysis to CO₂ and ammonia. Collection and assay of the CO₂ is usually performed over a 90 minute to 2 hour period. The test has been refined recently so that only one or two breath samples are required to provide an accurate diagnosis.^{17 18}

In the current investigation, we have devel-

TABLE I Effect of a single oral dose of De-Nol (8 mg/kg) on ¹⁴CO₂ recovery

Ferret no	Area under the curve			
	24 h before De-Nol	0.5 h after De-Nol	% Reduction	
15	688,680	491,385	28.7	Mean (SD)=47.5 (23.1)
17	805,815	365,295	54.7	
765	404,755	346,395	14.4	
800	715,605	284,865	60.2	
806	914,775	189,015	79.3	

TABLE II Effects of a single oral dose of flurofamide or acetohydroxamic acid on ¹⁴CO₂ recovery

Inhibitor	Dose (mg/kg)	Time of dose before test (h)	% Reduction of predose AUC
Flurofamide	5	0.5	98
	5	6	90
	5	24	53
	50	24	92
Acetohydroxamic acid	5	0.5	98
	5	6	15
	50	24	0

AUC=area under curve.

oped an analogous test using ¹⁴C-urea to diagnose ferrets colonised naturally or experimentally with *H. mustelae*. Calculation of the AUC proved a useful means of examining both inter- and intra-animal variation and of investigating the effects of various treatments. Using this method, evaluation of a population of ferrets with similar densities of gastric *H. mustelae* colonisation showed that the inter-animal breath test profiles were remarkably uniform. Furthermore, repeat testing of individual animals at various times showed only slight variations in response. It is possible that this uniformity of response relative to the human test reflects the identical background, breeding conditions, and exposure to *H. mustelae* experienced by the animals in the colony. Conversely, there is likely to be considerable diversity of *Helicobacter* strains and organism numbers seen clinically.

In human breath test studies, the presence of food in the stomach increases both the amount and duration of labelled CO₂ expiration.¹⁵ This occurs presumably by slowing of the gastric emptying rate thus increasing the retention time of the urea substrate in the stomach. Although there was little difference in response between fed and fasted ferrets, however, there did seem to be a slight increase in peak ¹⁴CO₂ release in the animals after fasting. It is possible that in the fasted ferret, despite a presumed decrease in gastric retention, the absence of food allows greater exposure of the *H. mustelae* urease to the substrate. In a recent clinical study, Marshall *et al*¹⁷ also proposed that food decreased the contact between isotope and gastric mucosa.

Data from *H. mustelae* challenge experiments performed in infant ferrets and treatment studies

in adult animals using bismuth with or without antibiotics showed that the greater the breath test response (that is, AUC), the higher the number of bacteria which could be isolated from the gastric mucosa. The relationship, at least in newly challenged infant animals, seemed to be linear in character.

The response of *H. mustelae* infections to bismuth was similar to that seen clinically, that is temporary clearance or suppression of the bacteria followed by recrudescence.^{4, 19-21} It was of interest that clearance of *H. mustelae* by bismuth alone, as gauged by gastroscopy after 4 weeks of therapy, only reduced, but did not totally eliminate, breath test activity. In contrast, a four week course of standard triple therapy comprising colloidal bismuth subcitrate plus amoxycillin and metronidazole as used clinically²¹⁻²³ and previously in ferrets,²⁴ resulted in eradication of the organism and almost total elimination of breath test activity. These observations suggest that culture negativity, at least in the ferret model, is not necessarily predictive of *Helicobacter* absence and indicates that in some cases breath testing may permit a better diagnosis of infection status. It is probable that inability to culture bacteria at the end of bismuth alone therapy, despite maintenance of a positive breath test response, represents an almost total but temporary clearance of *H. mustelae* from the stomach. It could, however, reflect conversion to a less easily cultured and less metabolically active form. In view of this, our results support the idea that 'suppression' rather than 'clearance' may be the preferred term for this effect.²⁵

A previous clinical study has reported that a single dose of bismuth administered just before testing may decrease breath test activity.¹⁵ A similar effect was seen in the current study using colloidal bismuth subcitrate. Since no detectable decrease in antral *H. mustelae* numbers was seen in biopsy specimens taken 3 days later, it is possible that the bismuth had either a very transient antibacterial action or exerted a direct inhibitory effect on the urease enzyme.²⁶

Experiments with acetohydroxamic acid and flurofamide, which are known inhibitors of *H. pylori* urease,²⁷ showed that at the lowest dose used (5 mg/kg) both compounds could inhibit breath test activity almost completely. Flurofamide, however, exerted a considerably longer period of inhibition which may correlate with published data showing its superior inhibitory activity over acetohydroxamic acid on the isolated enzyme.²⁷

In summary, a ¹⁴C-urea breath test for *H. mustelae* in ferrets is described which shows high reproducibility of response, is related linearly (at least in newly colonised infant animals) to the number of organisms on the gastric mucosa, and can be used to compare treatment efficacy. In the ferret *H. mustelae* model, the test is a useful non-invasive alternative to endoscopy which could also have applications in other animal models of *Helicobacter* infections.

Figure 7: Reduction of breath test area under the time radioactivity curve (AUC) in ferrets predosed with flurofamide (50 mg/kg; single dose): effects of time of administration.

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