In Vitro Assessment of Gastric Mucosal Transfer of Anti-Helicobacter Therapeutic Agents

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Received 12 April 1996/Returned for modification 12 October 1996/Accepted 17 March 1997

A novel animal model for studying antibiotic transfer across gastric mucosa was developed by using adult rats. Gastric corpus mucosa was mounted in an Ussing chamber system and bathed in oxygenated Krebs solution. Metronidazole flux from serosa to mucosa (\(J_{S-M}\)) was measured over 60 min under basal conditions and compared with mucosa-to-serosa flux (\(J_{M-S}\)). The effects of varying the chamber cross-sectional diameter and of stimulation by histamine and carbachol were assessed. Metronidazole \(J_{M-S}\) was measured with the mucosal pH at 2.2, 2.7, 3.2, and 7.4. Aminocillin \(J_{S-M}\) under basal conditions was also measured and compared with metronidazole \(J_{S-M}\). Metronidazole \(J_{S-M}\) was proportional to serosal concentration (\(P < 0.001\)) under basal conditions, being 3.98 nmol·h·cm\(^{-2}\) with a serosal concentration of 0.2 mmol/liter. Aminocillin \(J_{S-M}\) was significantly lower under similar conditions at 0.50 nmol·h·cm\(^{-2}\) (\(P < 0.001\)). Metronidazole \(J_{S-M}\) was not significantly different from \(J_{M-S}\) between chambers of different sizes, or following stimulation. When the mucosal pH was changed, \(J_{M-S}\) was proportional to the un-ionized concentration on the mucosal side (\(P < 0.001\)). Therefore, this model shows properties analogous to those of human gastric mucosa in vivo, with partitioning of metronidazole on the mucosal side according to pH, diffusion of metronidazole across the mucosa in both directions, and selectivity for different antibiotics, and it will be useful for the study of other therapeutic agents in the treatment of Helicobacter pylori.

Although the key role of Helicobacter pylori in the pathogenesis of peptic ulceration and gastric carcinogenesis is well recognized (13), and effective treatment is an important therapeutic goal, there has been little interest in the mechanisms of delivery of antibiotics within the gastric environment. Recently, however, both in vitro and in vivo studies have suggested that antibiotic delivery from blood to gastric mucosa is important in the success of therapeutic regimes (12), although the principles governing these processes have yet to be defined.

The development of appropriate animal models to investigate the physicochemical properties of antibiotics which govern transmucosal transfer will allow selection from the large number of as yet untried antimicrobials of those with the best potential to succeed. However, there are few such models, and those that have been developed (1, 18) suffer from two important flaws. Firstly, they rely on the measurement of antibiotic in homogenized mucosal tissue samples. This concentration cannot be equated to the amount of antibiotic that is available to the organism on the epithelial surface, as the distribution of antibiotics within tissues is not homogeneous, for example, due to accumulation within cells (1), trapping within the acid lumen of the stomach (18), or high levels of plasma protein binding. Cryomicrotomy, used to remove the superficial cell layer, has been proposed as an improvement (10), excluding contamination from interstitial tissue and plasma, but is still not ideal.

Secondly, control of the luminal and serosal conditions is difficult, especially with respect to pH, and therefore the role of such modifying factors in transfer cannot be accurately determined. To overcome these problems, we have designed an in vitro animal model which allows for both the accurate measurement of antibiotic available on the mucosal surface and control of the mucosal surface pH.

**MATERIALS AND METHODS**

Male Wistar rats weighing between 300 and 350 g, allowed food and water ad libitum, were sacrificed by cervical dislocation, and the stomach was immediately removed. Incisions were made along the lesser and greater curvatures, the corpus mucosa was washed and pinned out in oxygenated Krebs solution (140 mM Na\(^+\), 6 mM K\(^+\), 127 mM Cl\(^-\), 25 mM HCO\(_3\)\(^-\), 1.2 mM SO\(_4\)\(^-\), 1.2 mM HPO\(_4\)\(^-\), 1.2 mM Mg\(^2+\), 12 mM glucose) at pH 7.4 and 4°C, and the external muscle layers were removed by the method of Curtis and Gall (2). The tissue was then mounted in Lucite Ussing chambers (World Precision Instruments, Sarasota, Fla.) with a cross-sectional diameter of 1.2 cm, 12 ml of Krebs solution at 37°C was added to either side, and 5.95% CO\(_2\)-O\(_2\) was bubbled through to mix the solutions and oxygenate the mucosa. Tissue samples from the same stomach were used as controls for paired experiments.

The resting, open-circuit, potential difference (PD) generated by the mucosa was measured continuously (in millivolts) via calomel electrodes connected to the chambers by agar bridges (3 M KCl in 3% agar) with a voltage clamp (BioPac Systems, Goleta, Calif.). Additionally, the short-circuit current (ISC) was measured (in microamperes) at 0, 15, 30, 60, and 90 min following appropriate clamping, and conductance (G) was then calculated (in millisiemens per square centimeter) by using Ohm’s law.

Six milliliters of Krebs solution containing metronidazole (Sigma, St. Louis, Mo.) was added to one side of the mucosa (the reservoir chamber), after removal of 6 ml of Krebs solution at 30 min after mounting in the chamber such that the final concentration was between 0.2 and 0.4 mmol/liter (reservoir concentrations were varied in order to investigate the effect of serosal concentration on flux), i.e., of the same order as concentrations achieved in vivo. Four-hundred-microliter aliquots of solution were removed from both sides at 0, 15, 30, 45, and 60 min after the addition of test drug. These were centrifuged at 6,500 × g for 5 min, and the supernatant was stored in 300-μl vials at −70°C until analysis.

Antibiotic flux (\(J\)) (expressed as nanomoles per hour per square centimeter) for each experiment was calculated as \(|(\text{antibiotic}_{S} - \text{antibiotic}_{M})| \cdot \text{volume in chamber/area of chamber, where (antibiotic}_{S} and (antibiotic}_{M})\) were the antibiotic concentrations measured in the chamber opposite the reservoir chamber at 0 and 60 min, respectively.

Metronidazole flux was measured from serosal side to mucosal side (\(J_{S-M}\)) under baseline conditions (\(n = 8\), i.e., the serosal chamber was the reservoir chamber. Then, to test the hypothesis that metronidazole is actively secreted into the stomach, metronidazole \(J_{S-M}\) (\(n = 8\)) was compared both with metronidazole flux from mucosal side to serosal side (\(J_{M-S}\)) (\(n = 8\), i.e., the mucosal chamber was the reservoir chamber, and with metronidazole \(J_{S-M}\) following secretory stimulation with histamine and carbachol (both at 0.1 mmol/liter, 0.5 nmol/liter, and 0.2 μmol/liter for histamine and carbachol, respectively) and compared with mucosa-to-serosa flux (\(J_{M-S}\)). The effects of varying the chamber cross-sectional diameter and of stimulation by histamine and carbachol were assessed. Metronidazole \(J_{M-S}\) was measured with the mucosal pH at 2.2, 2.7, 3.2, and 7.4. Aminocillin \(J_{S-M}\) under basal conditions was also measured and compared with metronidazole \(J_{S-M}\). Metronidazole \(J_{S-M}\) was proportional to serosal concentration (\(P < 0.001\)) under basal conditions, being 3.98 nmol·h·cm\(^{-2}\) with a serosal concentration of 0.2 mmol/liter. Aminocillin \(J_{S-M}\) was significantly lower under similar conditions at 0.50 nmol·h·cm\(^{-2}\) (\(P < 0.001\)). Metronidazole \(J_{S-M}\) was not significantly different from \(J_{M-S}\) between chambers of different sizes, or following stimulation. When the mucosal pH was changed, \(J_{M-S}\) was proportional to the un-ionized concentration on the mucosal side (\(P < 0.001\)). Therefore, this model shows properties analogous to those of human gastric mucosa in vivo, with partitioning of metronidazole on the mucosal side according to pH, diffusion of metronidazole across the mucosa in both directions, and selectivity for different antibiotics, and it will be useful for the study of other therapeutic agents in the treatment of Helicobacter pylori.
TABLE 1. Effect of mucosal pH on the PD generated and conductance of gastric mucosa in an Ussing chamber at increasing time points*

<table>
<thead>
<tr>
<th>pH</th>
<th>0 min</th>
<th>30 min</th>
<th>60 min</th>
<th>90 min</th>
<th>30 min</th>
<th>60 min</th>
<th>90 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.2</td>
<td>-5.9 (1.4)</td>
<td>-18.4 (2.0)</td>
<td>-18.8 (2.0)</td>
<td>-17.0 (1.6)</td>
<td>16.4 (0.7)</td>
<td>16.8 (0.8)</td>
<td>17.8 (1.2)</td>
</tr>
<tr>
<td>2.7</td>
<td>-5.5 (0.6)</td>
<td>-14.2 (1.1)</td>
<td>-15.3 (1.1)</td>
<td>-13.4 (0.8)</td>
<td>12.2 (0.9)</td>
<td>12.7 (1.0)</td>
<td>13.4 (1.1)</td>
</tr>
<tr>
<td>3.2</td>
<td>-5.8 (0.6)</td>
<td>-21.8 (1.4)</td>
<td>-19.9 (1.5)</td>
<td>-17.2 (1.4)</td>
<td>13.5 (1.5)</td>
<td>13.1 (1.3)</td>
<td>13.7 (1.6)</td>
</tr>
<tr>
<td>7.4</td>
<td>-6.1 (0.5)</td>
<td>-27.3 (2.6)</td>
<td>-27.5 (1.7)</td>
<td>-24.4 (2.3)</td>
<td>13.7 (0.8)</td>
<td>13.5 (0.7)</td>
<td>13.1 (0.9)</td>
</tr>
</tbody>
</table>

* Values are expressed as means, with standard errors of the means in parentheses.

TABLE 2. Effect of chamber diameter on the PD generated and conductance of gastric mucosa at pH 7.4 in an Ussing chamber at increasing time pointsa

<table>
<thead>
<tr>
<th>Diam (cm)</th>
<th>0 min</th>
<th>30 min</th>
<th>60 min</th>
<th>90 min</th>
<th>30 min</th>
<th>60 min</th>
<th>90 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2</td>
<td>-5.8 (0.4)</td>
<td>-26.1 (1.7)</td>
<td>-27.8 (1.9)</td>
<td>-22.2 (1.9)</td>
<td>11.9 (0.5)</td>
<td>12.4 (0.5)</td>
<td>12.5 (0.4)</td>
</tr>
<tr>
<td>0.9</td>
<td>-5.3 (0.3)</td>
<td>-26.3 (1.2)</td>
<td>-27.9 (1.1)</td>
<td>-21.5 (1.1)</td>
<td>12.4 (0.5)</td>
<td>12.3 (0.6)</td>
<td>12.4 (0.8)</td>
</tr>
<tr>
<td>0.4</td>
<td>-1.7 (0.4)</td>
<td>-9.7 (4.1)</td>
<td>-9.7 (3.4)</td>
<td>-8.4 (2.5)</td>
<td>13.9 (1.3)</td>
<td>14.6 (1.5)</td>
<td>16.1 (1.6)</td>
</tr>
</tbody>
</table>

a Values are expressed as means, with standard errors of the means in parentheses.

Results

Electrical parameters were measured to ensure that the tissue was viable within the system and that tissues were comparable in their electrical characteristics. At pH 7.4, PD increased (became more negative) in the first 30 min of experiments as the tissue rewarmed, remained stable for the next 30 min, and fell slowly in the last 30 min (Table 1). Conductance remained stable during the time course of the experiments. Lowering mucosal pH was associated with a change in PD, though not in conductance (Table 1), except at pH 2.2, where significantly higher conductance values were observed (P < 0.01). This change in conductance coincided with the appearance of macromolecular erosions on the mucosal surface. Stimulation of the model with 0.1 mM histamine and carbachol resulted in a significant fall in PD of 6.8 (0.1) mV (standard errors of the means are indicated in parentheses throughout) over 15 min compared to controls of 2.2 (0.1) mV (P < 0.01). There was also a significant fall in conductance, which was 1.14 (0.05) mS cm⁻² compared to 0.04 (0.01) mS cm⁻² in controls (P < 0.01). The electrical characteristics of mucosae in 0.9- and 1.2-cm chambers were very similar, although reduction of chamber diameter to 0.4 cm resulted in a significant fall in PD (P < 0.01) and a significant increase in conductance (P < 0.05) (Table 2).

Under baseline conditions, metronidazole was easily detected in the mucosal chamber 15 min after addition to the serosal chamber (Fig. 1). Metronidazole J₃₋M was correlated with serosal concentration (P < 0.001), and although reservoir chamber concentrations varied between experiments from 0.20 (0.01) to 0.39 (0.01) mmol/liter, they did not differ between controls and test preparations within experiments. Metronidazole J₃₋M was not significantly different from metronidazole J₅₋S or from J₅₋S under stimulated conditions (Fig. 2). Use of 0.9- or 1.2-cm-diameter chambers had no significant effect on J₅₋-S, although flux was significantly higher with the 0.4-cm-diameter chamber (P < 0.01) (Fig. 2).

Metronidazole J₄₋-S fell as pH decreased (Fig. 3), while total mucosal concentration was constant at 0.80 mmol/liter. Metronidazole has a pKa of 5.25 (5), and hence the mucosal pH values of 2.2, 2.7, 3.2, and 7.4 will result in un-ionized mucosal concentrations of approximately 0.26, 0.48, 0.67, and 0.80 mmol/liter according to the Henderson-Hasselbalch equation. Metronidazole J₅₋-S was correlated with this un-ionized metronidazole concentration (P < 0.01), and when the results for pH 2.2 were excluded because of the macroscopic and conductance changes seen, the correlation strengthened further (P < 0.001).

Amoxicillin J₃₋-M was significantly (P < 0.01) less than metronidazole J₃₋-M, being 0.50 (0.07) nmol h⁻¹ cm⁻² and 3.98 (0.24) nmol h⁻¹ cm⁻², respectively, with a serosal concentration for both antibiotics of 0.20 mmol/liter.

Discussion

The results from this series of experiments show that this in vitro animal model can be used to assess the transfer of antibiotics across the gastric epithelium as well as the influence of intragastric pH on such transfer.

Resting electrical parameters were consistent with those shown by other investigators using a similar chamber system to investigate ionic transport (7, 14), suggesting that the mucosa is viable for the period under investigation and that normal epithelial ionic transport processes are present. The PD is the electrical gradient created as a result of numerous ionic movements across the epithelium, and where the concentrations of specific ions are changed (for example, by stimulation of a...
transport process), the observed PD will also change. Conductance is a measure of the integrity of the epithelial tight junctions, and increases in conductance imply epithelial damage. As the main purpose of the study was to investigate antibiotic transport, no attempt was made to identify the specific ion flux changes responsible for any changes in PD observed during the course of the experiments. It is therefore difficult to speculate as to the mechanisms behind the fall in PD seen with decreasing mucosal pH or with stimulation with histamine and carbachol. However, where large changes in PD were observed with significant increases in conductance, for example, with the 0.4-cm-diameter chamber and following the reduction of mucosal pH to 2.2, it can reasonably be assumed that significant epithelial damage has occurred, and antibiotic flux results under these conditions should be viewed with caution.

Several features of the model mirror the results from previous studies investigating antibiotic transfer across the human gastric mucosa. Firstly, metronidazole crossed the gastric mucosa easily and was readily detected (5, 15, 16). Secondly, metronidazole underwent ionic trapping on the mucosal side, a direct confirmation of the pH partition hypothesis (15) and a confirmation of previous findings with human volunteers (5, 17). Thirdly, amoxicillin J_{S→M} was significantly less than metronidazole J_{S→M}. Amoxicillin was chosen for comparison, as both antibiotics can be detected in human gastric juice after intravenous administration but in very different amounts (5), since amoxicillin penetrates the human gastric epithelium poorly (9). Thus, the two drugs can be used to demonstrate the selectivity of the model for different drugs.

No evidence was found for unidirectional active transport of metronidazole (17), implying that this drug crosses the gastric mucosa by simple diffusion, although this cannot be stated absolutely, as not only was equilibrium not reached, but increased acid secretion following stimulation was not demonstrated. Equilibrium was probably not reached because, unlike in vivo human studies where the mucosal volume-to-area ratio is small, only a small and poorly perfused area is available to a relatively large volume. Decreasing the volume-to-area ratio by using smaller volumes would compromise tissue viability by decreasing the availability of oxygen and glucose. Previous experiments using rat gastric mucosa under similar conditions have shown that stimulated acid secretion is around 2 to 5 μeq·h⁻¹·cm⁻² (3, 7) in unbuffered solution. If metronidazole were secreted in an equimolar manner, it is therefore likely that this would be detected in this model.

Another criticism of the model is that it is neither infected nor inflamed, as would be the case in vivo. However, as mice can be readily infected with Helicobacter species, an infected rat model could be designed to assess antibiotic transfer (since the mouse stomach has too small a surface area to be useful in this model). An alternative would be to use ferrets, which not only have a stomach with a large surface area but also harbor a natural Helicobacter infection (H. mustelae) causing gastritis and ulceration.

There are numerous applications for the model in the development of new treatments for H. pylori infection. There is now reasonable evidence that most antimicrobial agents (bismuth being the notable exception) reach the organism in its niche from the systemic circulation (5, 6, 12). Therefore, the model can be used to screen available antimicrobial agents to assess their ability to achieve this, prior to testing in humans. Although other models, such as gastric biopsy explants (11), can examine antibiotic uptake, because the present model can quantitatively compare transmucosal flux, it can be used to identify the physicochemical features of antibiotics which affect systemic delivery to the gastric mucosa. The elucidation of

FIG. 1. Mucosal chamber metronidazole concentration-time curve following application of 0.20 mmol of metronidazole/liter in the serosal chamber.

![Graph showing metronidazole concentration over time](image1)

FIG. 2. Metronidazole J_{S→M} in 1.2-cm-diameter chambers at pH 7.4 compared to J_{S→M} with reservoir chamber concentrations of 0.34 mmol/liter (a), J_{S→M} following stimulation with 0.1 mmol of histamine and carbachol/liter with serosal concentrations of 0.39 mmol/liter (b) and J_{S→M} in chambers of 0.9- and 0.4-cm diameters with serosal concentrations of 0.20 mmol/liter (c). **, P < 0.01.

![Graph showing metronidazole flux](image2)

FIG. 3. Metronidazole J_{S→M} in 1.2-cm-diameter chambers at varying mucosal pHs and a constant mucosal chamber metronidazole concentration of 0.80 mmol/liter. *, P < 0.05; **, P < 0.01.
these features will allow for better selection and even the
design of novel agents against *H. pylori*. This is an especially
pertinent problem in view of the increasing indications for *H.
pylori* eradication and the increase in antimicrobial resistance.

ACKNOWLEDGMENTS

This work was supported by a Clinical Research Fellowship from
Astra Hassle AB, Göteborg, Sweden.

We are grateful to Mohamed Jessa, Roger Knaggs, Dave Barrett,
Nick Shaw, Dave Reffin, Billy Stack and Chris Hawkey for their assis-
tance in these studies.

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model for antibiotic transport across gastric mucosa: inhibitory tissue con-
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