STUDIES ON THE MECHANISMS OF PEPTIC ULCERATION

Thesis submitted in accordance with the requirements of the University of London for the Degree of Doctor of Medicine

by

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ABSTRACT
The pathologic mechanisms behind two risk factors for peptic ulceration have been considered:

1. Non-steroidal anti-inflammatory drugs (NSAIDs), which are associated with gastric as well as duodenal damage, and
2. Infection of the gastric antrum with Helicobacter pylori (HP), which is strongly associated with duodenal ulcer (DU).

NSAIDs and the gastroduodenal mucosa
At low doses, the normal gastric and duodenal epithelia in man and the rat adapt to NSAID administration by increasing the rate of cell turnover. In man this is accompanied by an increase in gland and crypt duplication by fission. However, indomethacin exacerbates and reduces the rate of healing of experimentally induced gastric ulcers in the rat by specifically inhibiting the regenerative response at the ulcer edge. Likewise patients on NSAIDs with gastric and duodenal ulcers showed markedly lower rates of regeneration at the ulcer edge than patients with ulcers at the same locations not taking NSAIDs. This may explain the greater prevalence of ulcers in patients taking NSAIDs.

Large doses (300 μg/kg/d) of misoprostol, a prostaglandin El analogue, reversed the indomethacin-induced inhibition of regeneration at the ulcer edge in the rat. However, in usual therapeutic doses (approx. 10 μg/kg/d) misoprostol failed to influence gastroduodenal proliferation in rat or man.

Helicobacter pylori, duodenal ulcers and gastrin
We have also considered the relationship between antral HP colonisation, antral gastrin release and gastric acid secretion. I show that pentagastrin stimulated gastric acid secretion and postprandial plasma gastrin concentrations are significantly higher in HP+ve compared with HP-ve patients with DUs. Furthermore, eradication of antral HP significantly reduces postprandial plasma gastrin responses. These findings may explain the strong association of DUs with antral HP infection, and the lower relapse rates of DU following treatments which eradicate this organism from the antrum.
This Thesis is dedicated to my parents
CHAPTER 2
METHODOLOGY IN THE ASSESSMENT OF GASTROINTESTINAL PROLIFERATION IN THE RAT

Introduction 25

Methods 29
Animals 29
Cell production rate 29
Quantitative in vivo 3H-thymidine method 30
Quantitative in vitro 3H-thymidine method 31
DNA assay (fluorimetric method) 32
BRdU-anti-BRDU method 32

Results 32
Cell production rate 32
Quantitative in vivo 3H-thymidine method 33
Profile of gastrointestinal DNA labelling in vivo 33
3H-TdR disappearance curves 33
Quantitative in vitro 3H-thymidine method 33
Profile of gastrointestinal DNA labelling in vitro 33
Time course of in vitro labelling 33
Acid-base status of incubation mixture 34
Effect of volume of incubation mixture 34
Determination of non-specific background labelling 34
Effect of supporting metallic grid 35
BRdU-anti-BRDU method 35
CHAPTER 3
METHODOLOGY IN THE ASSESSMENT OF GASTROINTESTINAL PROLIFERATION IN MAN

Introduction

Methods
Ethical considerations
Endoscopies
Quantitative in vitro 3H-thymidine incorporation into human endoscopic gastric and duodenal biopsies
In vitro mitotic arrest in human endoscopic gastric and duodenal biopsies
Ki-67 monoclonal antibody immunohistochemical method as applied to human endoscopic gastric and duodenal biopsies
Microdissection of gastric glands and duodenal crypts in the estimation of gland and crypt morphometry and cell proliferative activity in endoscopic biopsies

Results
Quantitative in vitro 3H-thymidine incorporation into human endoscopic gastric and duodenal biopsies
In vitro metaphase arrest method
Ki-67 monoclonal antibody immunohistochemical method
Microdissection method

Discussion

CHAPTER 4
ACUTE EFFECTS OF INDOMETHACIN ON RAT GASTROINTESTINAL INTEGRITY AND PROLIFERATIVE ACTIVITY

Introduction

Methods
Preliminary pilot studies
In vivo proliferation study
Assessment of gastric mucosal erosions
Assessment of small intestinal and colonic lesions
Assessment of DNA specific radioactivity
CHAPTER 5
CHRONIC EFFECTS OF INDOMETHACIN AND MISOPROSTOL ON RAT GASTROINTESTINAL INTEGRITY AND PROLIFERATIVE ACTIVITY: A STUDY OF GASTRIC ADAPTATION

Introduction

Materials

Methods

Chronic indomethacin toxicity study
Effect of indomethacin on the rate of disappearance of 3H-TdR from the gastric corpus
Study of chronic effects of misoprostol

Results

Chronic indomethacin toxicity study
Effect of indomethacin on the rate of disappearance of 3H-TdR from the gastric corpus
Study of chronic effects of misoprostol

Discussion

CHAPTER 6
EFFECTS OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS AND MISOPROSTOL ON GASTRIC AND DUODENAL PROLIFERATION IN PATIENTS WITH ARTHRITIS

Introduction

Materials and Methods

Ethical considerations
Criteria for inclusion
Criteria for exclusion
Study design
Endoscopies, endoscopic lesion assessment, and biopsies
CHAPTER 7
EFFECT OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS AND MISOPROSTOL ON GASTRIC AND DUODENAL ULCER HEALING THROUGH REGENERATION

Introduction 88

Materials 89

Methods 89
  Animal studies: 89
    Ulcer induction 89
    Pilot studies 90
    Experimental design 90
    BRdU-anti-BRdU method for assessment of proliferative activity at the ulcer edge 91

  Human peptic ulcer study: 91
    Endoscopies and biopsies 91
    Analysis of proliferation in endoscopic biopsies 92

Results 92
  Animal studies 92
  Human peptic ulcer study 93

Discussion 95
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>156</td>
</tr>
<tr>
<td>2.2</td>
<td>157</td>
</tr>
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<td>2.3</td>
<td>158</td>
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<td>2.4</td>
<td>159</td>
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<tr>
<td>2.5</td>
<td>159</td>
</tr>
<tr>
<td>2.6</td>
<td>160</td>
</tr>
<tr>
<td>2.7</td>
<td>161</td>
</tr>
<tr>
<td>2.8</td>
<td>161</td>
</tr>
<tr>
<td>3.1</td>
<td>162</td>
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<tr>
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<td>165</td>
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<td>3.5</td>
<td>166</td>
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<td>3.6</td>
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<tr>
<td>3.7</td>
<td>168</td>
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<td>191</td>
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</tbody>
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The studies and experiments presented in this Thesis were designed by the author in conjunction with Dr. Humphrey Hodgson (Chapters 2-7), and Dr. John Calam (Chapter 8). The author performed all the animal experiments, directly ran the human studies, performed all the endoscopies in Chapter 2-6, two-thirds of the endoscopies in Chapters 7 and 8, analysed all the biopsy material personally, and processed the data. All the routine histological sections as well as the immunostains were examined by the author; Kate Beardshall performed the gastrin radio-immunoassays (Chapter 8); Sister M.L. Francis Reme and her endoscopy staff performed the studies of acid secretion and the standard meal tests (Chapter 8); Drs. Raymond Playford, William Foulkes, Prodyot Ghosh, Ian Swift and Lawrence Desa together provided one-third of the endoscopic biopsies for the studies in Chapter 8. In Chapter 7, the mechanistic studies presented were performed within the framework of a large U.K. multicentre study of the incidence of NSAID-associated complications in patients with arthritis, and the efficacy of this drug in the healing and prevention of NSAID-induced gastric and duodenal complications. This study was co-ordinated by the U.K. and
The following publications have arisen directly from work in this Thesis.


Material from this Thesis has been presented by the author at the following *oral presentations*:-
1. The British Society of Gastroenterology, Bradford, April 1989: Indomethacin toxicity may be explained by its effects on gastric and duodenal proliferation.


My thanks to the British Digestive Foundation for their financial support for these studies.

Thanks also to my fiancée Cherie for her constant encouragement and support.
In this Thesis I have attempted to achieve the following:

1. To familiarise myself with the methodology in the assessment of gastrointestinal proliferation in the rat, and develop techniques for the assessment of epithelial turnover in endoscopic biopsies of gastrointestinal mucosa in man;

2. To use this methodology to determine the effects of non-steroidal anti-inflammatory drugs (NSAIDs) and misoprostol (a stable prostaglandin E1 analogue), on gastric and duodenal epithelial turnover in the rat and in man;

3. To determine the effects of NSAIDs on the regenerative healing response to gastric and duodenal ulceration in the rat and in man; and

4. To examine the effects of colonisation of the human gastric antrum with Helicobacter pylori on gastrin release and acid secretion in patients with duodenal ulcer disease.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>AI</td>
<td>Adaptation index</td>
</tr>
<tr>
<td>BRdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>CCT</td>
<td>Cell cycle time</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
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<tr>
<td>CCPR</td>
<td>Crypt cell production rate</td>
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<td>C:V ratio</td>
<td>Crypt : Villus ratio</td>
</tr>
<tr>
<td>DC</td>
<td>Duodenal cap</td>
</tr>
<tr>
<td>D2</td>
<td>Second part of duodenum</td>
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<tr>
<td>DAB</td>
<td>Diaminobenzidine tetrahydrochloride</td>
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<tr>
<td>DAPI</td>
<td>4,6-diamidino-2-phenylindole.2HCl</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Minimal Essential Medium</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>EDTA</td>
<td>Ethylene diamine tetra-acetic acid</td>
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<td>Fc</td>
<td>'Fragment crystallizable'</td>
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<td>GF</td>
<td>Growth fraction</td>
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<td>HP</td>
<td>Helicobacter pylori</td>
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<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
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<tr>
<td>3H-TdR</td>
<td>Tritiated thymidine</td>
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<tr>
<td>indo</td>
<td>Indomethacin</td>
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<tr>
<td>i.p.</td>
<td>Intraperitoneally</td>
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<tr>
<td>kg</td>
<td>Kilogram</td>
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<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
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<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
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<tr>
<td>PAP</td>
<td>Peroxidase-anti-peroxidase</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<td>RI</td>
<td>Regeneration index</td>
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<td>s.c.</td>
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<td>uCi</td>
<td>MicroCuries</td>
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CHAPTER 1

GENERAL INTRODUCTION

Peptic ulcers of the stomach and duodenum are amongst the commonest diseases in gastroenterological practice. They result in considerable morbidity, and also a significant mortality from haemorrhage and perforation (Armstrong and Blower, 1987; Jick et al., 1987; Somerville et al., 1986).

Gastric and duodenal integrity is maintained by a complex set of mucosal 'defensive' properties. These properties usually successfully override the continual damaging effects of various 'aggressive' luminal and environmental factors. The balance between defence and aggression is, however, sometimes reversed in favour of the latter, with resulting mucosal damage and ulceration. In this thesis the pathologic mechanisms behind two different risk factors for peptic ulceration are considered:

1. Non-steroidal anti-inflammatory drugs (NSAIDs), which are associated with gastric as well as duodenal ulcers, and

2. Infection of the gastric antrum with Helicobacter pylori, which is strongly associated with duodenal ulceration.

NSAIDs are the mainstay in the treatment of arthritis. They are widely prescribed, often for chronic consumption for months or years, for patients with arthritis and other musculoskeletal disorders. The gastrointestinal tract bears the brunt of the side effects of these preparations. Gastric side effects, inflammation, ulceration and haemorrhage, have been recognised (Somerville et al., 1986; Clinch et al., 1987; Armstrong and Blower, 1987; McCarthy, 1989). Elderly persons, particularly females, appear to be at greatest risk (Lamy, 1987; Walt et al., 1986). Small intestinal side-effects - inflammation, ulceration, perforation and stricture formation - have also recently emerged (Bjarnason et al., 1984, 1986, 1987, and 1988).

In the United Kingdom alone there are currently 23 NSAIDs available for use in 64 proprietary preparations made by over 30
pharmaceutical companies. Aspirin as well as some non-aspirin NSAIDs are available for unregulated use "over the counter". An ulcerogenic potential appears to be common to all these agents. Several studies have indicated that patients with bleeding peptic ulcers are as much as 3 - 6.7 times as likely to be NSAID users than either their hospital or community controls. An overview of these studies follows.

Epidemiological evidence of the side effects of NSAID treatment have been obtained from four major sources:

1. Spontaneous reports of adverse drug events to official national agencies;
2. Retrospective case-control clinical enquiries;
3. Prospective (surveillance) clinical studies; and
4. Experimental evidence in human volunteers and patients.

Various official national agencies receive spontaneous reports from clinicians on adverse reactions to drugs. There are admittedly a number of limitations on the conclusions that can be drawn from data from this source, because of non-random sampling, low reporting rates, and a poorly defined target population. Nevertheless, it is clear that adverse reactions to NSAIDs are far more commonly reported to the United Kingdom Committee on the Safety of Medicines, as well as to the United States Food and Drug Administration, than for any another group of drugs (Griffin, 1986; Rossi et al, 1987). Most reports concern the elderly, in whom the use of NSAIDs is far more prevalent (Jick et al, 1987), and in whom the incidences of peptic ulceration (Glynn and Kane, 1985), and ulcer complications (Walt et al, 1986), have been increasing. Thus, for women over the age of 65, the annual number of prescriptions for NSAIDs increased 3-fold between 1967 and 1982, during which time the rates of perforation of duodenal ulcers doubled for those aged 65-74 years, and more than trebled for those aged 75 and over. For men over 65 years, prescriptions showed a similar increase, and although perforation rates actually fell for those aged 65-74 years, they sharply increased in those aged over 75 years. Perforation rates fell during this period in all other age groups, despite annual increases in the number of prescriptions (Walt et al, 1986). These findings suggest that, against a falling incidence of
peptic ulcer perforation in younger persons, particularly men, factors predisposing to perforation have been increasing in the elderly, and that the use of NSAIDs may be one of these.

Retrospective case-control clinical enquiries as well as prospective studies have provided more meaningful data on the gastrointestinal risks of NSAID treatment. The conclusions drawn from retrospective studies are, of course, often open to question because of inaccuracies and patient bias in retrospective symptom reporting, and because of the difficulty in obtaining suitably matched controls. Prospective (cohort surveillance) studies provide less biased information, but are costly and time-consuming. In order to detect small differences in risk, very large numbers of patients need to be studied. Nevertheless, taken together, the evidence from retrospective and prospective studies in support of the gastric and duodenal damaging effects of NSAIDs is convincing (Table 1).

Two large retrospective studies deserve mention. The first, a study in Australia, was conducted by McIntosh et al (1985). These authors found a strong association of gastric ulcer with NSAID consumption. In this study there were 104 patients and 208 controls, and the relative risk of gastric ulcer attributable to NSAIDs was 4.7. Clinch et al (1987) performed a further study of 175 patients and demonstrated a 6.7 fold increase in risk of peptic ulceration in NSAID users of either sex.

Various prospective studies, many of much larger magnitude, have also addressed the gastrointestinal risks of NSAID use (Guess et al, 1988; Inman 1985; Jick 1985; Jick et al 1987; Carson et al 1987a; Griffin et al, 1988). In the United Kingdom, Inman (1985) showed that NSAID users were more likely to experience dyspepsia, and to develop gastritis, than non-users, but peptic ulceration did not appear to be more common in NSAID users. However, the response rate to his questionnaires was just over 50%. Jick et al (1987), also in the United Kingdom, found an age- and sex-matched rate ratio of upper gastrointestinal perforation for NSAID users versus non-users of 1.6. Two studies (Guess et al, 1988; Griffin et al, 1988) addressing the risks of death from peptic ulceration in elderly NSAID users compared with age- and sex-matched controls, found identical
relative risks of 4.7, with confidence intervals of 1.7-13.6 and 3.1-7.2 respectively (Table 1).

It is incorrect and misleading, in my view, to take the following approach in determining the risk of gastric and duodenal ulceration in NSAID users. It seems clear that the crude prevalence estimate for GU in arthritic NSAID users is approximately 13% (reviewed by Langman, 1989 and by McCarthy, 1989). This has been compared with a GU prevalence estimate of 0.28% (Kurata, 1983) in the 'normal' population to yield a relative risk of x 46, i.e. a 46-fold greater risk of GU in the group. The crude prevalence estimate for DU of 11%, when compared with a prevalence of DU in the 'normal' population of 1.4% (Kurata, 1983), yields a relative risk for DU of x 8. However, I have serious doubts about this line of reasoning. Firstly, none of the studies quoted above were truly prospective. Secondly, the 'normal' population is an inappropriate control for patients with arthritis. Third, in some patients peptic ulcers may have antedated NSAID therapy; they may well have been activated by the disease that led to their use; or indeed may have occurred for entirely unrelated reasons (eg. smoking) during the period of NSAID therapy. For these reasons the computation of crude relative risks in this way probably over-estimates the true ulcer risk attributable to NSAID use.

A particularly alarming aspect of NSAID-associated peptic ulceration is that many of these patients present for the first time with a life-threatening complication. Thus, in one Canadian study, only 15% of patients with upper GI bleeding complained of abdominal pain (Bartle et al, 1986). In a large British study of 235 consecutive hospital admissions with life-threatening complications of peptic ulceration, the first sign of an ulcer in 58.2% of patients was the life-threatening complication itself - either bleeding or perforation (Armstrong and Blower, 1987). In this study, of the 78 patients who died, 78% were NSAID users. This represented a greater than 2-fold increase in mortality compared with non-users.

The most convincing data on the acute toxicity of aspirin and other NSAIDs have emerged from endoscopic studies of healthy human subjects. An extensive range of acute NSAID-induced lesions has
been noted in the stomach and duodenum, including subepithelial haemorrhages, erosions and superficial ulcers (Jiranek et al, 1989).

The mechanism of NSAID-induced gastric damage is not generally agreed, but is almost definitely multifactorial. The mucus-bicarbonate barrier is known to protect the gastric mucosa (Takeuchi, 1983). There is good evidence for a direct contact-induced disruptive action by NSAIDs, especially in the case of aspirin (Holt, 1960; Baskin et al, 1976; Lanza et al, 1979; Ivey et al, 1980). NSAIDs also inhibit mucosal cyclooxygenase, thereby reducing the production of protective prostaglandins (Whittle, 1981; Duggan, 1981; Dajani, 1986) and interfering with the process of 'cytoprotection' in the gastric mucosa. 'Cytoprotection' is a homeostatic mechanism, and may be defined as the ability of the gastric mucosa to resist injury through mechanism(s) independent of acid inhibition. 'Adaptive cytoprotection' is a term coined by Jacobson (1986); this is initiated by damage and limits its extent. Adaptive cytoprotection is abolished by indomethacin, a potent inhibitor of prostaglandin synthesis. Prostaglandin mediated cytoprotection of the gastric mucosa is thought to effected by a number of mechanisms, of which an increase in the mucus-bicarbonate barrier (Rees et al, 1983), maintenance of intercellular tight junctions (Meeroff, 1985), and an increase in mucosal blood flow (Leung et al, 1985) have been emphasised.

Recently it has been recognised that, in addition to their cytoprotective effects, prostaglandins may be important stimulators of gastrointestinal proliferation (Assad et al, 1985; Goodlad et al, 1989). This reversed earlier reports of no proliferative effects (Fich et al, 1985; Romain et al, 1987) or even decreased proliferative indices (Arber et al, 1985). Goodlad et al (1989) showed that misoprostol, a synthetic prostaglandin E1 analogue, induces hyperplasia in the gastric mucosa, and an increase in the number of mitotic cells per gland. This suggests that the effects of NSAIDs on mucosal cell renewal should also be considered. If NSAIDs did indeed reduce the mucosal turnover rate, as might be predicted by the depletion of mucosal prostaglandins, this would place these drugs in the unique position of inducing damage by direct disruptive effects as well as by inhibiting the natural mucosal regenerative response to this damage. These investigations have
already been initiated in the rat (St.John et al, 1973; Eastwood and Quimby, 1982; Baumgartner et al, 1986; Kuwayama and Eastwood, 1988), the dog (Hurley and Crandall, 1964; Levy et al, 1982; Hurley and Crandall, 1983) and in man (Kuwayama and Eastwood, 1988; Graham et al, 1983; Graham et al, 1988a), but uniform conclusions have failed to emerge. For example, the same group in different situations have found increases (Baumgartner et al, 1986) as well as decreases (Inauen et al, 1988) in gastric proliferation during indomethacin treatment, and there has been disagreement about the effects of NSAIDs on antral proliferation (Eastwood and Quimby, 1982; Baumgartner et al, 1986). This undoubtedly reflects the difficulties of assessing gastrointestinal turnover, which in man have made matters almost prohibitively difficult in this important area of gastrointestinal research.

Chapters 2-7 of this thesis are concerned with the effects of NSAIDs on gastric and duodenal integrity and proliferative activity. Chapters 2 and 3 are devoted to the studies of various direct and indirect methodological approaches in the assessment of gastrointestinal turnover in the Wistar rat, and in man, respectively. Epithelial turnover in the rat was assessed using a stathmokinetic method, as well as by quantitative in vivo and in vitro 3H-thymidine (3H-TdR) incorporation into mucosal DNA. Bromodeoxyuridine (BRdU) labelling in vivo was also used to assess proliferative activity as a monoclonal antibody to this nucleotide has enabled the detection of BRdU in tissue sections. In man, use was made of another monoclonal antibody, Ki-67, directed against an unidentified nuclear antigen in dividing human cells, to assess proliferative activity in endoscopic biopsies of the stomach and duodenum. Native gland and crypt microdissection, with counting of native mitoses combined with morphologic and morphometric assessments of gastric glands and duodenal crypts, is developed as a readily applicable research tool suitable for the study of proliferative activity in endoscopic biopsies in man.

Chapters 4 and 5 present studies of the effects of indomethacin and misoprostol, a new prostaglandin E1 analogue, on gastric and duodenal epithelial turnover in the Wistar rat. In Chapter 4 the acute effects of indomethacin in various doses are presented, and erosive damage is related to simultaneous effects on epithelial
turnover. In Chapter 5 the long term effects of indomethacin and misoprostol on gastric and duodenal proliferative activity are presented, and effects of these drugs on gastric adaptation through enhancement of epithelial turnover are considered.

Chapter 6 presents the results of clinical studies of gastric adaptation to NSAIDs, and here I also investigate whether misoprostol, in the relatively low doses tolerated by man, influences the gastric proliferative response to NSAIDs. Endoscopies were performed before and after specified periods of treatment with NSAIDs in the presence and absence of misoprostol. The effects of these agents on gastric and duodenal epithelial turnover were studied, using the microdissection methodology developed in Chapter 3.

Chapter 7 is concerned with the regenerative response of the gastric mucosa in an experimental gastric ulcer model. Gastric ulcers were induced by thermal injury, and rates of healing and the associated regenerative response at the ulcer edge studied in animals that had been pre-treated with indomethacin and/or trophic doses of misoprostol. These results prompted the investigation of regenerative rates at ulcer edges in man, in patients on and off NSAIDs, again using the microdissection methodology developed in Chapter 3.

Chapter 8 concerns Helicobacter pylori (HP), a spiral gram negative bacterium, which has a remarkable ability to colonise the human stomach. Since its isolation from the human gastric antrum by Barry Marshall, this organism has stimulated much interest and research, because of its very strong association with chronic active gastritis (Marshall, 1983; Warren, 1983; Warren and Marshall, 1984) and with duodenal ulceration (Warren and Marshall, 1984; Dooley and Cohen, 1988). Nearly 100% of patients with non-immune gastritis are colonised with HP (Hazell et al, 1987), and there are two reports in the literature of deliberate human ingestion of the organism leading to a persistent gastritis with antral colonisation (Marshall et al, 1985; Morris and Nicholson, 1987).

Even more intriguing is the relationship of this organism with duodenal ulcer disease, because HP can colonise only gastric
epithelium, and except in situations of duodenal gastric metaplasia, is not found in the duodenum.

Yet there is very strong epidemiological evidence to support a relationship between antral HP colonisation and DU disease. Firstly, HP colonisation occurs in the majority of patients with DU disease (Dooley and Cohen, 1988). Secondly, longer remissions of the disease are obtained with treatments that suppress or eradicate HP than with those that do not (Martin et al, 1981; Marshall et al, 1988; Smith et al, 1988). Finally, after eradication of HP, ulcers rarely occur before recolonisation with the organism (Coghlan et al, 1988; Marshall et al, 1988; Smith et al, 1988).

The important question, therefore, is how antral HP infection causes ulcers in the duodenum. The prevalent view (reviewed by Goodwin, 1988 and by Graham, 1989) stresses the importance of heterotopic gastric mucosa in the duodenal cap in patients with DU disease. HP, with an apparent requirement for gastric surface mucous cells, colonises these areas of duodenal gastric metaplasia, resulting in a localised patch of gastritis which progresses and eventually leads to a mucosal break at or adjacent to this site. In my view this sequence of events is at best of secondary importance. There is good evidence that the presence of gastric mucosa in the duodenum is an expected response to the increased duodenal acid load found in many patients with DU disease (Kreuning et al, 1978). This is supported by animal studies (Rhodes, 1964; Florey et al, 1939) where an increase in the prevalence and extent of gastric mucosa in the duodenum followed establishment of a chronic hyperacidic state or addition of gastric juice to intestinal contents. This may also be the case in man, as duodenal gastric metaplasia has been found in 84% of patients with DU disease with maximal acid outputs over 30 mmol/h (Patrick et al, 1974). A more plausible hypothesis is that gastric metaplasia in the duodenum occurs in response to duodenal injury, ie. it may reflect the fact that a DU has been present and has healed (Patrick et al, 1974). HP infection of this heterotopic gastric mucosa may then serve to perpetuate duodenal inflammation, and in this way determine the site of subsequent ulcers. A more fundamental question is why patients with DU disease have the increased acid secretion which leads to duodenal damage. Gastric acid secretion is fundamentally implicated in the pathogenesis of DU
disease. Firstly, it is established that mean basal, peak stimulated and meal stimulated gastric acid secretion rates are higher in patients with DU disease than in normal subjects (Wormsley and Grossman, 1965; Isenberg et al, 1975; Baron, 1978; Jalan et al, 1979; Lam and Ong, 1980). Secondly, DUs are rarely seen in individuals with low gastric acid secretion rates (peak acid output less than 15 mmol/hour) (Baron, 1982). Finally, suppression of gastric acid secretion by H2-receptor antagonists (Bardhan et al, 1979) and omeprazole (Lamers et al, 1984) leads to rapid healing of DUs.

In my studies I have considered the relationship between antral HP colonisation, antral gastrin release and gastric acid secretion. It is known that mean basal, peak pentagastrin-stimulated, and meal-stimulated gastric acid secretion rates are higher in patients with DU disease than in normal subjects (Walsh et al, 1975). Patients with DU disease also tend to have higher peak post-prandial plasma gastrin concentrations than normal subjects (Taylor et al, 1979 and 1981). At first sight the involvement of HP in DU disease might seem to be at variance with the established view that DU disease is due to the tendency to inappropriate gastrin release and increased gastric acid secretion. I therefore tested the unifying hypothesis that HP in the gastric antrum increases acid release and thereby acid secretion. In Chapter 8 I present my studies of gastrin release, gastric acid secretion, and the presence or absence of antral HP in patients with established DU disease. The effect of HP eradication on acid secretion and gastrin release is also presented.
**TABLE 1.1.** Relative risks of peptic ulcers and their complications in NSAID users

<table>
<thead>
<tr>
<th>Ulcer Occurrence</th>
<th>RR</th>
<th>95% CI</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric ulcer</td>
<td>5.0</td>
<td>1.4-26.9</td>
<td>Duggan <em>et al</em> (1986)</td>
</tr>
<tr>
<td></td>
<td>4.7</td>
<td>1.3-16.6</td>
<td>McIntosh <em>et al</em> (1985)</td>
</tr>
<tr>
<td>Duodenal ulcer</td>
<td>1.1</td>
<td>0.4-3.7</td>
<td>Duggan <em>et al</em> (1986)</td>
</tr>
</tbody>
</table>

**Ulcer complications**

<table>
<thead>
<tr>
<th>Complication</th>
<th>RR</th>
<th>95% CI</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleeding</td>
<td>3.8</td>
<td>2.2-6.4</td>
<td>Somerville <em>et al</em> (1986)(a)</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>1.1-4.9</td>
<td>Carson <em>et al</em> (1987a)(b)</td>
</tr>
<tr>
<td>Perforation</td>
<td>1.6</td>
<td>0.7-3.7</td>
<td>Jick <em>et al</em> (1987)</td>
</tr>
<tr>
<td></td>
<td>4.7</td>
<td>1.7-13.6</td>
<td>Guess <em>et al</em> (1983)(c)</td>
</tr>
</tbody>
</table>

RR, relative risk; CI, confidence interval; (a) hospital controls; (b) community controls; (c) risk was concentrated only in those without recent gastrointestinal symptoms.

**Note on statistical methods**

**Chapters 2-5**
In these studies, all tests of statistical significance were performed with parametric analysis, using the t-test.

**Chapters 6-8**
Adaptation and regeneration indices, being ratios, were analysed non-parametrically using the Wilcoxon test. In Chapter 8 the Wilcoxon matched pairs test was used to analyse differences in peak acid output and integrated plasma gastrin response. In this Chapter mean (SEM) are given for the purposes of illustration only.
CHAPTER 2

METHODOLOGY IN THE ASSESSMENT OF GASTROINTESTINAL PROLIFERATION IN THE RAT

INTRODUCTION

The gastrointestinal tract contains rapidly renewing epithelial tissue, and methodology in the study of gastrointestinal mucosal cell turnover has generated much debate with regard to the pros and cons of the various different approaches that are in use.

Cell renewal in the gastrointestinal epithelium occurs in more or less discrete reproductive zones, from which cells migrate, differentiate, and eventually die or are lost from the surface (Messier and Leblond, 1960). The reproductive zones in which cell renewal occurs are the neck and isthmus of the gastric glands (Willems, 1971; Eastwood, 1977) and the basal two-thirds of the colonic crypts and the crypts of Lieberkuhn of the small intestine (Cairnie et al, 1965; Sunter et al, 1979a; Sunter et al, 1979b).

The process of cell division consists of four continuous phases (G1, S, G2, M) which comprise the cell (renewal) cycle (Lipkin and Bell, 1968; Baserga, 1976). Interphase (G1) cells commence DNA synthesis (S phase), which is followed by the pre-mitotic gap (G2) and mitosis (M phase). Cells can 'rest' in either G1 or G2 (Lipkin, 1973), but usually do so in G1 and are then called G0 cells (Gelfant, 1977; Baserga, 1976). Cells in G1 may differentiate, thus leaving the cell cycle (Quastler and Sherman, 1959).

In the gastric gland or intestinal crypt, undoubtedly the best parameter of proliferative activity is the cell production rate per gland or crypt. There are three independent factors which determine the cell production rate (Wright, 1984a):-

1. The cell cycle time (CCT).
2. The growth fraction (GF), that is, the fraction of cells that are proliferating.
3. The crypt population size (CP).
There are additional, three dimensional considerations particularly in the case of the small intestine where it can been argued that since each villus receives new cells from several crypts (for example, the crypt : villus ratio is about 6 in the human), the final arbiter of cell production should be the net villus influx (Wright, 1984a). Thus, if CCPR is the crypt cell production rate,

$$\text{CCPR} = \frac{\text{GF} \times \text{CP}}{\text{CCT}}$$

Net villus influx = CCPR x C:V ratio

Cell cycle times clearly vary from one part of the gastrointestinal tract to the next. In the human jejunum the cell cycle time is about 48 hours (Wright et al, 1973b), whereas in the rat jejunum it is even more rapid, about 11 hours (Al Dewachi et al, 1974). A lengthening of the cell cycle time leads to a fall in the net cell renewal rate. This occurs, for example, in the rat, following a period of starvation (Al Dewachi et al, 1975; Hagemann et al, 1977).

The greater the proportion of a gland or crypt actively engaged in proliferation, the greater is the growth fraction, and the greater will be the cell production rate. In addition to a lengthening of the cell cycle time in starvation, there is a concomitant reduction in the crypt growth fraction, both of which contribute to the fall in CCPR after a period of starvation in the rat (Clarke, 1972). It is known that in the small intestine, proliferating cells are almost exclusively in the lower two-thirds of the crypt (Cairnie et al, 1965; Sunter et al, 1979a; Sunter et al, 1979b). In the gastric glands, however, most of the proliferative activity appears to be confined to neck and isthmus cells (Willems, 1971).

Thirdly, for obvious reasons, the CCPR is directly proportional to the total crypt cell population. A careful analysis of crypt population sizes shows that these are reduced in the rat after intestinal bypass or during starvation (Al Dewachi et al, 1975) and increased after intestinal resection (Gleeson et al, 1972), in the recovery phase of gut irradiation (Sato et al, 1972) and in the hyperproliferative crypts of patients with coeliac disease (Wright et al, 1973a and 1973b).
The factors just discussed are, therefore, all critically important determinants of proliferative activity in the gut. Measurements which reflect only one or two of these parameters may, therefore, give an inaccurate picture in situations where a parameter that has not been accounted for has changed.

There are a large variety of methods used to determine gut epithelial proliferative activity. Each has its own set of advantages and drawbacks. The techniques used and expertise required are diverse. Consequently each investigator has his own preferred method, and all the methods have their critics! The methods used in the assessment of epithelial proliferative activity may be classified as follows:

1. Direct or indirect estimation of cell production rate by metaphase-arrest, or stathmokinetic, techniques;

2. *In vivo* and *in vitro* incorporation of labelled DNA precursors, such as 3H-thymidine, followed by either:
   a) estimation of specific DNA radioactivity, or
   b) autoradiography; and

3. Immunohistochemical methods relying on the ability of antibodies to specifically bind to endogenous or exogenous nuclear antigen(s) in proliferating cells.

The metaphase arrest, or stathmokinetic, technique is the time-honoured standard in the study of intestinal proliferative activity. Used correctly, the method yields the rate of entry of crypt cells into mitosis (the 'birth rate' or 'mitotic rate'). The method involves administering an agent with metaphase arresting properties. The vinca alkaloids, vincristine and vinblastine, as well as colchicine and its less toxic derivative colcemid, have most commonly been used. Most investigators are agreed that vincristine is the best agent (Tannock, 1967). Following administration of vincristine intraperitoneally or intravenously, metaphases start to accumulate linearly between 15 minutes and 180 minutes after the injection. Metaphase counting is best done in microdissected crypts following appropriate whole-tissue metaphase staining. Between 10 and 20
crypts are counted, at each of six time points. The slope of the metaphase arrest line gives the rate of accumulation of arrested metaphases.

In experimental animal studies, the major alternative to the stathmokinetic technique lies in the use of 3H-thymidine labelling of DNA, followed by determination of DNA specific radioactivity and/or autoradiography. Autoradiography provides valuable information about the distribution of S-phase glandular and crypt cells, but requires about a month before results are obtained. The measurement of specific DNA radioactivity in this experimental system does, however, provide useful quantitative data quickly. It has been argued, however, that even when expressed as dpm per microgram of DNA, the measurement of 3H-thymidine incorporation into DNA may not reflect the DNA synthetic rate or the crypt cell production rate (Wright, 1984a). The criticisms relate to possible alterations in the activity of thymidine kinase, to possible changes in the intracellular thymidine pool size, and to undetermined extra-epithelial cellular DNA synthesis (for example, by proliferating lymphocytes in the lamina propria).

These arguments become particularly poignant when applied to methods involving *in vitro* 3H-thymidine incorporation into mucosal scrapes or sections of whole bowel wall. The additional problems of a non-physiological environment, non-epithelial and non-specific 3H-thymidine uptake, preferential "edge" labelling, and the release of nucleosides from damaged cells with consequent effects on the thymidine pool, it is argued, make the method less reproducible and, therefore, unreliable (Wright, 1984a).

New immunohistochemical methods, using monoclonal antibodies, have recently been used to identify proliferating cells in tissue sections. One such method, applicable in the experimental animal, has been studied. Bromodeoxyuridine (BRdU) is a thymidine analogue that is also taken up by S-phase cells during DNA synthesis. In 1982 Gratzner *et al* developed a monoclonal antibody against BRdU. Using this antibody it is possible to detect by immunohistochemistry on routine paraffin sections BRdU which was taken up into the nuclei of cells after *in vivo* or *in vitro* labelling. The results obtained from this technique are comparable to the
results from autoradiography using 3H-thymidine (Hamada, 1985; Tada et al, 1986). However, this BRdU-anti-BRdU method has the practical advantage of not requiring radioactive agents nor any special techniques other than standard immunohistochemistry. This method can be used without difficulty for analysing the cell kinetics of both diseased and normal tissues and has been applied to the analysis of cell kinetics in a variety of malignant tumours (Tada et al, 1986; Morstyn et al, 1983; Sasaki et al, 1986; Shimizu et al, 1988). Here I present this method as applied to gastrointestinal tissues, and use it later in Chapter 7 to analyse proliferative activity at the edge of experimentally induced gastric ulcers in the rat.

The aim of the studies presented in this Chapter is to compare the usefulness of these different approaches to the study of rat gastric and intestinal proliferative activity, and to determine to what extent the theoretical considerations outlined limit their usefulness.

**METHODS**

**Animals**
Animals used were adult male Wistar rats. In individual experiments body weights were always within 30 grams of the mean. The rats were kept in the Royal Postgraduate Medical School Zoology Department with free access to water and a standard diet, unless otherwise indicated. Daylight time was 05.00 to 17.00 hours. This is of particular importance with regard to the proliferation experiments, because of the circadian variation of gastrointestinal proliferative rates (Goodlad and Wright, 1984). Cages had wire bottoms to minimise coprophagia, as epithelial proliferative rates are known to vary profoundly depending on the state of starvation and feeding of the animals (Goodlad et al, 1983; Goodlad and Wright, 1984).

**CELL PRODUCTION RATE**
Rats were injected at 10.00 hours with vincristine sulphate 1mg/kg intraperitoneally following a 16 hour fast. They were then killed at timed intervals, and through a laparotomy incision the gastrointestinal tract was quickly dissected free of mesentery, flushed with 0.9% NaCl, and fixed in Carnoy's fluid for 4 hours. The
tissue was then transferred to and stored in 70% ethanol. For microdissection, stored tissue was rehydrated through 50% and then 25% ethanol, hydrolysed for 10 minutes in 1M HCl at 60 degrees C, and then stained with Schiff's reagent for 1 hour (this is the Feulgen reaction). Intestinal crypts and gastric glands were then microdissected in 45% acetic acid using a stereomicroscope and 21 gauge needles, and the number of arrested metaphases in 15 ileal or colonic crypts or 50 gastric glands was counted carefully by slowly altering the plane of focus in the crypt or gland being counted. The mean values were plotted against time after injection, and the slope of the line, fitted by least squares linear regression, gave the CPR and an estimate of its precision (Wright, 1982; Goodlad and Wright, 1982).

The stathmokinetic method is illustrated schematically in Figure 2.1.

**QUANTITATIVE IN VIVO 3H-THYMIDINE METHOD**

Rats were fasted for 16 hours with free access to water, and injected at 10.00 hours with 3H-thymidine intraperitoneally (125 uCi/kg in 0.1ml 0.9% NaCl). The animals were killed 1 hour later by a blow to the head, followed by cervical dislocation. The entire gastrointestinal tract from mid-oesophagus to lower rectum was quickly and carefully dissected from its mesentery and removed, then washed and flushed in 0.9% NaCl.

Tissue samples were obtained as follows. In the case of the stomach, this organ was cut along the greater curvature. Samples were obtained in triplicate from the anterior walls of the corpus or antrum, as required. Each sample included the entire gastric wall, free of omental fat and blood vessels, and weighed 50-70mg. In the case of small bowel and colon, the area of interest was removed "en bloc.", washed, cut and opened longitudinally and then cut transversely into pieces weighing 50-70mg.

The samples were weighed and then homogenised in cold 5mM Hepes (pH 7.4). DNA was precipitated by the addition of perchloric acid (HClO4, final concentration 0.4N) and centrifuged at 1200g for 30 minutes at 2 degrees C. The pellet is resuspended by ultrasonic disruption in 0.4N HClO4. The cycle of centrifugation and
ultrasonication was repeated three times. The DNA was then hydrolysed by incubating the final sediment in 1.5N HClO4 at 80 degrees C for 30 min. The supernatant was removed after centrifugation, and the hydrolysation procedure was repeated twice with the sediment.

For the determination of 3H-thymidine incorporation into DNA, aliquots were taken from the supernatants for

a) measurement of radioactivity by scintillation-counting, and
b) DNA assay (see below).

The results were expressed as disintegrations per minute per microgram of DNA. The DNA extraction procedure used here is a modification of that used by Baumgartner et al, 1986, the physico-chemical principles involved having been described by Kennel in 1967.

The in vivo DNA labelling method is outlined schematically in Figure 2.2.

**QUANTITATIVE IN VITRO 3H-THYMIDINE METHOD**
In these experiments rats were fasted for 16 hours with free access to water. The animals were killed by a blow to the head, followed by cervical dislocation. The gastrointestinal tract was dissected free of its mesentery, then washed and flushed with 0.9% NaCl. Tissue samples, taken as described for the in vivo method above, were then immediately incubated at 37 degrees C in Dulbecco's Minimal Essential Medium (DMEM, Gibco Ltd.) containing 3H-thymidine. Volume and time of incubation, concentration of isotope, bubbling with 95% O2 - 5% CO2, the use of a supporting metallic grid, and the type of container, are variables that were individually varied and studied in order to achieve optimal assay conditions.

The incubation was stopped by the addition of unlabelled carrier thymidine (final concentration 5mM) and immediate cooling to 0 degrees C. Individual mucosal samples were then weighed and washed four times in cold 0.9% NaCl. Subsequent storage and
determination of specific DNA radioactivity was as described for the 
*in vivo* method, above.

The *in vitro* DNA labelling method is illustrated schematically in Figure 2.3.

**DNA assay (fluorimetric method)**

This assay was used as described by Kapuscinski and Skoczylas (1977). 4,6-diamidino-2-phenylindole.2HCl (DAPI) forms a specific complex with DNA, and this fact is used for the quantitative estimation of DNA as low as 0.5 ng/ml.

**BRdU-ANTI-BRdU IMMUNOHISTOCHEMICAL METHOD**

S-phase gastrointestinal epithelial cells were pulse labelled *in vivo* with 5-bromo-2-deoxyuridine (BRdU) (100 μg/gram body weight) administered by a single intraperitoneal injection. One hour later the animals were sacrificed and full-thickness samples of gastrointestinal tissue taken for analysis from the sites of interest. This tissue was fixed in Carnoy's solution for 2-4 hours, then processed in chloroform, wax embedded, and 4 μm sections were cut. Endogenous peroxidase activity was removed by immersing sections in methanol 49.2 ml/H2O2 800 ul for 30 minutes. The sections were washed in running tap water for 5 minutes, and then immersed in 1M HCl (pre-heated to 60 degrees C) for 2 minutes to dissociate histones and partially denature DNA. The sections were again washed in running tap water for 5 minutes. Rabbit anti-BRdU antiserum (diluted 1/20) was then applied for 30 minutes at 21 degrees C. This was followed by a standard PAP immunolabelling procedure.

**RESULTS**

**CELL PRODUCTION RATE**

Examples of microdissected gastric glands and intestinal crypts are shown in Colour Plate 1. Cell production rates in the gastric corpus, antrum, duodenum, ileum and colon, as computed from the respective metaphase arrest lines, are as shown in Figure 2.4.
QUANTITATIVE IN VIVO 3H-THYMIDINE METHOD

Profile of gastrointestinal DNA labelling in vivo

Figure 2.5 shows the pattern of incorporation of 3H-TdR into DNA at the various sites studied. This pattern of DNA labelling was obtained reproducibly in several experiments. The similarity of Figure 2.5 to the gland and crypt cell production rates at the same sites in Figure 2.4 is striking.

3H-TdR disappearance curves

The in vivo 3H-TdR method was used in a different way to illustrate the different rates of turnover in the gastrointestinal tract. Rats received 3H-TdR on day 0, and were subsequently killed at intervals to determine the rates of disappearance of the isotope from the gastric, ileal and colonic epithelium. On day 9, the mean residual DNA radioactivity in the ileum, colon and gastric corpus was 6%, 26% and 32% (Figure 2.6). This is in keeping with the turnover rates being fastest in the ileum, slowest in the gastric corpus and intermediate in the colon.

QUANTITATIVE IN VITRO 3H-THYMIDINE METHOD

Profile of gastrointestinal DNA labelling in vitro.

Figure 2.7 shows the specific DNA radioactivity achieved by incubating rat gastric, duodenal, ileal and colonic wall in DMEM containing 3H-TdR at a concentration of 40 μCi/ml. In this experiment, the incubation mixture was continuously bubbled with a mixture of 5% CO2 and 95% O2, buffered with 0.02M Hepes, and kept at 37 degrees C. The pH was continually checked during the 3 hour incubation. Compared with the specific activity curve following in vivo 3H-TdR labelling, it can be seen that in these in vitro conditions the ileum and colon fail to achieve significantly greater DNA labelling than the stomach.

Time course of in vitro labelling

A typical time course of gastric DNA labelling is shown in Figure 2.8. For all the gastrointestinal tissues studied there was a variable 20-
40 minute lag phase before labelling became linear. This lag phase presumably represents the time required for diffusion of 3H-TdR into the mucosa prior to cellular uptake. The linearity of DNA labelling was maintained for 1-2 hours before a plateau was reached beyond which further labelling was unpredictable, particularly in the case of the ileum (data not shown).

**Acid-base status of incubation mixture**
A study was undertaken of the acid-base status of the incubation mixture during the 8 hour incubation. The mixture was pre-oxygenated once only at the start of the experiment. There was a continuous fall in pH during incubation of rat ileum, from 7.4 to 6.75 in the absence of 0.02M Hepes buffer (pH 7.4), and from 7.4 to 7.06 in the presence of the buffer. This confirmed the requirement for pH checking and correction (by adding appropriate quantities of a solution of potassium hydroxide) every 15 minutes, whether or not a buffer was used.

The drop in pO2 from 35 kPa to 11.5 kPa was identical with and without added buffer, as was the rise in pCO2 from 7.4 to 10.1 kPa.

**Effect of volume of incubation mixture**
The effect of volume of the incubation mixture on DNA labelling was also studied (data not shown). It was clear that volumes less than 1.0 ml resulted in greater variability of DNA labelling, and also made it more difficult to check and correct the pH during the incubation.

**Determination of non-specific background labelling**
Non-specific background labelling in this system was determined by first de-vitalising the tissue in one of 3 ways: (a) repeated freezing and thawing in liquid nitrogen, (b) heating to 80 degrees C for 5 minutes, or (c) pre-incubation with mitomycin C (1.0 mg/ml DMEM for 30 minutes. As expected, non-specific DNA labelling as determined by these methods was very low (less than 4% of untreated controls, data not shown).
Effect of a supporting metallic grid
A supporting metallic grid was used in an attempt to achieve better oxygenation of ileal samples in an atmosphere of 95% O2, 5% CO2. This system has been successfully used to study human ileal mucosal samples in vitro for periods of up to 24 hours (Paul Ciclitira, personal communication). The ileum continued to fail to achieve consistent or reproducible DNA labelling, even from one incubation well to the next in the same experiment (data not shown).

BRdU-ANTI-BRdU IMMUNOHISTOCHEMICAL METHOD
Using this method as described above, BRdU was clearly demonstrable in nuclei of columnar epithelial cells in the proliferation zone of the gastric glands, as well as in the lower half of intestinal crypts. Paneth cells at the base of the crypt, and stromal cells, did not stain positively. Control sections, stained with the same technique, but omitting the primary antibody, showed no nuclear staining. Quantitative results with this technique are presented in Chapter 7.

DISCUSSION
Many factors are involved in the choice of a cell kinetic method. The ideal method for the study of gastrointestinal epithelial turnover should be simple, quick, precise, and should take account of all the factors involved in epithelial replacement.

In the experiments presented, four methods for the assessment of gastrointestinal epithelial cell turnover have been studied in rats. The most reliable method, which relies on vincristine-induced metaphase accumulation in gastric glands and intestinal crypts, gives a reproducible pattern of cell production rate per gland or crypt. The CPR is lowest in the gastric corpus, greater in the gastric antrum, maximal in the small bowel and intermediate in the proximal colon (Figure 2.4).

The quantitative method for determination of in vivo 3H-thymidine incorporation into rat gastrointestinal DNA (Figure 2.5) shows an almost identical regional pattern of labelling as the cell production
rate. *In vitro* incubation of gastric and intestinal tissue with 3H-thymidine, however, gave unpredictable DNA labelling, especially in the cases of the ileum and colon where labelling was particularly variable from one experiment to another. A variety of modifications of the *in vitro* incubation conditions failed to improve the consistency of the labelling.

The metaphase arrest, or stathmokinetic, technique proved the most reliable method for the study of crypt proliferative activity. The method is theoretically sound, and used as above gives the cell production rate, and an estimate of its accuracy, by as direct a means as possible. Representing, as it does, the rate of entry of crypt cells into mitosis, the cell production rate is undoubtedly the best parameter of crypt proliferative activity. During microdissection, the C:V ratio can be estimated simultaneously if so desired.

However, there are some drawbacks to the stathmokinetic method. Some investigators (Tutton, 1973a; Tutton, 1973b; Klein, 1977) have chosen to count metaphases in paraffin sections, using either the whole crypt column or the bottom 10 cell positions as the denominator. Neither approach is sufficiently sensitive to changes in population size. Proper use of the stathmokinetic technique requires a dissecting microscope, as well as training in tissue microdissection and in the recognition and counting of metaphases (MacDonald, 1971). An excessive dose of vincristine, taking metaphase accumulation beyond 3 hours, or a failed intraperitoneal injection, may give rise to degenerated metaphases (in the former two situations) or anaphase escape (in the third mentioned situation). Technical difficulties (to be discussed later) in the microdissection of gastric glands may, in some cases, make this method completely unsuitable for the study of gastric mucosal turnover, unless the operator is experienced in microdissection. Properly used, however, this is the method of choice in the study of intestinal proliferative activity. By encompassing the cell cycle time, the growth fraction and population size in the CPR, the stathmokinetic method has earned its good reputation. With minor modifications this method can also be used to derive additional valuable information such as the cell turnover time, the mitotic
duration, the flux and birth rate per crypt cell position, and the cumulative birth rate curve (Cairnie et al, 1965).

The major alternative to the stathmokinetic technique in experimental animal studies lies in the use of in vivo 3H-thymidine incorporation into DNA, followed by determination of DNA specific radioactivity and/or autoradiography. The label is incorporated into newly synthesised DNA in cells that are in the S-phase of the mitotic cycle. Over the last 25 years the quantitative estimation of 3H-thymidine incorporation into DNA has proved the most popular method for assessing proliferative activity in epithelial tissues. There are several reasons for this. Firstly, very pure 3H-TdR of high specific activity is readily available (although it is expensive). Secondly, 3H-TdR is incorporated specifically into DNA alone. It is relatively straightforward to extract DNA and assay its radioactivity and quantity. Because minimal radioactivity is incorporated into cellular RNA and proteins, a rigorous separation of these molecular fractions prior to liquid scintillation counting is not required.

The technique has, however, had its vehement critics, from the relatively early days (Cleaver, 1967) as well as more recently (Maurer, 1981; Wright, 1984a and 1984b). Criticisms of the theory and practice of this method have been as follows. Firstly, incorporation of 3H-TdR into DNA is heavily dependent on the activity of one enzyme, thymidine kinase, which is the rate-limiting step in the entry of TdR into the cell prior to its use in the salvage pathway (Barlow and Ord, 1974). Therefore, variations in the activity of this enzyme alone could give misleading results as, for example, in the study of the effect of an alkylating agent, triethylene iminobenzoquinone, on Ehrlich and Yoshida ascites cells (Grunicke et al, 1975). Similar problems may arise from alterations in the intracellular ("cold") thymidine pool size. The method does not discriminate between a change in DNA labelling resulting from a change in the number of cells synthesising DNA, or to a change in the net incorporation per cell, ie. a change in the DNA synthetic rate itself. Finally, a very real consideration lies in the definition of the target population under study. Thus, in the small intestine, non-epithelial (lymphoid and stromal) cells are also proliferating, and this may account for as much as 10% of all mucosal mitoses (Clarke, 1971; Marsh and Trier, 1974). This could mean that changes in, say,
mucosal lymphocyte population size or proliferative activity may potentially affect the net mucosal DNA specific radioactivity in the absence of any change in the epithelial proliferative rate. This criticism does not apply to the stathmokinetic method, which has the advantage that, with care, the metaphases are counted only in the epithelial compartment.

These arguments are particularly applicable to methods involving in vitro 3H-thymidine incorporation into mucosal scrapes or sections of whole bowel wall. The additional problems of a non-physiological environment, non-epithelial DNA synthesis, and non-specific release of nucleotides from damaged cells with consequent effects on the intracellular TdR pool, make the method less reproducible and too unreliable, particularly in the study of small intestinal proliferative activity.

Intracellular "cold" thymidine pool size is a very difficult parameter to determine. While it is accepted that an alteration in this pool size could alone theoretically alter the degree of incorporation of 3H-TdR label into DNA, major changes in vivo in epithelial cell thymidine pool size in the experimental animal on a standard laboratory diet are unlikely, and have certainly not been demonstrated. This criticism is, however, more likely to be valid in vitro, for example when proliferative activity is being assessed in cell cultures. Here intracellular pool sizes may well vary enormously, depending on the availability of added nutrients, plasma membrane permeability, cell damage, crowding effects, and so on. Major alterations in the activity of thymidine kinase have also not been demonstrated in vivo. The only situation to my knowledge where this has been demonstrated is in the study of the toxic alkylating agent in vitro (vide supra, Grunicke et al, 1975).

Lymphoid and stromal cell proliferative activity may account for as much as 10% of mitoses in the small intestine (Clarke, 1971; Marsh and Trier, 1974). In a quantitative DNA labelling assay, this would represent the unknown 'background' radioactivity. It is always worth making sure, firstly, that no major change in intramucosal lymphocyte numbers has occurred, by inspecting histological sections. To be absolutely certain, however, that the figures obtained do not conceal this extraneous effect, autoradiography may
be used to determine the percentage of extra-epithelial DNA labelling in control and test animals. This would have the additional value of providing qualitative information on the pattern of epithelial labelling, should this be required. These checks were always made in the experiments presented in this Thesis, and in no experiment was a major change in intra-epithelial lymphocyte numbers or labelling noted.

The practical difficulties of the stathmokinetic method in the stomach deserve special mention. Without prolonged practice the glands in the gastric corpus, being long and slender, were extremely difficult to microdissect intact. When obvious breaks occurred, this was often in the region of the gland neck, at or near the proliferation zone. Mitotic activity may thus be underestimated. A further consideration is that the number of vincristine-arrested metaphases counted in gastric glands ranged between 0 and 7. Thus, to obtain an accurate mean, there was a need to dissect even larger numbers of glands, at each point on the metaphase arrest line, than is the case with intestinal or colonic crypts. These difficulties are not easily surmounted. The relative simplicity and reliability of in vivo 3H-thymidine labelling of DNA, therefore, makes it of value in the stomach, particularly as intramucosal lymphocyte numbers are smaller in this organ than in the remainder of the gastrointestinal tract.

In the animal experiments to be presented in this Thesis, the choice of proliferation methodology has been dictated by the nature of the experiment. In Chapter 3, where very rapid effects of indomethacin on gastrointestinal epithelial turnover are considered, use of the CPR was impractical and inappropriate. Use was therefore made of the quantitative in vivo 3H-thymidine method. In Chapters 4 and 5, longer term effects of indomethacin and misoprostol are determined by two methods, the CPR as well as the quantitative in vivo 3H-thymidine incorporation method, with similar results. In Chapter 7, where regeneration of gastric epithelium at the edge of experimentally induced gastric ulcers was studied, advantage was made of the BRdU-anti-BRdU method. This method was used to determine the number of labelled cells per gland at the ulcer edge, in preference to autoradiography, as former is quicker, considerably
cheaper, and does not involve the use of radioactivity. The quantitative *in vitro* method was discarded.
CHAPTER 3
METHODOLOGY IN THE ASSESSMENT OF GASTROINTESTINAL PROLIFERATION IN MAN

INTRODUCTION
The accurate assessment of cell turnover in man is vital for the understanding of a wide variety of physiological and disease processes. The gastrointestinal epithelium is a particularly useful model for study, because turnover rates are rapid, and because there are well defined anatomical divisions into reproductive and functional compartments.

In experimental animals the use of techniques which depend on labelling of dividing cells with nucleotide analogues, or on arrest of cells as they enter mitosis, is widespread. However, the use of radioactive or cytotoxic agents in humans is unethical, if not expensive and impractical. This has led to the use of in vitro methods for the assessment of turnover in human biopsy material (Hart Hansen et al, 1975), but these are of limited value due to the trauma of biopsy, problems of diffusion, and changes in intracellular nucleotide pool sizes. A further problem is that most measurements of cell proliferation, such as the labelling and the mitotic index, are expressed as a ratio of dividing to non-dividing cells. As gland and crypt size increase with adaptive responses, these proliferative indices can be confounded by concomitant changes in gland and crypt cell population. It is also vitally important to study well oriented, axially sectioned, glands and crypts; however, while this is possible in experimental animals, where relatively large blocks of well oriented tissue are available, this is rarely possible in man. Moreover, attempts to study human tissue in cross-section can lead to misleading results because of inability to account for changes in cell population, or expansion of the proliferative zone. This is highlighted by a recent study of the effects of prostaglandins on the dog stomach, where although no differences were noted in the labelling index, the labelling "per gland", and also the cell production rate "per gland" was almost doubled; this apparent paradox arose as a result of an increase in the gland cell population (Goodlad et al, 1989).
In this Chapter, I have studied and appraised a microdissection technique, used first by Ferguson et al (1977), to analyse proliferation in human gastrointestinal biopsies obtained at endoscopy.

I also examine the usefulness of two in vitro methods for the analysis of proliferation in endoscopic biopsies, by incubation with 3H-thymidine, and with vincristine sulphate.

Finally, I assess the usefulness and applicability of a monoclonal antibody, Ki-67, with specific affinity for an unidentified antigen in human mitotic cells, to study proliferation in snap-frozen endoscopic biopsies.

**METHODS**

**Ethical considerations**
The studies were approved by the Royal Postgraduate Medical School Ethical Committee. All patients gave informed consent to mucosal biopsy prior to endoscopy.

**Endoscopies**
All the endoscopies were performed between 09.00 and 10.30 hours. The endoscope used was an Olympus XQ10 (Keymed, UK), which has a 2.8 mm biopsy channel. The colonoscope used was a SE-34LH (Pentax, UK), which has a 4 mm biopsy channel. One biopsy was taken from each of the following sites: gastric fundus, gastric body and antrum; duodenal cap (D1) and second part of the duodenum (D2). From patients requiring colonoscopy for standard clinical indications, and who were found to be endoscopically normal, biopsies were taken from the terminal ileum, caecum, transverse colon, and rectum. Jejunal biopsies were obtained using a Crosby capsule in patients undergoing this procedure for standard clinical indications, and a small part of the sample obtained was used for these studies only if the tissue was architecturally and histologically normal. **Gastroscopies were performed for standard clinical indications, and gastric and duodenal biopsies were taken from endoscopically normal mucosa.**
Quantitative *in vitro* 3H-thymidine incorporation into human endoscopic gastric and duodenal biopsies

For these experiments endoscopic biopsies of the antrum and duodenum (weighing 5 - 12 mg) were immediately incubated for 1 hour at 37 degrees C in an atmosphere of 95% O2 - 5% CO2 in a solution of Dulbecco's Minimal Essential Medium (DMEM, Gibco Ltd.) containing 3H-thymidine (40 uCi/ml). Volume and time of incubation, and aeriation with 95% O2 - 5% CO2, are parameters that were individually varied and studied in order to achieve optimal assay conditions. Incubations were stopped by cooling on ice, washing 4 times in 0.9% saline, and tissue stored in liquid nitrogen prior to analysis. Specific mucosal DNA radioactivity was determined as described for rat gastrointestinal tissue in Chapter 2.

*In vitro* mitotic arrest in human endoscopic gastric and duodenal biopsies

The endoscopies were performed on 10-hour fasted patients at 09.00 hours under midazolam sedation. Endoscopic antral and duodenal biopsies weighing 5-12 mg from 6 endoscopically normal patients were immediately incubated for 3 hours in DMEM (corrected to pH 7.4) containing vincristine sulphate (at a concentration of 5 μg/ml). The incubator used contained 95% O2 and 5% CO2. Un-incubated ("time 0") biopsies and incubated (3 hour) biopsies were fixed in Carnoy's fluid for 4 hours, and then stored in 70% ethanol until metaphase staining and microdissection (as described below), in an attempt to define ex-vivo gland and crypt cell production rates in these mucosal samples.

Ki-67 monoclonal antibody immunohistochemical method as applied to human endoscopic gastric and duodenal biopsies

A. Handling of endoscopic biopsies: Each endoscopic biopsy was carefully placed on a narrow strip of nitrocellulose. Using a bright light and a magnifying lens, biopsies were oriented mucosal side up. A maximum of 2 biopsies from the same anatomic site were placed on each nitrocellulose strip. Four such strips were placed vertically in a 6 x 5 x 12 mm mould, which was immediately filled with OCT Compound, taking care to avoid air cavities. The block was then
snap-frozen in melting arcton (-146 degrees C) to avoid freezing artefact and transported to the laboratory for storage at -70 degrees C

B. Cryostat Sections: When required, blocks were cut by cryostat, taking 5 μm sections from areas of interest. The sections were picked up on poly-L-lysine coated slides, and dried at room temperature for 24 hours (no less).

C. Immunoperoxidase staining (peroxidase anti-peroxidase method): Sections were then fixed for 10-20 minutes in acetone at room temperature, and allowed to dry for 5 minutes, except for the area of the tissue section, which from here onwards was not allowed to dry (in order to prevent artefact).

A drop of heat-decomplemented non-immune goat serum, diluted 1/20 in 0.01M phosphate-buffered saline, pH 7.2 (PBS) was applied, to block non-specific protein binding sites and Fc receptors. After at least 10 minutes the normal serum was drained off, and the slides dried round the sections. The slides are placed in a damp chamber (a Petri dish containing wet cotton wool is adequate).

A drop of the primary antibody preparation, Ki-67, diluted 1/20, is then applied and left on the section for 2 hours at room temperature or overnight at 4 degrees C (to minimise evaporation). Antibody is then drained off and the slides rinsed three times in PBS (5 minutes each).

The slides are again dried, except for the area of the section, and replaced in a damp chamber. A drop of unconjugated goat anti-mouse immunoglobulin is applied and the slides left for 30 minutes at room temperature. The antibody is diluted 1/50 in PBS containing normal human serum (blood group AB) to absorb cross-reactivity with anti-human immunoglobulins that might produce background labelling.

A third layer of mouse peroxidase-anti-peroxidase complex (PAP) is applied, diluted 1/100 in PBS and left for 30 minutes. The slides are again rinsed in PBS as before.
Peroxidase is then developed in diaminobenzidine tetrahydrochloride (DAB), 0.025-0.05\% in PBS with 0.01-0.03\% hydrogen peroxide for 5-10 minutes. The end-point is a dark brown deposit at the site of immunoreaction.

The developed slides are rinsed in running water, and the nuclei are then counterstained lightly with haematoxylin.

**D. Positive Controls:** A positive control (human tonsil) was always run with each batch of slides. Anti-Ki-67 marks the nuclei of proliferating lymphocytes in this tissue.

**E. Counting:** All the counting was performed by myself in order to minimize inter-observer error. Cells with a brown nuclear reaction were regarded as being positive. The biopsy in section was examined and counted systematically from the epithelial surface to the base of the glands in the case of the stomach, and from the top to the base of the crypts in the case of the duodenum. Only the cells lining the glands and crypts were counted. A 'Ki-67 index' was obtained by dividing positively stained cells by the total number of cells in the area under study.

The antibody to Ki-67 was kindly provided for these studies by Professor N.A. Wright and Dr. R.A. Goodlad (source: Dako Ltd.). Goat anti-mouse immunoglobulin, mouse PAP and DAB were obtained from ICN Biomedicals Ltd., Dako Ltd. and Aldrich Chemical Co., respectively.

**Microdissection of gastric glands and duodenal crypts in the estimation of gland and crypt morphometry and cell proliferative activity in endoscopic biopsies**

Endoscopic biopsies, stored in 70\% ethanol, were hydrated in 50\% and then 25\% ethanol, hydrolysed in 1M HCl for 10 minutes, and then stained by the Feulgen reaction with Schiff's reagent. The tissue was then placed in 45\% acetic acid and gently teased apart under a dissecting microscope (at 25 x magnification). A coverslip was then gently placed on the wet tissue and pushed down until the glands or crypts began to separate.
Microdissected glands and crypts were examined under a compound microscope and the outline of the gland or crypt was traced using a Leitz drawing tube (which projects an image of a drawing board into the field of view of the microscope). The number of mitoses in each gland or crypt was also recorded. Cells in every active stage of mitosis were counted, the requisite morphologic criteria having been strictly defined. Each prophase, metaphase, anaphase pair and telophase pair were scored as 'one.'

Gland or crypt outlines were then traced (Figure 3.1) and digitized with a Macinitizer ADB (SSI Ltd., Pewsey, Wiltshire) graphics tablet connected to an Apple Macintosh SE personal computer running MacDraft software (IDD inc., Concord, California).

RESULTS

Quantitative in vitro 3H-thymidine incorporation into human endoscopic gastric and duodenal biopsies

Figure 3.2 shows the quantitative 3H-TdR incorporation into DNA in endoscopic biopsies of the gastric antrum and duodenal cap. It can be seen that there is an unacceptably large spread of in vitro labelling results from one patient to the next, for both the stomach as well as the duodenum. Reproducibility was not improved by increasing the concentration of isotope, volume and time of incubation, bubbling with oxygen, or altering the type of container used (data not shown).

In vitro metaphase arrest method

Baseline (unarrested) metaphase counts in 30 antral glands and 30 duodenal crypts from the same patient were 3.1 (0.6) and 4.5 (0.7) (mean (SEM)), respectively. After 3 hours of incubation with vincristine sulphate (5 μg/ml), the metaphase counts were 7.4 (1.1) and 20.0 (3.2), respectively. This gives estimated mean cell production rates of 1.43 and 5.17 cells per hour, respectively, for antral glands and duodenal crypts.

These rates are certainly smaller than expected, for the following reason. Colour Plate 2 shows duodenal crypts microdissected 3
hours after incubation with vincristine. The crypt clearly contains cells which have escaped into anaphase, suggesting that \textit{in vitro} mitotic arrest was incomplete. Incompletely arrested crypts were encountered more frequently deeper inside the sample of incubated tissue, suggesting that the diffusion of vincristine sulphate from the medium through the tissue was rate limiting. This effect was also noted in gastric antral mucosa. Higher concentrations of vincristine sulphate (10 $\mu$g/ml and 25 $\mu$g/ml) were used to try to minimize the consequences of this diffusion effect, without success. At a concentration of 25 $\mu$g/ml of vincristine sulphate the 3 hour incubation yielded fragmented and friable tissue, and crypts which broke easily during microdissection.

\textbf{Ki-67 monoclonal antibody immunohistochemical method}

In the stomach, only glandular cells in the proliferative zone were positive for nuclear reaction with Ki-67. Surface epithelial cells, parietal and chief cells showed no nuclear reaction. Figure 3.3 shows the 'Ki-67 index' at the 6 upper gastrointestinal sites studied in 6 patients. This index is defined here as the ratio (expressed as a percentage) of positively stained cells to the total number of cells counted in the area of interest. It can be seen that the index is lowest in the fundus, body and greater curve of the stomach, somewhat higher in the lesser curve of the antrum, and higher still in the duodenum. It should be noted that, as applied to gastrointestinal tissue positively stained cells were rather more difficult to identify with certainty than is the case with lymphoid tissue; that the monoclonal antibody, despite storage as recommended by the manufacturers, rapidly aged in its ability to bind to the nuclei of dividing cells; that non-epithelial 'background' staining was considerable; and that there was considerable variation in the Ki 67 index at the same site from one patient to the next. The latter is reflected in the large size of the standard error bars in Figure 3.3.

\textbf{Microdissection method}

Figure 3.4 shows the profiles of numbers of mitotic cells per microdissected gastric gland and duodenal crypt at the 11 gastrointestinal sites studied. Figure 3.5 shows the cross-sectional areas of these structures. It can be seen that the number of mitoses
increases progressively and reproducibly proximo-distally till the caecum, which has the largest number of mitoses per crypt. From the caecum, the number of mitoses per crypt starts to decrease towards the rectum. Figure 3.6 shows a plot of the number of mitoses per gland or crypt versus the cross-sectional area. There is a very good straight-line correlation between these two variables ($r = 0.893$, $P = 0.001$), confirming the relationship between proliferative rate and compartment size in steady state systems.

The lengths of microdissected gastric glands and intestinal crypts are shown in Table 3.1. The longest gastric glands were located in the corpus. Crypt length progressively increases proximo-distally, the longest crypts being in the rectum.

Table 3.2 shows the degree of gland and crypt duplication, by fissuring. Fissuring is the process by which glands and crypts duplicate and divide. A fissure, or cleft, occurs at the distal end of the gland or crypt, and proceeds towards the proximal end, thus producing two glands or crypts. Fissuring may also occur in the 'daughter limbs' of a duplicating gland, thus resulting in a gland with three, or more, limbs. Fissuring of gastric glands occurred most frequently in the antrum. Crypt fissuring occurred most frequently in the duodenal cap, and was extremely infrequent in the colon.
**TABLE 3.1** Lengths of microdissected human gastric glands and small and large intestinal crypts. 'n' is the number of endoscopically normal patients studied. Results are expressed as mean (SEM).

<table>
<thead>
<tr>
<th>Site</th>
<th>'n'</th>
<th>Length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric fundus glands</td>
<td>5</td>
<td>0.44 (0.03)</td>
</tr>
<tr>
<td>Gastric corpus glands</td>
<td>5</td>
<td>0.36 (0.02)</td>
</tr>
<tr>
<td>Antral glands</td>
<td>19</td>
<td>0.39 (0.02)</td>
</tr>
<tr>
<td>D1 crypts</td>
<td>22</td>
<td>0.32 (0.01)</td>
</tr>
<tr>
<td>D2 crypts</td>
<td>5</td>
<td>0.33 (0.01)</td>
</tr>
<tr>
<td>Jejunal crypts</td>
<td>4</td>
<td>0.33 (0.03)</td>
</tr>
<tr>
<td>Terminal ileal crypts</td>
<td>5</td>
<td>0.37 (0.04)</td>
</tr>
<tr>
<td>Caecal crypts</td>
<td>5</td>
<td>0.51 (0.03)</td>
</tr>
<tr>
<td>Transv. colon crypts</td>
<td>4</td>
<td>0.52 (0.05)</td>
</tr>
<tr>
<td>Rectal crypts</td>
<td>4</td>
<td>0.54 (0.04)</td>
</tr>
</tbody>
</table>

**TABLE 3.2** Degree of gland and crypt division by fission at various gastrointestinal sites in man. '％ of fissured glands or crypts' refers to the numbers of these structures containing one or more fissures. 'n' refers to the number of endoscopically normal patients on which the mean (SEM) are based.

<table>
<thead>
<tr>
<th>Site</th>
<th>'n'</th>
<th>％ of fissured glands or crypts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastric fundus</td>
<td>5</td>
<td>17.1 (4.6)</td>
</tr>
<tr>
<td>Gastric body</td>
<td>5</td>
<td>27.4 (5.0)</td>
</tr>
<tr>
<td>Gastric antrum</td>
<td>19</td>
<td>28.0 (4.9)</td>
</tr>
<tr>
<td>Duodenal cap (D1)</td>
<td>22</td>
<td>12.9 (3.8)</td>
</tr>
<tr>
<td>Descending duodenum (D2)</td>
<td>5</td>
<td>3.1 (1.1)</td>
</tr>
<tr>
<td>Jejunum</td>
<td>4</td>
<td>none noted</td>
</tr>
<tr>
<td>Terminal ileum</td>
<td>5</td>
<td>none noted</td>
</tr>
<tr>
<td>Colon</td>
<td>13</td>
<td>0.54 (0.23)</td>
</tr>
</tbody>
</table>
DISCUSSION
The quantitative estimation of gastrointestinal proliferation in man presents the investigator with unique problems relating to accessibility of the tissue of interest, and ethical constraints on his methodology.

Ideally, a measure of the rate of entry of cells into mitosis is required. However, this inevitably requires the in vivo administration of radioactive nucleotides (to label S phase cells) or cytotoxic agents such as vincristine (to arrest mitotic cells in metaphase). In a few severely ill patients cell proliferation in the gastric mucosa has been investigated by means of autoradiography after labelling in vivo with 3H-thymidine (Theml et al, 1967 and 1973; Lipkin and Bell, 1968; Trepel and Schick, 1976). It was shown that the general pattern of epithelial cell renewal in the gastrointestinal tract in man is similar to that in laboratory animals. However, ethical considerations prohibit the use of such agents in man. Consequently, in vitro methods have been developed for the assessment of gastrointestinal turnover in human biopsy material (Hart Hansen et al, 1975), but these are of limited value due to the trauma of biopsy, problems of diffusion, and changes in intracellular nucleotide pool sizes. Consequently, the cell kineticist is unable to obtain a measure of the rate of entry of cells into the mitotic cycle, and must be content with measures of state instead. These are less desirable than measures of rate because they provide a static view of a dynamic process.

Our experiments using in vitro mitotic arrest in endoscopic biopsies have confirmed that inadequate diffusion of vincristine sulphate through the tissue lead to unequal and inadequate mitotic arrest (with anaphase escape) in deeper parts of the tissue, resulting in an underestimate of the cell production rate. Likewise, in vitro labelling with 3H-thymidine in gastric and duodenal endoscopic biopsies resulted in highly variable degrees of DNA labelling, presumably relating once again to problems of diffusion, tissue trauma, or changes in intracellular nucleotide pool sizes. These methods have therefore not been used in the human studies to be described later in this Thesis.
Recently, a new method of measuring proliferative activity in human tissues was described by Miyachi et al (1978): they identified an autoantibody in the sera of patients with systemic lupus erythematosus that reacted with nuclear antigens exclusively present in proliferating cells. Following on this theme, Gerdes et al (1983) have produced a mouse monoclonal antibody, designated Ki-67, that recognises a nuclear antigen associated with cell proliferation. Ki-67 was obtained in studies aimed at the production of monoclonal antibodies to nuclear antigens specific to lymphoma cells in patients with Hodgkin's disease, including the Reed-Sternberg cells. The existence of such antigens was recently suggested (Stein et al, 1981) by the reactivity of conventional rabbit antiserum raised against the Hodgkin's disease derived cell line L428 (Schaadt et al, 1980). While most of the monoclonal antibodies generated against nuclei of the Hodgkin's cell line were reactive with nuclear structures of all cells tested, Ki-67 reacted with a nuclear antigen restricted to proliferating cells. Expression of this antigen occurs preferentially during late G1, S, G2 and M phases of the cell cycle, while cells in the G0 phase consistently lack the antigen (Gerdes et al, 1984). My studies with this monoclonal antibody in the assessment of gastrointestinal turnover have, however, been disappointing. The problems related to non-reproducibility, antibody aging, positive staining that was more difficult to discern than is the case with lymphoid tissue, and excessive non-epithelial background staining. Most importantly, the requirement to express results as an 'index' means that the method is likely to be confounded by changes in gland or crypt population (vide infra). For these reasons this method was not used in the human studies to be described later in this Thesis.

Determination of the 'mitotic index,' or the percentage of cells in mitosis, has been one of the most commonly used methods for the analysis of proliferative activity in gastrointestinal epithelia. This can be determined from tissue sections under the microscope. The method does, however, have its drawbacks. It is tedious and time consuming. Aherne, Camplejohn and Wright (1977) have calculated that 2300 interphase cells should be counted to distinguish between a mitotic index of 0.5% and 1.0% at a level of significance of 0.05. The criteria used to distinguish mitotic figures (Macdonald, 1971) are often not stated clearly; it is often difficult to distinguish mitotic
figures from pyknotic cells. Different investigators have used different denominators for the index: some have taken the non-mitotic cells in the proliferative compartment as the denominator, whereas others have taken the total cell count in the proliferative compartment (Goodlad and Wright, 1982). Some do not limit the cell counting to the proliferative compartment (see Aherne, Camplejohn and Wright, 1977). Most importantly, the mitotic index cannot detect changes in cell population due to increased gland or crypt size; the method also cannot distinguish between changes in the proportion of cells in mitosis and changes in the duration of mitosis (Goodlad and Wright, 1982).

The use of microdissection methodology as described here to define the number of mitotic cells per gland or crypt has several distinct advantages over the conventional mitotic index. Firstly, relatively small quantities of tissue are adequate. In my studies the method was shown to be feasible even with single small endoscopic biopsies. Each biopsy of the stomach and duodenum yielded approximately 30 glands or crypts suitable for counting. Furthermore, the problem of correct tissue orientation for sectioning does not apply here, so the method is much quicker. For gastric glands with a mean row count of 10, there are 10 times more cells per gland than may be seen on a single perfectly oriented gastric gland in cross-section. Therefore, studying 30 microdissected glands yields information equivalent to counting mitoses and cells in 300 perfectly oriented gastric glands in section. This argument is illustrated in Figure 3.7. A further consideration is that in microdissected tissue stained by the Feulgen reaction, individual mitotic cells in the various stages of mitosis are easier to recognise, and to distinguish from pyknotic cells, than in tissue sections stained routinely with Haematoxylin and Eosin. In sectioned tissue mitotic counting is further confounded by the movement of mitotic cells towards the axis of the gland or crypt, requiring correction by Tannock's factor in order to correct for tissue geometry (Wright et al, 1972; Sunter et al, 1979b).

The crypt dissection procedure utilised has an additional advantage over histological sections in that large numbers of crypts and glands can be individually scored in three dimensions, whereas histological sections are scored in one plane only. This is highly relevant here because gastric glands and intestinal crypts duplicate by fissuring
and bifurcating. This process, which was first described by Clarke (1972), is an important adaptive process which has received little attention. It has been postulated that crypt fission is involved in the growth and regeneration of the intestinal mucosa (Clarke, 1972; Cairnie, 1976; Maskens, 1978; Maskens and Dujardin-Loits, 1981). It has been shown by Maskens (1978) that crypt fission is high in the neonatal rat colon, but then decreases as the animal ages. Totaflurno, Bjerknes and Cheng (1987) proposed a model in which crypts grow until they bifurcate. Their studies of mouse intestinal epithelial kinetic responses to irradiation and partial resection suggested that most, if not all, crypts in the adult mouse are cycling asynchronously and independently. This is the 'crypt cycle,' which is analogous to the cell cycle. Thus, in addition to the brisk turnover of intestinal epithelial cells, there is a continuous production of new crypts. I find that most of the fissures in human gastric glands and intestinal crypts extend no further than the deeper one-third of the gland or crypt, suggesting that once the fissure extends beyond this point, fissure extension proceeds at an accelerated rate. The rate limiting factor is therefore likely to be in the lower portion of the crypt or gland, which is known to have a lower cellular turnover rate than cells above it in the proliferation zone (Wright, 1984a,b). Fissuring also occurs in the new 'daughter' limbs of the gland or crypt, resulting in triplication, quadruplication, and so on. In Chapter 6 it is shown that an increase in crypt duplication through fissuring represents one aspect of the adaptive response to NSAID therapy in humans, its occurrence correlating well with the accompanying increase in epithelial turnover in the stomach and duodenum. These morphological changes are impossible to assess accurately even in well oriented histological sections.

However, the most important advantage of the microdissection method described here is that it enables one to express the numbers of mitoses on a 'per gland' and 'per crypt' basis. The mitotic index, which expresses the number of mitoses as a fraction of the total number of cells counted, is far less sensitive in the detection of changes in proliferation status. This is because adaptive and growth responses, by their very nature, involve not simply an increase in the total number of dividing cells, but also increases in the size of the proliferation zone and in the total gland or crypt population. This is exemplified by a recent study of the effects of prostaglandins
on the dog stomach, where although no differences were noted in
the labelling index, the cell production rate "per gland" was almost
doubled (Goodlad et al, 1989). Earlier studies (Fich et al, 1985;
Romain et al, 1987; Arber et al, 1985), which relied solely on mitotic
and labelling indices, had failed to show the proliferative effect of
prostaglandins on the gastric mucosa. Neither method, however, can
distinguish between changes in the proportion of cells in mitosis and
changes in the duration of mitosis.

The microdissection method as described here has therefore been
used to analyse cell turnover in endoscopic biopsies in the human
studies to be presented in Chapters 6 and 7.
CHAPTER 4

ACUTE EFFECTS OF INDOMETHACIN ON RAT GASTROINTESTINAL INTEGRITY AND PROLIFERATIVE ACTIVITY

INTRODUCTION
The mechanism of NSAID-induced gastric damage is not generally agreed, but is likely to be multifactorial. There is good evidence for a direct contact-induced disruptive action, especially in the case of aspirin (Holt, 1960; Baskin et al, 1976; Lanza et al, 1979; Ivey et al, 1980). NSAIDs also inhibit mucosal cyclo-oxygenase, thereby reducing the production of prostaglandins (Whittle, 1981) and interfering with the process of 'cytoprotection' in the gastric mucosa. 'Cytoprotection' refers to protection of the mucosa against injury through mechanism(s) independent of acid-inhibition, and includes 'adaptive cytoprotection', which is initiated by damage and limits its extent (Jacobson, 1986). Prostaglandin mediated cytoprotection of the gastric mucosa is probably effected by a number of mechanisms, of which an increase in the mucus-bicarbonate barrier (Wilson et al, 1986) maintenance of intercellular tight junctions (Meeroff, 1985), and an increase in gastric mucosal blood flow (Leung et al, 1985) have been emphasised.

Recently, however, it has been recognised that prostaglandins may be important regulators of gastrointestinal proliferation, negating earlier views to the contrary (Fich et al, 1985). Thus, in the dog treated with exogenous prostaglandin E1, although there is no significant change in the mitotic and labelling indices, there is an impressive increase (by 40%) in gastric gland length, with a corresponding increase in the number of mitoses per gland. These studies suggest that the effects of NSAIDs on mucosal cell renewal should also be considered. These investigations have already been initiated in the rat (St. John et al, 1973; Eastwood and Quimby, 1982; Baumgartner et al, 1986; Kuwayama and Eastwood, 1988), the dog (Hurley and Crandall, 1964; Levy et al, 1982; Hurley and Crandall, 1983) and in man (Kuwayama and Eastwood, 1988; Graham et al, 1983; Graham et al, 1988), but uniform conclusions have failed to emerge, and acute effects have been ignored. For example, the same group in different situations have found increases (Baumgartner et
as well as decreases (Inauen et al., 1988) in gastric proliferation during indomethacin treatment, and there is disagreement about the effects of NSAIDs on antral proliferation (Eastwood and Quimby, 1982; Baumgartner et al., 1986). In the following experiments acute indomethacin toxicity is delineated at various gastrointestinal sites, and related to simultaneous effects on epithelial turnover.

**METHODS**

*Animals* used in this experiment were adult male Wistar rats weighing 272-309 gm.

*Indomethacin* powder was kindly supplied by Merck Sharp and Dome Ltd. For experimental use indomethacin was dissolved in a solution of 5% sodium bicarbonate, and used within 30 minutes of preparation.

**Preliminary pilot studies**

Pilot experiments were conducted to determine the dose- and time-dependent macroscopic gastrointestinal ulcerative damage following single subcutaneous injections of indomethacin. The doses of indomethacin studied ranged from 0-40 mg/kg. The macroscopic pathologic effects on the gastrointestinal tract were determined at time periods of 3-72 hours.

**In vivo proliferation study**

Twenty rats were studied. The animals were divided into four groups of five. Each rat was fasted for 16 hours and then received a single subcutaneous injection of indomethacin or control vehicle (5% sodium bicarbonate) at 09.00 hours, as indicated in the following table:

<table>
<thead>
<tr>
<th>Group</th>
<th>Subcutaneous injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>5% NaHCO3</td>
</tr>
<tr>
<td>II</td>
<td>Indomethacin 2 mg/kg</td>
</tr>
<tr>
<td>III</td>
<td>Indomethacin 5 mg/kg</td>
</tr>
<tr>
<td>IV</td>
<td>Indomethacin 30 mg/kg</td>
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</table>
Six hours following the injection, each animal received 3H-TdR at a dose of 125 μCi/kg intravenously. The animals were sacrificed 1 hour after receiving 3H-TdR.

**Assessment of gastric mucosal erosions**
The gastrointestinal tract was dissected, washed and flushed. The stomach was removed and opened along the greater curvature. The mucosal and serosal surfaces were carefully examined under bright light and magnifying lens for evidence of linear and punctate erosions. The presence of erosive damage was confirmed histologically.

**Assessment of small intestinal and colonic lesions**
The entire small bowel and colon was washed in cold saline and cut longitudinally. Mucosal ulceration and oedema, and the presence of serosal perforation, were recorded for each site.

**Assessment of DNA specific radioactivity**
Samples of gastric fundus, corpus, antrum, duodenum, jejunum, ileum, proximal colon and rectum (each weighing 50-70 mg) were frozen at -20 degrees C for subsequent analysis of specific DNA labelling (using the methods outlined in Chapter 2).

The experimental plan is summarised in Figure 4.1.

**RESULTS**

**Pilot study findings**
In these experiments gastric damage, consisting of erythema, and linear erosions equally distributed in the glandular mucosa of the corpus and antrum, was first detectable 3 hours after a single subcutaneous injection of indomethacin (7.5 mg/kg) (n = 6 animals). This damage fully heals by 24 hours. The minimum single dose of indomethacin required to induce macroscopic gastric damage was 7.5 mg/kg.

Small intestinal damage consisted of oedema, nodule formation and ulceration, followed by adhesions, perforation (Colour Plate 3) and a
frank purulent peritonitis. These lesions were noted predominantly in the ileum, being much less marked in the duodenum and jejunum. Intestinal ulcers tended to occur at or close to Peyer's patches. The threshold single doses of indomethacin below which no damage occurred was 10 mg/kg, respectively (n = 10 animals). The earliest small bowel damage (patchy oedema and erosion) was noted 20 hours after doses of indomethacin over this threshold. Perforation occurred at single doses of indomethacin above 20 mg/kg, usually between 48 - 72 hours (n = 12 animals, two killed daily following commencement of indomethacin). With repeated once daily injections of indomethacin at a dose of 5 mg/kg, 5 out of 12 rats died between days 9 and 12 from multiple ileal perforation and peritonitis.

Colonic damage did not occur at single doses of indomethacin in the 0 - 40 mg/kg range. However, repeated administration at 5 mg/kg/day subcutaneously resulted in significant colonic erythema and inflammation, but no ulceration, in surviving animals by day 9.

Effects of indomethacin on gastrointestinal mucosal DNA synthesis in vivo.

Figure 4.2 A shows the acute effects of indomethacin on gastric mucosal DNA synthesis in vivo, in the three doses tested. There was no macroscopic damage, nor effect on DNA synthesis in the fundus at any of the three doses tested. The gastric body and antrum, however, showed 60% and 30% mean inhibition of DNA synthetic rates, respectively, at the 30 mg/kg dose (the only dose to achieve gastric corpus and antral damage at this time point) (P <0.05 in each case). Jejunal and ileal DNA synthesis remained unaltered by each of the doses of indomethacin tested (Figure 4.2 B). There was no small intestinal damage at this time point.

Proximal colonic DNA labelling is also shown in Figure 4.2 B. It can be seen that there was stimulation of DNA synthesis by the 5 mg/kg as well as the 30 mg/kg doses of indomethacin, by 21% and 50%, respectively, but this stimulation was not statistically significant at the P < 0.05 level. No colonic damage was noted macroscopically at this time point.
DISCUSSION
In these studies it has been demonstrated that single subcutaneous
doses of indomethacin (7.5 mg/kg) can induce gastric damage within
3 hours. The gastric damage induced by relatively low doses of
indomethacin heals spontaneously, the macroscopic appearance of
the stomach being normal again within 48 hours of the insult.

In contrast, indomethacin-induced small intestinal damage is slower
to appear, being noted first at 20 hours, and often progressing
within 3 days to ulceration and perforation with death of the
animals from peritonitis. The colon was notable for its apparent
lack of immediate NSAID mucosal toxicity even at the large doses
tested in this study.

It has also been shown here that indomethacin, in gastric ulcerative
doses, decreases gastric corpus and antral DNA synthesis within 7
hours. At this time neither damage nor effect on DNA synthesis was
noted in the small intestine. In the colon a stimulation of DNA
synthesis was noted, although this was not statistically significant at
this time point. Stimulation of DNA synthesis in the colon is usually
apparent after 24 hours, and is sustained in the long term (Chapter
5).

A direct toxic effect of NSAIDs such as indomethacin on isolated
gastric glands in vitro has been unequivocally demonstrated
(Tarnawski et al, 1988). These investigators showed that
indomethacin significantly reduced the viability of gastric glands,
increasing lactate dehydrogenase release into the medium, and
producing obvious ultrastructural damage. The latter consisted of
gland shrinkage, plasma membrane disruption, cell detachment and
disintegration. Significantly, this damage was prevented by pre­
treating the gastric glands with 16,16-dimethyl prostaglandin E2. A
direct local toxic effect of aspirin on the gastrointestinal mucosa in
vivo has also been established in several studies of its contact
induced and ultrastructural effects (Holt, 1960; Baskin et al, 1976;

The response of the gastric mucosa to injury is complex, and is likely
to be different in the presence of NSAIDs. In man, gastric erosions,
in the absence of concurrent NSAID administration, are associated with an increase in DNA synthesis in the damaged gastric mucosa (Myszor and Hodgson, 1989). Presumably, this reflects increased mucosal repair, new cells being generated at an accelerated rate to replace damaged surface cells. In the same study, however, similar gastric erosions in the presence of NSAID treatment were not associated with this 'adaptive' enhancement of DNA synthesis, suggesting that normal repair was not occurring. This led to the suggestion that failure to enhance epithelial cell generation in response to injury may be an important mechanism leading to persistence of mucosal damage.

The reason for the reduction of DNA synthesis by ulcerative doses of indomethacin is unknown. At least two explanations can be offered. Firstly, the S-phase DNA synthetic machinery may be disrupted by the destruction and damage of large numbers of mucosal cells, including those in the proliferative zone in the upper half of the gastric glands. In all cell lines studied in culture, indomethacin does not stimulate but in fact inhibits proliferation, for example hepatocyte, fibroblast and chondroblast cultures (Bayer et al, 1979; Kirkpatrick et al, 1983; Hial et al, 1977). Indomethacin appears to arrest some cells in the G1 phase of the cell cycle (Bayer et al, 1979). There are, however, no pure gastric cell lines to study.

Secondly, it is possible that the powerful cyclo-oxygenase inhibiting effects of both aspirin and indomethacin greatly reduce the mucosal levels of cytoprotective prostaglandins. Recent evidence of a direct stimulatory effect of exogenous prostaglandin E1 on gastric epithelial cell proliferation (Goodlad et al, 1989), reversing former suggestions (Fich et al, 1985) that they decrease cell turnover, implies a role for endogenous prostaglandins in mucosal regeneration. There is good supporting evidence for this view. Thus, in one study indomethacin markedly inhibited gastric mucosal cyclo-oxygenase, as measured by prostacyclin biosynthesis, and this was accompanied by the development of gastric erosions, although after 48 hours both inhibition of cyclo-oxygenase and gastric erosions were no longer apparent (Whittle, 1981). One interpretation of this finding is that when the inhibition of cyclo-oxygenase is relaxed, renewed prostaglandin generation stimulates mucosal regeneration and healing. This view is supported by other
work, where topical administration of exogenous prostaglandins prevented the aspirin-induced fall in DNA synthesis while protecting the mucosa from ulcer formation (Konturek et al., 1981). The role of prostaglandins in the latter situation, however, is likely to be complex, and to include true cytoprotection (blunting NSAID-induced direct toxic damage) as well as effects on regeneration. Interestingly, other growth stimulating agents, such as epidermal growth factor, also have anti-ulcerogenic properties combined with the ability to prevent the NSAID induced fall in mucosal DNA synthesis (Konturek et al., 1981).

The relative contributions of the above mechanisms towards inhibition of gastric epithelial cell proliferation in the presence of NSAIDs is not clear. It is known that the ability of indomethacin to inhibit cyclo-oxygenase is dose-dependent in the 1.25 - 10 mg/kg dose range (Whittle, 1981). At a dose of 10 mg/kg indomethacin induces striking 95 + 1% and 86 + 3% reductions in cyclo-oxygenase activity in the gastric mucosa and ileum, respectively. Nevertheless, even doses as low as 1.25 mg/kg, which are not acutely ulcerogenic, achieve significant inhibition of prostacyclin formation in the gastric mucosa (by 77 + 3%) and ileum (by 55 + 8%) in the same study. As direct toxicity against gastric glandular cells would also be expected to be dose-dependent, these findings should not be interpreted as favouring either mechanism as the major cause of erosive damage in the stomach.

Our results, and those of others, therefore lead us to propose that acute indomethacin-induced gastric ulceration in rats results from the combined ability of this drug to induce direct toxic damage, as well as to simultaneously inhibit gastric epithelial cell renewal. In the ileum, where no effect on DNA synthesis is noted, there is indeed no damage noted either, at this time point. The colon is peculiar in its ability to increase mucosal turnover quickly in response to indomethacin. This increase in turnover is maintained in the long term (Chapter 5). This property may explain, in part, the relative resistance of the colon to indomethacin damage.
INTRODUCTION
In Chapter 4 the acute gastrointestinal damaging effects of indomethacin were discussed in relation to the known direct toxic effects of this drug, and its ability to inhibit the gastric epithelial proliferation in high doses. The sequence of events following repeated administration of low doses of indomethacin was not addressed. While a stimulation of cell turnover in the gastric corpus of the rat after chronic indomethacin therapy is well known, effects on the antrum are less clear and some of the findings at this site have been conflicting (Eastwood and Quimby, 1982; Baumgartner et al, 1986; Kuwayama and Eastwood, 1988). Effects of misoprostol, a prostaglandin E1 analogue, on gastrointestinal proliferation have also been the subject of debate and disagreement (Fich et al, 1985; Goodlad et al, 1989).

It is well known that the gastric mucosa has the ability to minimise the damaging effects of repeated injury (Hurley and Crandall, 1964; Levy et al, 1972; St. John et al, 1973; Eastwood and Quimby, 1982; Graham et al, 1983; Baumgartner et al, 1986; Graham and Smith, 1986; Graham et al, 1988a). This phenomenon is known as gastric mucosal adaptation (Jacobson, 1986). Most of the studies on gastric mucosal adaptation have been in experimental animals, using injurious agents such as ethanol (Robert 1981 and 1984), salicylates (Levy et al, 1972; St. John et al, 1973;) and other non-steroidal anti-inflammatory drugs (Baumgartner et al, 1986). There are, in addition, a small number of studies in human subjects (Graham et al, 1983; Graham et al, 1988a).

There is some experimental evidence that the damaging effect of chronic indomethacin administration on the stomach is paralleled by an increase in cell proliferation, especially in the gastric corpus (Eastwood and Quimby, 1982; Baumgartner et al, 1986; Kuwayama
and Eastwood, 1988). Exogenous prostaglandins, however, also have the ability to stimulate gastric cell proliferation (Halter et al, 1984; Dembinski and Konturek, 1985; Goodlad et al, 1989). There is thus an apparent paradox, that inhibition of natural prostaglandin production by NSAIDs, as well as administration of exogenous prostaglandins, has the same end result on gastric mucosal turnover. A resolution of this paradox has been offered by Baumgartner et al (1986). This group have argued on morphologic grounds that the increased cell turnover in response to indomethacin administration may not be a primary effect. It is suggested that indomethacin, even in relatively low doses, increases gastric cell shedding with a consequent homeostatic increase in regeneration. When an equilibrium is reached in the chronic state, therefore, the relative numbers of chief and parietal cells in the gastric corpus increase significantly, but the numbers of surface mucus cells, which were shed as well as replaced at a faster rate, were unchanged.

The above explanation may apply to the simultaneous increases in proliferative activity in the colon already noted in short and long term aspirin and indomethacin administration studies (Craven et al, 1983; Craven et al, 1988; DeRubertis et al, 1985 and Chapter 4). There is adequate evidence of mild and moderate colonic toxicity in animals (Chapter 4; Craven et al, 1988; DeRubertis et al, 1985) as well as in man (Rampton and Sladen, 1981; Schwartz, 1981; Hall et al, 1983; Phillips et al, 1983; Ravi et al, 1986; Kaufman and Taubin, 1987; Tanner and Raghunath, 1988; Sheers and Williams, 1989), although some of the evidence in man is admittedly circumstantial.

The present experiments were designed to study and quantify the effects of repeated administration of low doses of indomethacin and misoprostol on macroscopic mucosal morphology and proliferative activity in the gastrointestinal epithelium of rats. Two different techniques were used to determine proliferative activity and cell turnover rates: in vivo 3H-TdR incorporation, and the cell production rate.

**MATERIALS**

*Animals* used in this experiment were young adult male Wistar rats weighing 285-323 gm. Indomethacin powder was kindly supplied
by Merck Sharp and Dome Ltd. For experimental use indomethacin was dissolved in a solution of 5% sodium bicarbonate, and used within 30 minutes of preparation.

Misoprostol oil was kindly supplied by G. D. Searle and Co. Ltd. (batch no 3P11200). The preparation supplied was of high purity (98.5%). It was stored at -20 degrees C prior to use. For these experiments 50 mg of misoprostol was brought to room temperature and dissolved in absolute ethanol (Analar grade). The resulting solution was immediately divided into aliquots and stored at -20 degrees C in amber glass containers covered in foil. Once a batch was thawed for use, any remaining misoprostol was discarded. For administration to rats by gavage, the misoprostol solution was diluted with sodium phosphate buffer (pH 7.4).

METHODS

Chronic indomethacin toxicity study
At 10.00 each day for 8 weeks 15 rats each received a subcutaneous injection of indomethacin in a dose of 2 mg/kg. Control animals each received a daily injection of 5% sodium bicarbonate (vehicle).

The animals were allowed free access to food and water throughout the experiment until day 55 when food was withdrawn 24 hours prior to gastrointestinal proliferation assessments.

Gastrointestinal proliferative activity was determined on day 56 by two methods:

1. Vincristine metaphase-arrest (using 10 animals), and
2. Quantitative in vivo 3H-TdR incorporation (using 5 animals).

Effect of indomethacin on the rate of disappearance of 3H-TdR from the gastric corpus
In this experiment 15 animals were pre-labelled at 10.00 hours on day -1 by an intraperitoneal injection of 3H-TdR (200 uCi/kg). Five animals were sacrificed on day 0 for determination of gastric corpus
DNA radioactivity (using the methods in Chapter 2). Between days 0 and 8, five of the remaining animals each received a subcutaneous injection of indomethacin (5 mg/kg/day) at 10.00 hours. The other 5 (control) animals received the vehicle alone (5% sodium bicarbonate). On day 8 at 12.00 hours both groups of animals were sacrificed for determination of residual specific DNA radioactivity in the gastric corpus.

**Study of chronic effects of misoprostol**
Twenty animals were divided into two groups of 10. One group received misoprostol by gavage once daily (300 μg/kg/day) at 10.00 hours for 14 days; the other (control) group received just phosphate buffer containing 0.1% ethanol (the vehicle). Animals received an intraperitoneal injection of vincristine sulphate prior to sacrifice, in order to determine the cell production rate in the gastric corpus and antrum as described in Chapter 2.

This experiment was repeated with misoprostol given at a lower dose of 10 μg/kg/day by the same route.

**RESULTS**

**Chronic indomethacin toxicity study**
Indomethacin administration for 8 weeks at 2 mg/kg was well tolerated by the animals, with no mortality and no macroscopic or histologic gastrointestinal damage. Figure 5.1 shows that in vivo 3H-TdR incorporation was significantly increased at every gastrointestinal site studied. Thus specific DNA labelling was increased by 56%, 27%, 26%, 43% and 121%, in the gastric corpus, antrum, duodenum, ileum and colon, respectively (P < 0.05 in each case). In the cases of the duodenum, ileum and colon, the increase in cell turnover was confirmed by determination of crypt cell production rates which were increased by 20%, 33%, and 57%, respectively (P < 0.05 in each case) (Figure 5.2).

There were no significant accompanying changes in the length of glands in the gastric corpus (from 485 (16), control, versus 463 (18) μm, indomethacin (mean (SEM)), or in the gastric antrum (210 (14), control, versus 199 (12) μm, indomethacin).
Effect of indomethacin on the rate of disappearance of $3\text{H}-\text{TdR}$ from the gastric corpus

Figure 5.3 shows that indomethacin significantly accelerated the rate of disappearance of $3\text{H}-\text{TdR}$ radioactivity from the gastric corpus. Taken with the findings above, this confirms an accelerated rate of shedding and replacement of rat gastric glandular cells during indomethacin therapy.

Study of chronic effects of misoprostol

Misoprostol, at 300 $\mu$g/kg/d for 14 days, induced a 27% increase in the length of glands in the gastric corpus ($P < 0.05$), and a 54% increase in the length of antral glands ($P < 0.05$) (Figure 5.4). Misoprostol also significantly increased the cell production rate in the gastric corpus and antrum, as shown in Figure 5.5.

Misoprostol at a dose of 10 $\mu$g/kg/d failed to induce any significant changes in corpus or antral gland length, or indeed in the crypt cell production rate. Cell production rates (cells per gland per hour) in gastric glands were 1.85 (0.20) (corpus) and 3.15 (0.41) (antrum) in the control animals, and 1.91 (0.34) (corpus) and 3.36 (0.46) (antrum) in the misoprostol group (mean (SEM), $n = 10$ in each group). Gland lengths (pm) were 495 (10) (corpus) and 201 (11) (antrum) in the control animals, and 501 (14) (corpus) and 207 (12) (antrum) in the misoprostol group (mean (SEM)).

The proliferative effects of misoprostol are therefore dose dependent.

DISCUSSION

In these experiments it has been demonstrated that chronic low-dose indomethacin treatment in rats produces no macroscopic gastrointestinal damage and results in a generalised increase in gastrointestinal proliferative activity, most marked in the colon. Indomethacin administration resulted in no significant changes in the lengths of gastric glands in the gastric corpus or antrum. Misoprostol administration in high doses, however, resulted in lengthening and hyperplasia of corpus and antral glands, and an
accompanying increase in the cell production rate in the gastric corpus and antrum.

Effects of chronic NSAID administration on gastrointestinal proliferative activity have been previously studied in three species: rats, dogs and man. Some of the results have been conflicting, particularly in the rat antrum.

Studies in each of the above species have shown that proliferation in the fundus and corpus, but not antral, mucosa is increased by indomethacin (Baumgartner et al., 1986; Kuwayama and Eastwood, 1988) as well as by aspirin (Eastwood and Quimby, 1982; St. John et al., 1973). For example, Kuwayama and Eastwood (1988) examined the effects of chronic indomethacin ingestion in rats as well as humans. Here, rats received indomethacin at 3 mg/kg/day for 4 weeks and human volunteers 2 mg/kg/day for 2 weeks. Using autoradiography following in vivo labelling with 3H-thymidine in rats, and in vitro labelling of human endoscopic biopsies, these investigators demonstrated stimulation of fundic epithelial proliferation but no effect on antral proliferation in both rats and in humans. From these observations they conjectured that the failure of antral epithelial proliferation to respond to indomethacin may account for the ulcerogenic action of this drug.

Likewise, Eastwood and Quimby (1982), studying the effects of chronic aspirin ingestion in rats, demonstrated stimulation of fundic and proximal duodenal, but not antral, epithelial proliferation using autoradiography following intravenous 3H-thymidine. In the duodenum, aspirin increased proliferative activity in the lowest four crypt-cell positions, indicating an increase in stem cell production.

However, in a similar study of rat mucosal turnover, Baumgartner et al. (1986) showed increased DNA synthesis in gastric corpus as well as antral mucosa. However, they used in vitro 3H-thymidine labelling of mucosal samples followed by estimation of DNA-bound radioactivity as the estimate of proliferative activity at these two gastric sites. They then, however, used sequential autoradiography to "chase" the labelled cells up the gland on days 1 and 3 of indomethacin treatment, elegantly demonstrating more rapid migration resulting from an increased turnover rate of the glandular
cells. While cell shedding also increased at both sites, only in the corpus (but not the antrum) did proliferation exceed shedding, thus leading to increased corpus mucosal volume. The latter was due to a selective hyperplasia of parietal cells (by 15%, P < 0.05) and chief cells (by 45%, P <0.01) noted after 14 days of indomethacin. My findings are in line with this group of investigators, but not with those of Eastwood and Quimby (1982), as far as the antrum is concerned.

It is worthy of note that mucosal ulceration did not occur in any of the studies of chronic NSAID therapy using doses twice those used in the therapy of arthritis in man, given in my study for as long as 8 weeks. The proliferative effects of NSAIDs may help explain the previously observed phenomenon of gastric mucosal adaptation in rats (St. John et al, 1973; Eastwood and Quimby, 1982), dogs (Hurley and Crandall, 1964; Levy et al, 1982; Hurley and Crandall, 1983) and in humans (Graham et al, 1983; Smith et al, 1984; Graham et al, 1988a) after repeated exposure to NSAIDs.

It is not clear whether NSAID-induced stimulation of gastrointestinal epithelial proliferative activity is a primary effect, or whether it is a compensatory homeostatic response consequent on increased cell damage and shedding. I believe the latter to be true although the evidence to support this view is largely indirect.

First, in all cell lines studied in culture, indomethacin does not stimulate but in fact inhibits proliferation, for example hepatocyte, fibroblast and chondroblast cultures (Bayer et al, 1979; Kirkpatrick et al, 1983; Hial et al, 1977). Indomethacin appears to arrest some cells in the G1 phase of the cell cycle (Bayer et al, 1979). There are, however, no pure gastric cell lines to study.

Second, isolated human gastric glands (from human surgical specimens) are damaged by indomethacin. Thus Tarnawski et al (1988) assessed the viability of the glandular cells by the fast green exclusion method, by measuring the release of lactate dehydrogenase (LDH) into the medium, and by assessing ultrastructural damage by scanning and transmission electron microscopy. By excluding vascular and extraglandular neural as well as hormonal factors in this simple experimental system, the
The third line of evidence comes from studies of aspirin, an NSAID well known for its local irritant and toxic effects on the gastric mucosa. Given orally, aspirin causes gastric erosions (Holt, 1960), bleeding (Ryan et al., 1987), and ultrastructural changes in surface epithelial cells (Baskin et al., 1976). The erosive effects of aspirin as well as non-aspirin NSAIDs correlate poorly with potency of cyclooxygenase inhibition, and in the case of aspirin are not seen if the oral preparation is buffered (to pH<5) or given intravenously (Ivey et al., 1980). A wealth of evidence therefore supports the conclusion that oral aspirin has an immediate direct damaging effect on gastric epithelial cells, yet in the long term adaptation of the gastric mucosa is known to occur in animals (Hurley and Crandall, 1964; St. John et al., 1973; Eastwood and Quimby, 1982; Levy et al., 1982) as well as in humans (Graham et al., 1983; Smith et al., 1984; Graham et al., 1988a), in part through increased epithelial cell proliferation.

Finally, it is instructive to compare the effects of NSAIDs and prostaglandins on mucosal morphology and turnover. There is an apparent paradox here, because I have shown that both inhibition of endogenous synthesis of prostaglandins, as well as administration of exogenous prostaglandin in large doses, stimulate gastric proliferation. There are, however, clear cut differences in gastric epithelial morphology after treatment with indomethacin or prostaglandin. In contrast to the selective effect of indomethacin on parietal and chief cells (Baumgartner et al., 1986), exogenous prostaglandins induce growth that is most pronounced in surface and foveolar mucous cells of corpus and antral mucosa (Halter et al., 1984; Goodlad et al., 1989). Since the target of a proliferative stimulus seems to be a single gastric epithelial stem cell (Willems and Lehy, 1975), the differences in morphology resulting from
various proliferative stimuli may be better explained by invoking differential cell loss, without then having to propose altered cellular differentiation. Accordingly, prostaglandin-induced proliferation, accompanied as it is with protection of surface cells, leads, as I have shown, to an increase in gastric gland length. Conversely, the indomethacin-induced increase in proliferation, which is paralleled, it is proposed, by increased loss of surface cells, leads to a relatively localised hyperplasia of deeper glandular cells such as the parietal and chief cells (Baumgartner et al, 1986), with no major effects on net mucosal volume once equilibrium is reached.

The sequence of events in the small intestine and colon may well be similar to that in the stomach. Small intestinal damage from NSAIDs is well known in the experimental animal (see Chapter 4, and also: Somogyi et al, 1969; Robert and Asano, 1977; Robert, 1979; Whittle, 1981; Del Soldato, 1985; Del Soldato et al, 1987) as well as in humans (Bjarnason et al, 1984, 1986, 1987, 1989). Human volunteers, as well as patients with rheumatoid arthritis and osteoarthritis, treated with NSAIDs, develop increased small intestinal permeability to ethylene diamine tetraacetic acid (EDTA) (Bjarnason et al, 1984 and 1986). This suggests that there is a significant disruption of the low-capacity intercellular pathway for large hydrophilic substances. Patients with arthritis treated with a range of NSAIDs commonly show evidence of small intestinal inflammation, as detected by isotope-labelled autologous leucocyte scanning (Bjarnason et al, 1988). Furthermore, there are small numbers of patients on long term NSAIDs who have clearly developed specific diaphragm-like fibrous small intestinal strictures (Bjarnason et al, 1988).

The association of NSAID treatment and colonic disease is also described in animals (Chapter 4; Craven et al, 1988; DeRubertis et al, 1985) and in patients with arthritis (Rampton and Sladen, 1981; Schwartz, 1981; Hall et al, 1983; Phillips et al, 1983; Ravi et al, 1986; Kaufman and Taubin, 1987; Tanner and Raghunath, 1988). Thus, indomethacin at 5 mg/kg/day given subcutaneously for 9 days induced colonic erythema and inflammation in rats, although obvious ulceration was not noted (Chapter 4). In patients with arthritis, diarrhoea and colitis have been reported, particularly with mefenamic acid and indomethacin (Kaufmann and Taubin, 1987).
Tanner and Raghunath (1988) found that of 43 new cases of proctocolitis over an 18-month period (excluding Crohn's colitis), 4 patients were taking NSAIDs. In these 4 patients discontinuation of NSAID resulted in rapid resolution of the symptoms, and two patients experienced immediate relapse of their symptoms upon inadvertent rechallenge. These authors suggest that about 10% of cases of newly diagnosed colitis may be NSAID related. Sheers and Williams (1989) report two patients who developed an unusual variety of colonic stricture (characterised by circumferential and spiral scarring), following NSAID therapy.

Therefore, in the small intestine and colon, as well as in the stomach, there is good evidence from more than one species that NSAIDs induce toxic damage at all levels of the gastrointestinal tract. This damage is likely to be the basis of what I propose is an adaptive increase in mucosal turnover at each of these sites.
INTRODUCTION
The evidence that non-steroidal anti-inflammatory drugs (NSAIDs) damage the gastric and duodenal mucosae in man is now convincing. The relative risk of gastric ulcer occurrence in NSAID users versus non-users is approximately 5.0 (Duggan et al, 1986; McIntosh et al, 1985). NSAIDs appear to confer a 3.8-fold increase in the risk of peptic ulcer bleeding (Somerville et al, 1986), a 1.6-fold increase in the risk of peptic ulcer perforation (Jick et al, 1987), and a 4.7-fold increase in the risk of death from complications of peptic ulcers (Griffin et al, 1988; Guess et al, 1983). The elderly appear to be at particularly increased risk of NSAID induced peptic ulcers and their complications (Lamy, 1987; Somerville et al, 1986; Walt et al, 1986).

No NSAID is free from these long term complications. Thus, although it has been possible to develop NSAIDs that have been shown endoscopically to produce markedly less mucosal damage than existing agents (Lanza, 1984; Graham et al, 1985; Osnes et al, 1979; Hradsky, 1981; Halvorsen, 1973; Lehtola and Sipponen, 1977; Chernish et al, 1979), all of the newer drugs in retrospect are similar in their propensity to cause chronic gastroduodenal damage (Larkai et al, 1987). Therefore, acute studies have clearly failed to predict the effects of chronic NSAID use.

Attempts have also been made to reduce the gastric damaging effects of aspirin and other NSAIDs, without much success. For example, methods have been developed to reduce contact between aspirin and the gastric mucosa through enteric coating, the use of microspheres, administration of "pro-drugs" (such as sulindac) and the alteration of the pH of the preparation. Although acute lesions occur less commonly, none of these measures has had documented success in the long term. Sulindac, ironically, has emerged as the
leading cause of upper gastrointestinal bleeding within 30 days of starting NSAID therapy (Carson et al, 1987b).

NSAID induced gastric injury may be 'minor' or 'major.' Major NSAID induced injury involves damage to deeper glandular structures, including the proliferative zone. Recent evidence in experimental models using aspirin as well as ethanol favours increased vascular permeability below the superficial epithelium as an early critical event (Szabo et al, 1986; Guth, 1986; Woods et al, 1988). Such injury is endoscopically apparent, and is observed to lessen over a period of days, despite continued presence of the damaging agent. This mucosal adaptation, which is slower than restitution, may involve a variety of mucosal defence mechanisms. One of these includes an augmented regenerative rate, with enhanced cell production by the proliferative zone (Baumgartner et al, 1986). There is good evidence that mucosal adaptation occurs in man, although much of the evidence for this is indirect. Thus, while almost 100% of volunteers receiving aspirin develop acute mucosal lesions (Jiranek et al, 1989), one-quarter to one-third of patients taking NSAIDs in the long term have an endoscopically normal gastric mucosa (Larkai et al, 1987). However, the ability of the gastric mucosa to adapt is limited. Thus, it takes markedly longer for adaptation to occur when the daily dose of aspirin is doubled from 1300 mg to 2600 mg (Graham et al, 1988a).

The role of prostaglandins in mucosal adaptation is not clear. Prostaglandins of the E and I series have a range of mucosal protective properties, including enhancement of gastric mucosal blood flow (Sato et al, 1987), increase in gastric mucus synthesis (Wilson et al, 1986), stimulation of duodenal bicarbonate production (Selling et al, 1985), and strengthening of intercellular tight junctions (Meeroff, 1985). These properties confer on prostaglandins the ability to cytoprotect, ie. the ability to reduce or prevent major mucosal damage by a range of noxious substances, including NSAIDs. Prostaglandins, as well as sulphydryl-containing compounds, have the ability to prevent major acute gastric mucosal injury by a variety of damaging agents, including NSAIDs (Silverstein, 1986; Silverstein et al, 1987; Ryan et al, 1988). Interestingly, patients with duodenal ulcer disease in the absence of
NSAID therapy appear to have diminished mucosal prostaglandin synthesis (Ahlquist et al, 1983).

Until relatively recently, the effects of prostaglandins on gastric proliferation were ignored. Goodlad et al (1989) recently showed an impressive increase in gastric glandular proliferative activity in dogs treated with misoprostol. This resulted in a 30% increase in gland length. The meticulous studies of this group of investigators reversed earlier findings, based on mitotic and labelling indices (the limitations of which are discussed in Chapter 3) to the contrary (Fich et al, 1985; Arber et al, 1985; Romain et al, 1987). It is therefore possible that exogenous prostaglandins may protect in part by augmenting the mucosal adaptive process through enhancement of glandular regeneration. This possibility has not so far been tested in man. The need to do this has been highlighted by earlier studies in man in this department showing that NSAIDs may blunt the normal proliferative response of the antral epithelium to superficial damage (Myszor and Hodgson, 1989).

No study to date has considered either the gastric or duodenal mucosal proliferation status during misoprostol therapy for the healing and prevention of gastroduodenal damage by NSAIDs. The present double-blind placebo-controlled clinical study was therefore undertaken with the following aims:

1. To obtain detailed endoscopic, kinetic and proliferative data relating to the mechanisms of mucosal damage by, and adaptation to, NSAIDs; and
2. To establish whether the mucosal protective effects of misoprostol in NSAID users may be attributed to a stimulation of regeneration in the gastric and duodenal epithelium.

The duodenal cap and the lesser curve of the antrum were chosen for study because these are commonly the sites of NSAID-associated lesions. In contrast to the studies in Chapter 7, however, where ulcer edges were biopsied, in this study only endoscopically normal tissue was biopsied. This enables the study of 'field' changes in the mucosa resulting from NSAID and misoprostol administration. Biopsies of lesions would have confounded this issue, because, as I
show in Chapter 7, proliferation at the sites of damage is inhibited by NSAIDs.

**MATERIALS AND METHODS**

**Ethical considerations**
This study was approved by the Royal Postgraduate Medical School Ethical Committee.

**Criteria for inclusion**
1. Males, or females not of childbearing age (unless there was a documented history of a sterilisation procedure). This is because misoprostol, like its parent prostaglandin, has the ability to increase uterine tone and thereby induce abortion.
2. Age over 18.
3. The presence of an arthritic condition requiring NSAIDs on a regular basis.
4. No anti-ulcer medication (except antacids) in previous week.
5. The ability to give informed consent.

**Criteria for exclusion**
1. Patients with a recent history (within 3 months) of gastric or duodenal surgery.
2. Patients with the following medical conditions: oesophageal varices, inflammatory bowel disease, any malignancy, Zollinger-Ellison syndrome, renal or hepatic impairment.
3. Patients taking anti-ulcer medication (except antacids), anticoagulants or steroids.
4. Patients with a history of alcoholism.

**Study design**
Patients with arthritis requiring chronic NSAID therapy underwent an initial one-week washout period at the beginning of which their NSAID was withdrawn and pain relief provided by paracetamol (maximum 8 x 500 mg per day). For patients who had NSAID associated gastrointestinal symptoms at entry to the washout phase, an antacid, aluminium hydroxide (maximum 8 x 500 mg per day) was provided.
At the end of the washout phase patients were reassessed and an endoscopy was performed. Provided ulcers were not present, patients were then randomised (in approximately a 1:1 ratio) to receive either misoprostol or placebo with the relevant NSAID. The dose of NSAID was that required to achieve the desired anti-inflammatory effect (but not exceeding the manufacturers recommendations). The dose of misoprostol used was 200 μg t.i.d. At the end of the two-week period a second endoscopy was performed.

If at the first endoscopy gastric or duodenal damage was noted, the patient was entered into the appropriate lesion healing study as follows: patients with bleeding lesions and/or erosions were entered into a two week randomised placebo-controlled trial in which they received the NSAID in addition to either misoprostol or placebo. The dose of misoprostol used was 200 μg t.i.d. These patients were also re-endoscoped after 2 weeks. After this endoscopy the randomisation code was broken. Ten patients were randomised to receive NSAID placebo. An equal number were randomised to receive NSAID plus misoprostol.

A third group of patients (n=4) with superficial gastric antral ulcers was also studied. These patients discontinued their NSAID treatment for 4 weeks, during which they received a healing dose of misoprostol (200 μg q.i.d). At the end of this treatment period the patients were re-endoscoped to determine the degree of ulcer healing by misoprostol, and to determine whether any changes induced by misoprostol on proliferative status of the endoscopically normal antral and duodenal mucosae had occurred.

Endoscopies, endoscopic lesion assessment, and biopsies
The endoscope used was the Olympus XQ10 which is a standard forward-viewing scope, with a 2.8 mm biopsy channel. Erosive lesions and mucosal ulcers were defined as follows for the purposes of this study.

Erosive lesions: Erosions are shallow, often multiple, mucosal breaks, which fail to penetrate the muscularis mucosae, and are not associated with scarring. For the purposes of this study, an erosion is defined as a mucosal break, containing a purulent or
haemorrhagic exudate, which is less than 5mm in maximum diameter.

**Ulcers**: Endoscopic studies of this nature must accurately define the criteria used to distinguish 'erosions' from 'ulcers'. Before the introduction of gastrointestinal endoscopy ulcers were defined by pathologists, based on operative or autopsy material, as losses of epithelial surface penetrating through the muscularis mucosae. However, the endoscopist, who views ulcers en face, can have no accurate perception of true lesion depth (Butt *et al*, 1988). The definition of what constitutes an ulcer has therefore changed, but there is still some disagreement. For the purposes of this study, the definition of an ulcer is that used by Larkai *et al* (1987), who state: "ulcers are three-dimensional, circumscribed mucosal defects, associated with an exudate, and having a diameter of 5 mm or more".

Two mucosal pinch biopsies from endoscopically normal tissue were taken for kinetic and histologic analysis from each of the following sites:

1. lesser curve of gastric antrum
2. duodenal cap

**Processing of endoscopic biopsies**

Biopsies were immediately placed in a solution of Carnoy's fixative, and transferred 4 hours later to 70% ethanol for storage.

The Carnoy's fixed biopsies were used for the microdissection analysis of morphometry and cell proliferation in gastric glands and duodenal crypts.

**Determination of morphometry and cell proliferation in gastric glands and duodenal crypts**

The microdissection of gastric glands and duodenal crypts was performed as described in Chapters 2 and 3.

Microdissected glands and crypts were examined under a compound microscope and the outline of the gland or crypt was traced using a Leitz drawing tube (which projects an image of a drawing board into
the field of view of the microscope). The number of mitoses in each gland or crypt was also recorded. Cells in every active stage of mitosis were counted. Prophases, metaphases, anaphase pairs and telophase pairs were respectively recorded as 'one.'

Gland and crypt outlines were then digitized with a Macinitizer ADB (SSI Ltd., Pewsey, Wiltshire) graphics tablet connected to an Apple MacIntosh SE personal computer running MacDraft software (IDD inc., Concord, California). The frequencies of the different states of fission (that is, duplication, triplication, and so on) of each gland and crypt were also recorded.

RESULTS
Tables 6.1 - 6.3 show the characteristics of the patients studied in each of the limbs of the study, as well as the endoscopic findings.
TABLE 6.1 Characteristics of, and endoscopic findings in, 10 patients randomised to receive NSAID plus placebo for 2 weeks following initial 'washout.' Patients in this group had previously used NSAIDs regularly for a mean of 3.65 years.

<table>
<thead>
<tr>
<th>Mean age</th>
<th>49.90 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age range</td>
<td>23 - 70 years</td>
</tr>
<tr>
<td>Type of arthritis</td>
<td>Osteoarthritis n = 4&lt;br&gt;Rheumatoid arthritis n = 3&lt;br&gt;Other musculoskeletal disorders n = 3</td>
</tr>
<tr>
<td>Lesions noted at 1st endoscopy</td>
<td>None n = 5&lt;br&gt;Antral erosions n = 4&lt;br&gt;Duodenal erosions n = 1&lt;br&gt;Gastric ulcers n = 0&lt;br&gt;Duodenal ulcers n = 0</td>
</tr>
<tr>
<td>Range of NSAIDs used</td>
<td>Indomethacin n = 3&lt;br&gt;Ibuprofen n = 2&lt;br&gt;Piroxicam n = 2&lt;br&gt;Naproxen n = 1&lt;br&gt;Diclofenac n = 1&lt;br&gt;Fenbufen n = 1</td>
</tr>
<tr>
<td>Lesions noted at 2nd endoscopy</td>
<td>None n = 6&lt;br&gt;Antral erosions n = 2&lt;br&gt;Duodenal erosions n = 0&lt;br&gt;Gastric ulcers n = 2&lt;br&gt;Duodenal ulcers n = 0</td>
</tr>
</tbody>
</table>
**TABLE 6.2** Characteristics of and endoscopic findings in, 10 patients randomised to receive NSAID plus misoprostol (200 µg t.i.d.) for 2 weeks following initial 'washout.' Patients in this group had previously used NSAIDs regularly for a mean of 4.30 years.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Description</th>
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<tbody>
<tr>
<td>Mean age</td>
<td>55.36 years</td>
</tr>
<tr>
<td>Age range</td>
<td>26 - 88 years</td>
</tr>
<tr>
<td>Type of arthritis</td>
<td></td>
</tr>
<tr>
<td>Osteoarthritis</td>
<td>n = 4</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>n = 5</td>
</tr>
<tr>
<td>Other musculoskeletal disorders</td>
<td>n = 1</td>
</tr>
<tr>
<td>Lesions noted at 1st endoscopy</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>n = 5</td>
</tr>
<tr>
<td>Antral erosions</td>
<td>n = 5</td>
</tr>
<tr>
<td>Duodenal erosions</td>
<td>n = 0</td>
</tr>
<tr>
<td>Gastric ulcers</td>
<td>n = 0</td>
</tr>
<tr>
<td>Duodenal ulcers</td>
<td>n = 0</td>
</tr>
<tr>
<td>Range of NSAIDs used</td>
<td></td>
</tr>
<tr>
<td>Indomethacin</td>
<td>n = 4</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>n = 2</td>
</tr>
<tr>
<td>Piroxicam</td>
<td>n = 2</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>n = 1</td>
</tr>
<tr>
<td>Naproxen</td>
<td>n = 1</td>
</tr>
<tr>
<td>Lesions noted at 2nd endoscopy</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>n = 8</td>
</tr>
<tr>
<td>Antral erosions</td>
<td>n = 1</td>
</tr>
<tr>
<td>Duodenal erosions</td>
<td>n = 1</td>
</tr>
<tr>
<td>Gastric ulcers</td>
<td>n = 0</td>
</tr>
<tr>
<td>Duodenal ulcers</td>
<td>n = 0</td>
</tr>
</tbody>
</table>
**TABLE 6.3**  Characteristics of 4 patients with gastric ulcers who discontinued NSAID therapy and who were treated with misoprostol (200 μg q.i.d) for 4 weeks. Patients in this group had previously taken NSAIDs for a mean of 2.8 years.

<table>
<thead>
<tr>
<th>Mean age</th>
<th>61.3 years</th>
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<tbody>
<tr>
<td>Age range</td>
<td>48 - 72 years</td>
</tr>
<tr>
<td>Type of arthritis</td>
<td>Osteoarthritis n = 2</td>
</tr>
<tr>
<td></td>
<td>Rheumatoid arthritis n = 2</td>
</tr>
<tr>
<td>Range of NSAIDs used</td>
<td>Indomethacin n = 2</td>
</tr>
<tr>
<td></td>
<td>Piroxicam n = 1</td>
</tr>
<tr>
<td></td>
<td>Naproxen n = 1</td>
</tr>
<tr>
<td>Ulcer sizes at 1st endoscopy</td>
<td>patient 1 0.9 cm</td>
</tr>
<tr>
<td></td>
<td>patient 2 0.6 cm</td>
</tr>
<tr>
<td></td>
<td>patient 3 0.9 cm</td>
</tr>
<tr>
<td></td>
<td>patient 4 1.0 cm</td>
</tr>
<tr>
<td>Ulcer sizes at 2nd endoscopy</td>
<td>patient 1 healed</td>
</tr>
<tr>
<td></td>
<td>patient 2 healed</td>
</tr>
<tr>
<td></td>
<td>patient 3 0.4 cm</td>
</tr>
<tr>
<td></td>
<td>patient 4 0.8 cm</td>
</tr>
</tbody>
</table>
Figure 6.1 A shows the changes in the number of mitoses per antral (lesser curve) gastric gland in 10 patients before and after two weeks of treatment with NSAID. Each of these patients received placebo tablets in addition to the NSAID. There were increases in glandular proliferation following NSAID therapy in each of the patients studied. The ratio of proliferative activity prior to commencement of the NSAID to the proliferative activity at the end of treatment has been denoted the 'Adaptation Index' (AI). The mean number of mitoses per gland prior to treatment was 4.45 (0.57) (mean (SEM)). The mean number of mitoses per gland at the same site after completion of two weeks of NSAID therapy was 7.69 (1.08). The AI for lesser curve antral mucosa following NSAID therapy is, therefore, 1.73 (0.06) (mean (SEM), P<0.05). 

Figure 6.1 B shows the changes in the number of mitoses per antral (lesser curve) gastric gland in 9 patients before and after two weeks of treatment with an NSAID plus misoprostol. Antral biopsies from the tenth patient were unsuitable for analysis because of drying of these biopsies on the walls of the storage container. There were similar increases in glandular proliferation following this combination therapy in each of the 9 patients studied. The AI for lesser curve antral mucosa following combination therapy with NSAID plus misoprostol was 1.84 (0.18) (mean (SEM), P<0.05). 

Figure 6.2 A shows the changes in the number of mitoses per duodenal crypt (D1) in 9 patients before and after two weeks of treatment with an NSAID. Each of these patients received placebo tablets in addition to the NSAID. Biopsies from the tenth patient were unsuitable for analysis because of drying of the biopsies on the walls of the storage container. There were increases in crypt proliferation following NSAID therapy in each of the patients studied. The AI for duodenal cap mucosa following NSAID therapy was 1.62 (0.23) (mean (SEM), P<0.05). 

Figure 6.2 B shows the changes in the number of mitoses per duodenal crypt (D1) in 10 patients before and after two weeks of treatment with an NSAID plus misoprostol. There were increases in crypt proliferation following this combination therapy in 9 of the 10 patients studied. The AI for lesser curve antral mucosa following
combination therapy with NSAID plus misoprostol was 1.50 (0.18) (mean (SEM), P<0.05).

NSAID therapy for two weeks also resulted in an increase in the relative number of fissured glands and crypts, such that duplicates, triplicates, and higher levels of gland and crypt division increased in frequency (Figures 6.3 and 6.4).

Figures 6.1 C and 6.2 C show the changes in the number of mitoses per antral gland and duodenal crypt, respectively, in patients before and after a month of treatment with misoprostol. It can readily be seen that there is no significant net proliferative effect of misoprostol alone in the gastric antrum or in the duodenum in these patients. Thus the adaptation indices for the antrum and duodenal cap were 1.08 (0.13) and 0.93 (0.14) (mean (SEM)), respectively, neither of which is significantly different from unity. Misoprostol therapy on its own did not alter the state of fissuring or duplication of gastric glands or duodenal crypts (data not shown).

**DISCUSSION**
The following conclusions are possible from the studies presented in this Chapter.

1. Patients with arthritis develop gastric and duodenal erosive lesions following administration of NSAIDs for two weeks.
2. These patients respond to NSAID therapy by increasing gastric and duodenal epithelial turnover rates.
3. Misoprostol failed to augment this adaptive response to NSAID therapy, even in patients in whom lesion occurrence was prevented, and in whom pre-existing lesions were healed.
4. Misoprostol therapy in 4 patients with gastric ulcers had no effect on gastric or duodenal cell turnover, despite ulcer healing.

Previous studies of gastric adaptation in man have been limited to healthy volunteers (Graham *et al*, 1983 and 1988a). One study showed that aspirin (2.6 gm daily) induced endoscopically visible gastric injury (erosions and bleeding) which was maximal within 3 days, and then tended to resolve despite continued administration
of aspirin (Graham et al, 1983). This study also showed that the healing time was accelerated in those who received aspirin for 7 days compared with those who had received it for one day. In an attempt to study this process more closely in humans, Graham et al (1988a) used gastric deoxyribonucleic acid (DNA) recovery as a marker of cellular exfoliation and regeneration. It was found that DNA recovery increased significantly just before the time of resolution, when, on average, it more than doubled. These authors concluded that gastric adaptation to chronic injury may involve increased mucosal cellular turnover. However, reliable direct histologic and mucosal kinetic data in support of this claim were lacking. No study to date has considered the proliferative effects of NSAIDs in patients with arthritis.

The crypt dissection procedure utilised in my studies has several advantages over the examination of histological sections, as discussed in detail in Chapter 3. Here I have shown that large numbers of crypts and glands can be individually scored in three dimensions, whereas histological sections are scored in one plane only. This is particularly relevant to my work because gastric glands and intestinal crypts duplicate by fissuring and bifurcating. This process, which was first described by Clarke (1972), is an important adaptive process which has received little attention. It has been postulated that crypt fission is involved in the growth and regeneration of the intestinal mucosa (Clarke, 1972; Cairnie, 1976; Maskens, 1978; Maskens and Dujardin-Loits, 1981). This increase in crypt duplication through fissuring represents one aspect of the adaptive response to NSAID therapy in humans, its occurrence correlating well with the accompanying increase in epithelial turnover in the stomach and duodenum. There are at least two precedents for this phenomenon in the literature: Cairnie and Millen (1975), studying the response of the mouse intestine to moderate doses of X-radiation, showed an increase in crypt number through budding and fission of repopulated crypts which had become larger than normal (i); later, Cheng, McCulloch and Bjerknes (1986) showed that following a 30% intestinal resection, villi as well as crypts grow in size, as does the number of proliferating cells per crypt (ii). My microdissection studies indicate that gland and crypt division by fission is an important component of the adaptive response to NSAID treatment in man.
The experiments presented earlier in this Thesis (Chapter 5), and by other investigators in recent literature (Goodlad *et al.*, 1989), confirm that misoprostol increases the gastric gland cell production rate in animals. In the study of Goodlad *et al.*, the augmented gastric gland cell production rate resulted in mucosal hyperplasia, with a preferential increase in the mucous secreting foveolar cell compartment of the gastric pits, and a consequent relative reduction in the acid-secreting parietal cells and pepsin-secreting chief cells. The increase in size of the protective functional compartment, and the relative decrease in size of the acid and pepsin secreting compartment, would reasonably be expected to favour mucosal healing and resistance to erosive insult. At the high doses used (300 μg/kg/day) such resistance to exogenous insults is certainly seen. However, it is also clear in animals (Robert, 1979) as well as man (Roth *et al.*, 1989) that the protective and healing properties of prostaglandins occur at much lower doses (approximately 10 μg/kg/day in humans). At this dose, no proliferative effect of misoprostol is noted in the four patients with arthritis studied. Indeed, no proliferative effect was noted at this dose in rats (Chapter 5). Therefore, the proliferative and trophic effects of prostaglandins may well be purely pharmacological effects, noted in animals at high doses during the course of routine pre-clinical toxicology studies. Thus, while the recent heated debate about the trophic effects of prostaglandins has been a fascinating exercise in proliferation methodology, these effects are likely to be of little clinical significance.

The facts are, however, that misoprostol is not only effective in the prevention of gastric ulceration in NSAID users (Agarwal *et al.*, 1987a), but appears to have a clear advantage over H2-receptor antagonists (Ehsanullah *et al.*, 1988) and sucralfate (Caldwell *et al.*, 1987) in this respect. Misoprostol also heals established NSAID-induced lesions, including ulcers, in patients with rheumatoid arthritis despite continuation of NSAID therapy (Agarwal *et al.*, 1987b; Roth *et al.*, 1989).

The protective and healing properties of prostaglandins in sub-trophic doses must therefore reside in their 'cytoprotective' (Dajani and Nissen, 1985; Wilson, 1987) and anti-secretory (Bauer, 1985)
functions. These effects are certainly operative at the low doses which humans can tolerate without significant gastrointestinal side effects. The anti-secretory activity of misoprostol is thought to be mediated through a class of high affinity E-type prostaglandin receptors on the gastric parietal cell surface (Tsai et al, 1986). In preparations of enriched canine gastric parietal cells misoprostol, as well as its major acid metabolite (SC-30695), binds to these receptors saturably, reversibly, and stereospecifically. This receptor-binding activity correlates well with the ability of misoprostol, and of its acid metabolite, to inhibit histamine-stimulated acid production by the parietal cells, possibly through inhibition of histamine-sensitive adenylate cyclase. In the recommended therapeutic doses, the drug achieves significant reductions in unstimulated nocturnal gastric acid secretion, as well as in basal, food-, histamine-, pentagastrin-, betazole- and coffee-stimulated gastric acid secretion (Akdamar et al, 1982; Nakamura et al, 1986; Wilson et al, 1986; Wilson, 1987; Davis et al, 1988; Mutoh et al, 1988).

The mechanisms underlying the mucosal cytoprotective effects of misoprostol are less clear. Cytoprotection may be defined as the ability to protect the gastrointestinal mucosa against damage by injurious agents through mechanism(s) independent of acid inhibition. It is believed to result from an enhancement of physiological mucosal defence mechanisms that are normally achieved by endogenous mucosal prostaglandins. These activities include:

1. Stimulation of gastric mucus secretion.
2. Stimulation of duodenal bicarbonate secretion.
4. Other activities, including the strengthening of intercellular tight junctions.

Misoprostol achieves each of the first three foregoing functions in human volunteers. Thus, it increases gastric mucus secretion (Wilson et al, 1986) and duodenal bicarbonate secretion (Selling et al, 1985) dose dependently. In addition, endoscopic organ reflectance spectrophotometry shows a 10-25% increase in mucosal
blood volume measured at 20 different gastric locations after a 200 μg dose (Sato et al, 1987).

Cytoprotection has been confirmed against a range of injurious agents, including alcohol, hypertonic solutions and thermal injury. Most notably, however, cytoprotection appears to be conferred against damage by the non-steroidal anti-inflammatory drugs (NSAIDs). As cyclo-oxygenase inhibitors, NSAIDs inhibit the production of mucosal prostaglandins. Exogenous prostanoids, such as misoprostol, may therefore be the logical choice in the protection against such damage and indeed, studies using aspirin and a range of non-aspirin NSAIDs confirm the ability of misoprostol to prevent as well as heal gastric and duodenal damage in healthy subjects (Cohen et al, 1985; Silverstein, 1986; Aadland et al, 1987; Silverstein et al, 1988; Lanza et al, 1988a and 1988b) as well as in patients with arthritis (Graham et al, 1988b; Roth et al, 1989).

The studies and conclusions in this Chapter have been concerned solely with the effects of NSAIDs and misoprostol on endoscopically normal mucosa. A deliberate effort was made to biopsy 'non-lesional' mucosa. The situation may well be very different at sites of major mucosal damage, such as erosions and ulcers. Such damage calls for augmented regenerative rates to achieve healing of mucosal defects. Chapter 7 addresses the effects of NSAIDs and misoprostol on the healing responses at the edges of ulcers in the rat, and in patients with gastric and duodenal ulcers.
CHAPTER 7
EFFECT OF NON-STEROIDAL ANTI-INFLAMMATORY DRUGS AND MISOPROSTOL ON GASTRIC AND DUODENAL ULCER HEALING THROUGH REGENERATION

INTRODUCTION
It is generally accepted that non-steroidal anti-inflammatory drugs (NSAIDs) are associated with acute and chronic gastric and duodenal damage (Langman, 1989). The range of lesions seen includes subepithelial haemorrhages, erosions and frank ulceration (Jiranek et al, 1989). The gastric and duodenal mucosae possess a range of complex physiologic functions that protect against such injury. These include the elaboration of a protective mucus-bicarbonate barrier (McGuigan, 1980), which confers protection against luminal irritants, and epithelial restitution (Svanes et al, 1982; Critchlow et al, 1985; Ito and Lacy, 1985; Silen and Ito, 1985; Lacy, 1987), the rapid movement of glandular cells to restore epithelial integrity following damage. Surprisingly, however, epithelial regeneration has received little attention as a repair function. Gastric ulcers heal by a brisk glandular regenerative response at the ulcer edge. This can be readily studied in experimental models of gastric ulceration. Interestingly, prostaglandins of the E series, which have recently received much attention as mucosal protective and ulcer healing agents, appear to have the ability to heal and prevent NSAID induced gastro-duodenal damage (reviewed by Konturek and Pawlik, 1986). Misoprostol, a prostaglandin E1 analogue, shares these protective (Silverstein et al, 1987) and healing (Agarwal et al, 1987a and 1987b) properties, and has convincingly been shown to stimulate gastric glandular proliferation (Goodlad et al, 1989 and Chapter 5). As cyclo-oxygenase inhibitors, NSAIDs reduce mucosal prostaglandin levels. We therefore hypothesised that NSAIDs predispose to gastric and duodenal ulceration by inhibiting reparative regeneration at the ulcer edge. Misoprostol would be expected to accelerate gastric ulcer healing in the presence of NSAIDs by augmenting the regenerative response. We tested this hypothesis in rats and in man. In rats, gastric ulcers were induced by a cryo-probe, using a modification of a method developed by Inauen et al (1988), and proliferative activity in the regenerative zone at ulcer edges was studied using the in vivo bromo-
deoxyuridine labelling method. In man, regenerative activity in the healing edges of gastric and duodenal ulcers was studied directly by microdissection of gastric glands and duodenal crypts in endoscopic biopsies from users and non-users of NSAIDs.

**MATERIALS**

Animals used were young adult male Wistar rats weighing 282-319 gm.

Indomethacin powder was kindly supplied by Merck Sharp and Dome Ltd. For experimental use indomethacin was dissolved in a solution of 5% sodium bicarbonate, and used within 30 minutes of preparation.

Misoprostol oil of high purity (98.5%) was supplied by G. D. Searle and Co. Ltd. (batch no 3P11200). It was stored at -20 degrees C prior to use. For these experiments 50 mg of misoprostol was brought to room temperature and dissolved in absolute ethanol (Analar grade). The resulting solution was immediately aliquoted into amber glass containers covered in foil for storage at -20 degrees C. Once a batch was thawed for use, any remaining misoprostol was discarded. For administration to rats by gavage, the misoprostol solution was diluted with sodium phosphate buffer (pH 7.4), the final concentration of ethanol being 0.1%.

**METHODS**

**ANIMAL STUDIES**

**Ulcer induction**

Gastric ulcers were induced by the application of a cryo-probe to the external (serosal) surface of the stomach. The cryo-probe used is shown in Colour Plate 4. It was designed by the author and produced specifically for this purpose by the Royal Postgraduate
Medical School Engineering Department. It is made of stainless steel with a rounded tip and an insulating handle.

Unfasted animals were anaesthetised at 09.00 by a 75 µl intramuscular injection of 'Hypnorm' (fentanyl citrate 0.315 mg/ml, fluanisone 10 mg/ml, Janssen Pharmaceuticals), and the stomach exposed through a 3 cm laparotomy incision. The cryo-probe was cooled to equilibrium in liquid nitrogen for 5 minutes and then applied firmly to the serosal surface of the anterior wall of the corpus of the stomach for 45 seconds (in preliminary experiments ulcer sizes were too small and variable with probe application times shorter than 30 seconds). This procedure was performed at 2 separate sites 8 mm apart. The laparotomy incision was then closed. The animals were recovered on a warmed mat, and then allowed free access to rat chow and water.

Pilot studies
Thirty seconds after removal of the cryo-probe from the serosal surface of the stomach, this site became hyperaemic and oedematous, and remained so for 2 days. The first histological evidence of mucosal ulceration (defined as a break in the muscularis musosae) at this site is seen after 24 hours. Ulcers become well demarcated macroscopically on day 3, enabling a determination of ulcer area in a pinned out stomach by square counting under a dissecting microscope. Ulcer healing occurs rapidly after this (Figure 7.1).

Experimental design
32 animals were divided into groups of 8 and received the following treatment daily at 09.00 hours for 10 days prior to ulcer induction and continued after ulcer induction until the animals were sacrificed for analysis.

Group A Controls (gavage and subcutaneous injection of phosphate buffer pH 7.4 containing 0.1% ethanol, and 5% bicarbonate, respectively).

Group B Indomethacin 2 mg/kg sc, and gavage vehicle.

Group C Indomethacin 2 mg/kg sc and misoprostol 300 µg/kg by gavage.

Group D Misoprostol 300 µg/kg by gavage, indomethacin vehicle.
On days 3 and 6 four animals from each group received an intraperitoneal injection of BRdU (100 μg/gram body weight) and was sacrificed an hour later for determination of gastric ulcer sizes and proliferative activity at the ulcer edges.

**BRdU-anti-BRDU method for assessment of proliferative activity at the ulcer edges**

Gastric ulcers with 2 mm of surrounding mucosa were carefully mounted in paraffin so that sections revealed the ulcers in cross-section with the glands cut longitudinally. The BRdU-anti-BRDU immunohistochemical staining technique used was as described in Chapter 2. Processed slides were analysed by counting labelled cells in 20 consecutive gastric glands in the regenerative zone at each ulcer edge.

**HUMAN PEPTIC ULCER STUDY**

**Endoscopies and biopsies**

Thirty four patients with gastric and duodenal ulcers attending the Hammersmith Hospital Endoscopy Unit were studied. All endoscopies were performed for standard gastroenterological indications. Patients were studied if they were found to have either a gastric or a duodenal ulcer. For the purposes of this study, the definition of an ulcer was that used by Larkai *et al* (1987), who state: "ulcers are three-dimensional, circumscribed mucosal defects, associated with an exudate, and having a diameter of 5 mm or more". Two pinch biopsies were carefully taken from the ulcer edge, taking care to align the axis of the forceps on an ulcer diameter, and excluding ulcer slough. For gastric ulcers two 'control' biopsies were taken 2 cm away from the ulcer, making sure that the mucosa here was endoscopically normal. Control biopsies for duodenal ulcers, all of which were in the duodenal cap, were taken from the opposite side of the cap, again making sure that the duodenal mucosa from this biopsy site was endoscopically normal.

Biopsies were immediately placed in Carnoy's solution, and then transferred to and stored in 70% alcohol until analysis by microdissection as described in Chapter 3.
The patients' drug histories were carefully noted by direct enquiry prior to endoscopy, when consent to the study was obtained. Regular use of NSAIDs, including aspirin, through until the day before endoscopy, was noted. Patients taking steroids, anti-neoplastic drugs, warfarin or excessive alcohol were excluded from the study. Patients with rheumatoid arthritis, and critically ill patients with stress ulcers, were also excluded from the study.

**Analysis of proliferation in endoscopic biopsies**

Using the microdissection method described in Chapter 3, gastric glands and duodenal crypts were microdissected in the biopsies taken. The whole of each biopsy was microdissected. This yielded approximately 30 suitable glands or 50 suitable crypts for counting of mitoses. Proliferative activity was expressed in mitoses per gland or crypt in the tissue obtained from the ulcer edge, as well as in the control sample. The ratio of these two figures provides a useful index of regenerative activity at the ulcer edge (the Regeneration Index, see below).

**RESULTS**

**ANIMAL STUDIES**

Figure 7.2 shows the gastric ulcer sizes on days 3 and 6 in each of the groups studied. Figures 7.3 A, B and C show the regenerative responses at the ulcer edges on day 3. Figures 7.4 A, B and C show the regenerative responses on day 6.

Rapid ulcer healing (Figure 7.2) was achieved in the control animals. Indomethacin treatment resulted in significantly larger ulcers (10.1 (1.3) mm²) compared with controls (4.9 (0.6) mm²) (mean (SEM), P <0.05); indomethacin also reduced by 3.1-fold the peak regenerative activity at the ulcer edge on day 3 (peak BRdU labelling per gland falling from 25 (6) to 8 (3) (P<0.05, Figures 7.3 A and B). On day 6 the indomethacin treated animals showed partial recovery of ulcer edge regeneration compared with control animals (peak labelling 14 (4) indomethacin, versus 16.6 (5) control, Figures 7.4 A and B).
Misoprostol treatment did not by itself alter the course of these gastric ulcers (Figure 7.2), but significantly reduced the indomethacin-induced increase in ulcer sizes (P <0.05). This was accompanied by partial restoration of the regenerative activity (to peak labelling of 14 (2) cells per gland on day 3 (Figure 7.3 C), and 20 (6) on day 6 (Figure 7.4 C). Misoprostol alone had no significant effects on the brisk regenerative response to ulcer healing (data not shown), suggesting that the control response is either maximal or near maximal.

At first sight it may seem surprising that misoprostol alone did not accelerate ulcer healing. However, the ulcers produced in this experimental system are very small, and heal very rapidly; the system is therefore not ideally suited to a study of acceleration of healing, but is suited to study of agents which slow healing.

In similar experiments, omeprazole (a powerful acid-inhibitory agent) was also studied, and showed no effect on the regenerative responses at the ulcer edge either alone or in combination with indomethacin (data not shown). This suggests that the misoprostol-induced enhancement of regeneration in indomethacin-treated animals is independent of the ability of this drug to inhibit acid secretion.

Representative histological sections of the gastric ulcers are shown in Colour Plates 5, 6 and 7.

HUMAN PEPTIC ULCER STUDY
Of the 35 patients studied, 17 had gastric ulcers and 18 had duodenal ulcers. Eight patients with gastric ulcers, and 10 patients with duodenal ulcers were regular users of NSAIDs for the treatment of osteoarthritis and other musculoskeletal disorders excluding rheumatoid arthritis. The mean age of the patients with duodenal ulcers was 45.4 years (range 21 - 68) (no-NSAID group) and 49.9 years (range 28 - 74) (NSAID group). The mean age of the patients with gastric ulcers was 53.5 years (range 39 - 79) (no-NSAID group) and 56.1 years (range 33 - 74) (NSAID group).
The range of NSAIDs used by these patients was as follows: in the gastric ulcer / NSAID group 6 patients used indomethacin, 1 used piroxicam, and 1 used aspirin. In the duodenal ulcer / NSAID group, 7 patients used indomethacin, 2 used piroxicam, and 1 used ibuprofen.

Figure 7.5 shows the number of mitoses per gastric gland in the no-NSAID group at each of the two locations biopsied: the endoscopically normal mucosa near the gastric ulcer, and the mucosa at the ulcer edge. All 9 patients showed significant increases in proliferative activity at the ulcer edge. The ratio of proliferative activity at the edge of the ulcer to the proliferative activity in the mucosa nearby has been denoted the 'regeneration index' (RI). The mean (SEM) number of mitoses per gland at the ulcer edge was 11.60 (1.37). In these patients the mean number of mitoses per gland in the adjacent mucosa was 3.75 (0.37). The RI in the no-NSAID gastric ulcer group was therefore 3.1 (0.61).

Figure 7.6 shows the number of mitoses per gastric gland in the NSAID group at each of the two locations biopsied. Seven of the 8 patients showed relatively small increases in proliferative activity at the ulcer edge. The RI in the NSAID gastric ulcer group is 1.49 (0.16) (mean (SEM)).

It is therefore clear that the regenerative response at the gastric ulcer edge is greater in the no-NSAID group than in the NSAID users. The difference in RI is significant (P=0.014, Wilcoxon).

Figure 7.7 shows the number of mitoses per duodenal crypt in the no-NSAID group at each of the two locations biopsied. All 8 patients showed increases in proliferative activity at the ulcer edge. The RI in this no-NSAID duodenal ulcer group is 3.64 (0.99) (mean (SEM)).

Figure 7.8 shows the number of mitoses per duodenal crypt in the NSAID group at each of the two locations biopsied. Eight of the 10 patients showed relatively small increases in proliferative activity at the ulcer edge. The RI in the NSAID duodenal ulcer group is just 1.20 (0.12) (mean (SEM)).
It can be seen that the regenerative response at the duodenal ulcer edge is greater in the no-NSAID group than in the NSAID users. The difference in RI is significant (P=0.014, Wilcoxon)

**DISCUSSION**
Damage of the gastric and duodenal epithelia is followed by two major types of reparative response: restitution (reconstitution) and regeneration. Of these, the former has received the most attention. Restitution, which is not affected by prostaglandins or by NSAIDs, is the process by which surface epithelial defects are covered by the migration of deeper, uninjured, cells over an intact basal lamina (Svanes et al, 1982; Critchlow, 1985; Ito and Lacy, 1985; Lacy, 1987). This movement of cells necessarily results in an epithelial surface which is thinner, and therefore more vulnerable, than normal, until such time that cell replication, initiated in the proliferation zone, restores normal cell numbers and mucosal thickness.

In this Chapter the effects of indomethacin and misoprostol on the healing of experimental gastric ulcers in the rat have been studied. Patients with gastric and duodenal ulcers in the presence and absence of NSAID therapy were also studied with regard to regenerative responses at the healing edges of the ulcers.

The following conclusions may be drawn from the rat experiments presented in this Chapter.

1. Controlled application of a cold cryo-probe to the serosal surface of the rat stomach results in well-defined and reproducible gastric ulcers. These ulcers heal over a period of days, as a result of a brisk regenerative response at the ulcer edge.

2. Indomethacin treatment leads to the formation of larger cryo-ulcers, and significantly impairs the healing of these ulcers. This results from a dramatic inhibition of reparative regeneration at the ulcer edge.
3. Co-treatment with indomethacin and trophic doses of misoprostol effectively reverses the indomethacin-induced slowing of ulcer healing. This is accompanied by partial restoration of the regenerative response at the ulcer edge.

The following conclusions may be drawn from the human studies presented in this Chapter.

1. In the absence of NSAID therapy patients with gastric and duodenal ulcers show regenerative responses at the ulcer edge. This is a normal feature of the ulcer healing process.

2. Patients with gastric and duodenal ulcers who are regular users of NSAIDs show significantly impaired regenerative responses at the ulcer edge.

Other investigators have now also shown that administration of indomethacin significantly delays the natural healing of experimental gastric ulcers in rats. Wang et al (1989) produced gastric ulcers by injecting 20% acetic acid (0.03 ml) into the submucosal layer of the gastric wall of the antral-oxyntic border. The natural healing of the acetic acid-induced ulcers was extensively delayed by administering indomethacin (1 mg/kg) subcutaneously once daily for 2 or 4 weeks. Subcutaneous administration of natural prostaglandin E2 (1 or 3 mg/kg) twice daily for 2 and 4 weeks, together with indomethacin, significantly prevented the delay of ulcer healing. Prostaglandin E2 (3 mg/kg) administered twice daily for 2 weeks also significantly accelerated the natural healing of the ulcers in the absence of indomethacin therapy. These results suggested that endogenous prostaglandins play an important role in the healing of gastric ulcers. However, the mechanistic basis of these observations was not explored. Inauen et al (1989) did study the effect of indomethacin and prostaglandins on 3H-thymidine labelling index at the edges of experimentally induced gastric ulcers in the rat, but this method for the assessment of proliferation ignores effects of the healing process on gland size and length (discussed in detail in Chapter 3). Thus, these investigators concluded that indomethacin reduced the labelling index at the ulcer edge by 30%, but curiously also found that large doses of prostaglandin E2, which in the same study were shown to
be trophic, had no significant effect on the labelling index. This suggests that in this study the proliferation methodology was confounded by its inability to account for the change in population size of the growing gastric glands. The effect of indomethacin and prostaglandin co-administration was not studied; neither was the effect of drug therapy prior to ulcer induction.

No study to date has examined the effect of NSAIDs on the ulcer healing process in man. There has been no adequate explanation of the much greater prevalence of gastric and duodenal damage in patients regularly using NSAIDs, a finding confirmed by several retrospective as well as prospective studies (reviewed by Langman, 1989; McCarthy 1989; and in Chapter 1). Over 10% of patients receiving NSAIDs in the long term will have a gastric ulcer on any given day (reviewed by McCarthy, 1989). Several studies have implicated NSAIDs with upper gastrointestinal bleeding and perforation of both gastric and duodenal ulcers, particularly in women (Lamy, 1987; Walt et al, 1986). NSAID users are overrepresented in patients who bleed from peptic ulcers (Somerville et al, 1986), perforate their peptic ulcers (Armstrong and Blower, 1987), and die from complications of peptic ulcers (Armstrong and Blower, 1987). Clearly, NSAIDs must have a fundamental influence on either the rate of generation or the rate of healing of peptic ulcers (or both). It would be extremely difficult, if not impossible, to determine from epidemiological studies which of these two possibilities is actually operative. This highlighted the need for studies of the effects of NSAIDs on gastric mucosal function, and in particular their effects on healing.

Our finding that treatment with a range of NSAIDs appears to blunt the normal regenerative response at the ulcer edge is consistent with the greater prevalence of ulcers in NSAID users, as ulcers in these patients would be expected to heal spontaneously at a significantly lower rate. There is evidence in man to suggest that NSAIDs retard the healing of gastric damage by H2-receptor antagonists (Bianchi Porro et al, 1987). NSAID users may well also have a greater predisposition to ulcer formation, but our human studies do not shed any light on this additional possibility.
It is not clear why NSAIDs should inhibit the normal regenerative function of the gastric and duodenal mucosae at sites of mucosal damage. The situation at sites of mucosal damage appears to be very different from other apparently 'normal' areas of mucosa, where adaptation to continuous low grade damage of superficial structures leads to an effective adaptive increase in gland cell turnover (discussed in Chapters 5 and 6). It is possible that the mucosal adaptive process may fail focally at sites which are then rendered more vulnerable to erosive damage. What is clear is that no NSAID is free from long term gastrointestinal complications (Committee on The Safety of Medicines Updates 1 and 2, 1986). The property of cyclo-oxygenase inhibition is common to all NSAIDs, and is believed to be an important mechanism underlying their toxicity (Duggan, 1981; Dajani, 1986). My experimental findings, and those of others (Wang et al, 1989), suggest an important role for prostaglandins E1 and E2, respectively, in the augmentation of ulcer healing through regeneration. This is entirely in keeping with their known trophic and proliferative effects on the gastric mucosa (Goodlad et al, 1989; and Chapter 5). As cyclo-oxygenase inhibitors NSAIDs significantly lower mucosal prostaglandin synthesis. For example, endogenous prostaglandin E2 levels in the gastric mucosae of rats were significantly reduced for at least 12 h after single doses or repeated administration of indomethacin (1 mg/kg) (Wang et al, 1989). It is therefore not surprising that NSAIDs inhibit reparative regeneration. It is also not surprising that prostaglandin co-administration with indomethacin, as we have shown, prevents the delay of ulcer healing. This is presumably because prostaglandin levels revert significantly toward control levels after administration of exogenous prostaglandin; however, this requires large doses of exogenous prostaglandin. In the study of Wang et al, 3 mg/kg of prostaglandin E2 was required to adequately replenish gastric mucosal levels for brief periods. It has been shown in Chapter 6 that the relatively small doses of misoprostol tolerated by humans (approximately 10 μg/kg/day) are not trophic to either the gastric or duodenal mucosae. This dose of misoprostol would therefore not be expected to augment reparative regeneration at sites of ulcers. Indeed, the early clinical experience with misoprostol shows it to be no more effective than H2-receptor antagonists in acceleration of gastric or duodenal ulcer healing (Brand et al, 1985; Agrawal et al, 1985): their ulcer healing effects are likely, as with the H2-
antagonists, to reside predominantly in their gastric anti-secretory properties and/or other mucosal effects, and not, at the recommended therapeutic doses, in their growth promoting effects.
CHAPTER 8

HELCOBACTER PYLORI AND DUODENAL ULCERS: THE GASTRIN LINK

INTRODUCTION
Helicobacter pylori (HP) is a microaerophilic, spiral gram negative bacterium, which has a remarkable ability to colonise the hostile environment of the human stomach. Since its isolation from the human gastric antrum by Marshall in 1983, this organism has stimulated much interest and research with regard to its relationship to human disease.

HP colonises only gastric mucosa and prefers the antrum (Steer, 1985; Blaser 1987; Malfertheiner and Ditschuneit, 1988). Its prevalence in normal individuals rises from 24% in medical students (Langenberg et al, 1984) to 75% in the over-seventies (Graham et al, 1987; Graham et al, 1988c; Mitchell et al, 1988; Perez-Perez et al, 1988; Kosunen et al, 1989), although the age-adjusted prevalence clearly differs between various geographic areas (Graham et al, 1988c; Mitchell et al, 1988; Kosunen et al, 1989). How normals become colonised is not clear. Although it has been speculated that coccoidal forms of the bacterium may be important in its transmission (Megraud, 1989), there is still no firm evidence of the mode of transmission. Abattoir workers are more frequently seropositive (Vaira et al, 1988), though no animal reservoir has been identified. Recent studies suggest that the molecular basis of the unique preference of HP for gastric epithelium, and especially the antrum, lies in its ability to specifically bind to a glycerolipid present in gastric mucosa (Lingwood et al, 1989).

HP colonisation is diagnosed after gastroscopy by microscopy (Warren, 1983), culture (Goodwin et al, 1985) or a urease test on antral biopsies (McNulty and Wise, 1985).

The Warthin-Starry silver stain, conventionally a technique for demonstrating spirochaetes in tissue sections, was used by Warren
and Marshall in their original work (Warren, 1983; Marshall, 1983). Although this shows HP particularly clearly, the staining technique is technically demanding, and prone to producing artefactual silver deposits which may mimic bacteria (Wyatt and Gray, 1989). Such stains as Giemsa, Gimenez, half-Gram, Brown-Hopps, cresyl fast violet, and acridine orange (with fluorescence microscopy) are all suitable, and have advantages of speed and simplicity over the Warthin-Starry silver stain (Wyatt and Gray, 1989). However, none of the stains described above is specific for HP but instead demonstrate the characteristic appearance of the organism which allows HP to be recognised.

HP grows best in a microaerobic atmosphere of 5% O$_2$ with 5-10% CO$_2$ (Goodwin et al., 1985); it will not grow in the complete absence of oxygen (anaerobic culture). A variety of basal media with agar and added blood have been used for the isolation of HP from clinical specimens (Tompkins, 1989). On these solid media, incubated at 37°C, small colonies (1mm in diameter) are seen usually after incubation for 3-4 days.

The urease test is based on HP's production of a powerful urease, first described by Langenberg et al., in 1984, and later partially characterised by Mobley et al., in 1988. The latter group found the HP urease to have a molecular weight of 480,000 daltons but metabolically active subunits of 240,000 and 120,000 daltons were also found later (Megraud, 1989b). Mobley et al. (1988) obtained a value of 510,000 daltons for the molecular weight, and 5.9 for the pHi. The nucleotide sequence of two genes from HP, encoding for urease subunits, has now been obtained (Clayton et al., 1990). Urease, which has a narrow substrate specificity, hydrolyses one molecule of urea to produce one molecule of carbon dioxide and two molecules of ammonia, with net production of alkali. This may enable HP to survive gastric acid by neutralising the microenvironment around the organism. All strains of HP so far isolated possess urease at the time of isolation. Urease negative strains have only been found in subcultures (Megraud, 1989).
HP may also be diagnosed by the urea breath test (Marshall and Surveyor, 1987) which also detects urease. New serological tests based on anti-HP IgG are more specific for HP than earlier immunological tests (Newell et al, 1988).

HP infection is strongly associated with chronic active gastritis (Marshall, 1983; Warren, 1983; Marshall and Warren, 1984) and duodenal ulceration (Dooley and Cohen, 1988). Nearly 100% of patients with non-immune gastritis are colonised with HP (Hazell et al, 1987), and there are two researchers who deliberately ingested the organism and developed a persistent gastritis with antral colonisation (Marshall et al, 1985; Morris and Nicholson, 1987).

Even more intriguing is the relationship of this organism with duodenal ulcer disease, because HP can colonise only gastric epithelium (Steer, 1985; Malfertheiner and Ditschuneit, 1988), and is found in the duodenum only if gastric metaplasia is present.

Yet there is very strong epidemiological evidence to support a relationship between antral HP colonisation and DU disease. Firstly, HP colonisation occurs in the majority of patients with DU disease, compared with a minority of the normal population (Dooley and Cohen, 1988). Secondly, longer remissions of the disease are obtained with treatments that suppress or eradicate HP than with those that do not (Martin et al, 1981; Marshall et al, 1988; Smith et al, 1988; Coghlan et al, 1988). Finally, after eradication of HP, ulcers rarely occur before recolonisation with the organism (Coghlan et al, 1988; Marshall et al, 1988; Smith et al, 1988).

An important question, therefore, is how antral HP infection causes ulcers in the duodenum. The prevalent view (reviewed by Goodwin, 1988 and by Graham, 1989) stresses the importance of heterotopic gastric mucosa in the duodenal cap in patients with DU disease. HP, with an apparent requirement for gastric surface mucous cells, colonises these areas of duodenal gastric metaplasia, resulting in a localised patch of gastritis which progresses and eventually leads to a breach in the mucosa at or adjacent to this site. The author's view
is that this sequence of events is at best of secondary importance. There is good evidence that the presence of gastric mucosa in the duodenum, which allows HP to colonise the duodenum, is an expected response to the increased duodenal acid load found in many patients with DU disease (Kreuning et al, 1978; Wyatt et al, 1987). This is supported by animal studies (Rhodes, 1964; Florey et al, 1939) where an increase in the prevalence and extent of gastric mucosa in the duodenum followed establishment of a chronic hyperacidic state or the addition of gastric juice to intestinal contents. This may also be the case in man, as duodenal gastric metaplasia has been found in 84% of patients with DU disease with maximal acid outputs over 30 mmol/h (Patrick et al, 1974). It would appear, therefore, that gastric metaplasia in the duodenum occurs in response to duodenal injury, i.e. it may reflect the fact that a DU has been present and has healed (Patrick et al, 1974). HP infection of this heterotopic gastric mucosa may then serve to perpetuate duodenal inflammation, and in this way determine the site of subsequent ulcers. The organism may achieve this via a local cytotoxic effect diminishing mucosal resistance to luminal aggressive factors, including acid (Goodwin, 1988).

A more fundamental question is why patients with DU disease have the increased acid secretion which leads to duodenal damage. Gastric acid secretion is fundamentally implicated in the pathogenesis of DU disease. Firstly, it is established that mean basal, peak stimulated and meal stimulated gastric acid secretion rates are higher in patients with DU disease than in normal subjects (Wormsley and Grossman, 1965; Isenberg et al, 1975; Baron, 1978; Jalan et al, 1979; Lam and Ong, 1980). Secondly, DUs are rarely seen in individuals with low gastric acid secretion rates (peak acid output less than 15 mmol/hour) (Baron, 1982). Finally, suppression of gastric acid secretion by H2-receptor antagonists (Bardhan et al, 1979) and omeprazole (Lamers et al, 1984) leads to rapid healing of DUs.

In my studies I have considered the relationship between antral HP colonisation, antral gastrin release and gastric acid secretion. It is
known that mean basal, peak pentagastrin-stimulated, and meal-stimulated gastric acid secretion rates are higher in patients with DU disease than in normal subjects (Walsh et al, 1975). Patients with DU disease also tend to have higher peak post-prandial plasma gastrin concentrations than normal subjects (Taylor et al, 1981). Gastrin is the most potent known stimulant of gastric acid secretion and appears to be responsible for most of the increase in gastric acid secretion which occurs after eating (Maxwell et al, 1984). At first sight the involvement of HP in DU disease might seem to be at variance with the established view that DU disease is due to the tendency to inappropriate gastrin release and increased gastric acid secretion. I therefore tested the unifying hypothesis that HP in the gastric antrum increases gastrin release and thereby acid secretion. Here I present my studies of gastrin release, gastric acid secretion, and the presence or absence of antral HP in patients with established DU disease. The urease test on antral biopsies has been used to detect HP. This is because it seemed logical, in this study, to use a test which depended on ammonia production for a positive result, and had the added advantage of speed of results. The urease test has a sensitivity of between 94-98% (Hazell et al, 1987; McNulty et al, 1989; Vaira et al, 1988b). However, although false positives are a potential problem with this test, the specificity is near 100% (McNulty, 1989). It is accepted that the urease test, as described by McNulty et al (1989), is not as sensitive as some of the other methods for detecting the presence of HP (vide supra). Future studies in our laboratory are therefore using the urease test in conjunction with culture and histology.

METHODS

Patients, endoscopies, and urease test
The ethics committee approved the protocol and 51 patients gave informed consent. None had any other significant medical condition or had undergone gastric or vagal surgery. None had taken histamine H2-receptor antagonists for at least 3 days, or colloidal bismuth subcitrate ('Denol', Gist-Brocades, Holland) for at least 8
weeks. Patients with renal failure and gastrinoma were excluded from this study. DUs were seen at endoscopy in all patients and 2 antral biopsies were taken for determination of HP status by the urease method, as described and validated by McNulty and Wise (McNulty and Wise, 1985; McNulty et al, 1989): briefly, biopsies are crushed in a solution containing urea and phenol red (modified Christensen's urea broth) and incubated at 21 degrees C. The presence of a pink colour after 24 h is taken to be positive.

**Acid secretion and gastrin studies**

Studies of gastric acid secretion and antral gastrin release were performed within 4 days of endoscopy, when patients had not taken H2 antagonists for at least 4 days. Basal and peak gastric acid secretion, respectively, were determined before and during an infusion of pentagastrin (6 μg/kg/h) for 105 min, as described previously (Baron, 1978). Pyloric losses of gastric juice were corrected for by determining the phenol red recovery in aspirated gastric juice. Pyloric reflux was also corrected for by the estimation of the sodium concentration in the aspirated gastric fluid, as follows. The sodium concentration (mmol/L) was measured in each 10 minute sample of gastric aspirate by flame photometer. The sodium output (QNa) is then calculated, enabling the volume of reflux (VR) from the duodenum to be derived using the following formula:

\[ VR = [7.34 \times QNa] - [0.0712 \times VC] - 1.281 \]

where VC is the volume corrected for pyloric losses. The estimated volume of gastric juice produced is then VC - VR. The principles behind these calculations have been obtained from Whitfield and Hobsley (1977).

Venous blood for gastrin assay was collected into tubes containing EDTA before and after a standard meal consisting of 2 eggs, 2 beef
extract cubes ('Oxo', Brooke Bond Oxo Ltd, Croydon) in 200 ml water and 2 rounds of dry toast. Gastrin was measured by radioimmunoassay (Bryant and Adrian, 1982) using antiserum G179 donated by Professor S.R. Bloom.

Suppression of antral HP
Ten HP +ve patients were treated with colloidal bismuth subcitrate (120 mg qid) for 4 weeks, and metronidazole (400 mg tid) for the first 2 weeks. A similar regime was recently shown to eradicate HP in 75-80% of colonised patients with duodenal ulcers (O'Riordan et al., 1989). Endoscopies, urease tests, and acid and gastrin studies were repeated on completion of this treatment.

Gastric acid secretion and plasma gastrin concentrations were determined by workers who were unaware of the patients' HP status. Statistical analysis was by Wilcoxon's rank sum and matched pairs tests, and results are expressed as mean + SEM. Integrated postprandial gastrin responses were calculated over the first 40 minutes after commencement of the meal.

RESULTS

HP status, acid secretion and gastrin studies
Seven of the patients had a negative urease test (HP-ve) and 44 had a positive urease test (HP+ve). The HP-ve group comprised 3 men and 4 women aged 31 - 67 years, with a mean age of 49 years. The HP+ve group comprised 22 men and 22 women aged 15 - 60 years, with a mean age of 46 years.

Mean basal rates of gastric acid secretion were similar in the HP+ve (5.74 (0.90)) and in the HP-ve (6.01 (2.00)) patients (mean (SEM)). However, peak pentagastrin stimulated acid secretion (mmol/h) was 45.18 (3.0) in the HP+ve compared with 29.7 (4.0) in the HP-ve patients (mean (SEM), p <0.05) (Figure 8.1). The HP+ve patients also produced significantly greater volumes of gastric juice during pentagastrin infusion than the HP-ve patients (Figure 8.2).

Mean basal and postprandial plasma gastrin concentrations were also higher in the HP+ve compared with the HP-ve patients (Figure 8.3). Integrated meal-stimulated plasma gastrin responses
(pmol.min/1) were 1564 (267) in the HP+ve and 965 (248) in the HP-ve patients (mean (SEM), p <0.05).

**Effects of suppression of antral HP**

Of the 10 HP+ve patients treated with colloidal bismuth subcitrate 120 mg qid for 4 weeks and metronidazole 400 mg tid for 2 weeks, 9 were HP-ve following treatment. Changes in integrated gastrin responses in individual patients are shown in Figure 8.4 A. Integrated meal-stimulated gastrin responses fell from 1184 (350) to 498 (117) pmol.min/l (mean (SEM), P = 0.005, Wilcoxon matched pairs test) (Figure 8.5). It is of interest that mean basal and mean peak acid secretion rates remained unchanged after treatment (Figure 8.4 B). Mean PAO before treatment was 45.27 (7.48) and after treatment was 45.56 (4.37) (mean (SEM)).

**DISCUSSION**

In this study I have demonstrated that pentagastrin stimulated gastric acid secretion and postprandial plasma gastrin concentrations are significantly higher in HP+ve compared with HP-ve patients with DU disease. Furthermore, eradication of antral HP led to a significant drop in postprandial plasma gastrin response.

The relationship between colonisation of the gastric antrum with HP and DU disease has been demonstrated by epidemiology. HP colonisation is present in the great majority of patients with DU disease, compared with about one third of the general population (Dooley and Cohen, 1988). Treatments which suppress or eradicate HP produce longer remissions of DU disease than treatments which do not (Martin et al, 1981). Moreover, after eradication of HP, ulcers rarely return before re-colonisation occurs (Coghlan et al, 1988; Marshall et al, 1988; Smith et al, 1988). For example, Marshall et al (1988) showed that treatment which failed to eradicate HP resulted in a 61% healing rate and 84% relapse rate as assessed by sequential endoscopies over 12 months. When HP was successfully cleared from the antrum, using either colloidal bismuth subcitrate or tinidazole, 92% of ulcers healed (P <0.001) and only 21% (P <0.0001) relapsed during the 12 month study period. Thus eradication of HP significantly improves the healing rate as well as the length of
remission of DU disease. So far, the basis of these well established observations has been elusive.

It is established that mean basal, peak stimulated and meal stimulated gastric acid secretion rates are higher in patients with DU disease than in normal subjects (Wormsley and Grossman, 1965; Isenberg et al, 1975; Baron, 1978; Jalan et al, 1979; Lam and Ong, 1980). In addition there is evidence that patients with DU disease secrete a greater proportion of their PAO after meals (Lam and Ong, 1980; Malagelada et al, 1977; Soll, 1989), suggesting that their parietal cells are stimulated more, or for longer, after eating.

Patients with DU disease also tend to have higher peak postprandial plasma gastrin concentrations than normal subjects (Taylor et al, 1979; Taylor et al, 1981). DU patients with both postprandial hypergastrinaemia and increased gastric secretion rates have been described as having antral G-cell hyperfunction (Taylor et al, 1981). Gastrin release is also inappropriate in patients with elevated gastrin and normal acid secretion or normal gastrin and elevated acid secretion, because intragastric acid normally inhibits gastrin release (Walsh et al, 1975). Indeed, Walsh et al. (1975) have demonstrated, using intragastric titration experiments, that a low intragastric pH inhibits antral gastrin release less effectively in patients with DU disease than in normal subjects. This has been confirmed in other studies by Hirschowitz et al (1985) who showed exaggerated release of gastrin with bombesin (which is structurally almost identical with gastrin releasing peptide), as well as a defective inhibition of acid and pepsin secretion with higher doses of bombesin.

There are several possible explanations for the respective roles of HP colonisation, postprandial hypergastrinaemia and hypersecretion in the aetiology of DU disease. Duodenal ulceration has been regarded as the common end result of several different abnormalities, but epidemiological evidence now suggests that HP plays a major role in most cases (Coghlan et al, 1988; Marshall et al, 1988; Smith et al, 1988). It may seem reasonable to argue that DUs occur if an individual has a sufficiency of different adverse factors, including HP colonisation and gastric hypersecretion. However if this were
the case I might have expected to find hypersecretion in my HP-ve patients, whereas I have found the reverse.

On the basis of my present results it is proposed that HP in the gastric antrum increases antral gastrin release. HP attaches to epithelial cells on the surface and within the pits of the gastric antrum (Price et al, 1985; Dooley and Cohen, 1989). Gastrin is released from specialised G-cells located in the antral glands. The powerful urease that HP produces (McNulty and Wise, 1985; Dooley and Cohen, 1988) splits urea which is present in the stomach, to produce one molecule of carbon dioxide and two molecules of ammonia, with net production of alkali (Marshall, 1986). We postulate that the ammonia elevates the pH within the mucus layer which overlies the gastric antrum (Price et al, 1985), resulting in impairment of the normal inhibition of gastrin release by intraluminal acid, as observed by Walsh et al (1975) in patients with DUs (Figure 8.5). Increased gastrin release increases gastric acid secretion both by directly stimulating parietal cell acid secretion and by its trophic effect on the gastric secretory cell mass (Crean et al, 1969; Johnson, 1976; Lam and Ong, 1980). Increased acid secretion then causes duodenal ulcers by producing a low intraduodenal pH (Malagelada et al, 1977). My intramucosal acid-neutralisation hypothesis is illustrated in Figure 8.6.

Our observed reduction in meal stimulated plasma gastrin responses following suppression of HP could explain the reduced likelihood of ulcer relapse in patients treated with agents that suppress or eradicate HP, as observed in several studies (Coghlan et al, 1988; Marshall et al, 1988; Smith et al, 1988). This fall in meal stimulated plasma gastrin levels, which is not seen after treatment with H2-receptor antagonists (Forrest et al, 1979) and is therefore not simply a result of ulcer healing, would be expected to result in a diminished meal stimulated secretory drive on the gastric parietal cells. Another predictable long term result of the lower gastrin levels is a reduction of the parietal cell number, due to a reduction of the trophic effect of gastrin on these cells (Crean et al, 1969; Johnson, 1976; Lam and Ong, 1980). Peak acid output, which reflects the parietal cell mass, did not fall significantly in my patients after one month of treatment to eradicate HP, despite the lower gastrin levels. Presumably this reflects the need for a longer period of time to
elapse before a significant reduction of parietal cell numbers occurs. Longer term studies are required in patients who remain free of the organism to reveal an effect on maximal acid secretory capacity.

An alternative explanation of my present results deserves comment: HP colonisation might occur in acid hypersecretors if a low pH encourages growth of the organism. However, this seems unlikely because HP grows well in vivo at neutral pH (Dent and McNulty, 1988). In addition, HP colonisation is uncommon in patients with hypersecretion due to the Zollinger Ellison syndrome (Graham, 1989). The fall in postprandial gastrin responses after eradication of HP in my studies disproves this alternative theory.

The relationship between HP colonisation and antral gastrin release is now also being considered by other investigators. Thus, Smith et al (1989) have shown 24-hour hypergastrinaemia in normal asymptomatic individuals with HP antibodies in their serum, but patients with DU were not studied. Brady et al (1988), studying acid secretion in a heterogeneous group of patients, found fasting hypergastrinaemia in a small number of patients found to have HP, but attributed this variously to co-existing chronic renal failure, vagotomy, total parenteral nutrition, and 'possible antral G-cell hyperfunction.' This group investigated whether HP could survive a wide range of acid conditions, and concluded that it could. Oderda et al (1989) have shown falls in fasting gastrin levels in children with HP-associated gastritis treated with amoxycillin plus tinidazole, but untreated controls, required here as some of the children may well have been studied during an acute infection, were lacking in this study. I stress that HP induced hypergastrinaemia with gastric hypersecretion in DU disease is likely to result from chronic colonisation with the organism. It is clear that the situation following acute infection may be very different. There is good evidence that acute infection actually induces hypochlorydria (Morris and Nicholson, 1987), and I note with interest that this has been attributed to the direct effect of a protein produced by HP on the gastric parietal cells (Cave and Vargas, 1989).

Other investigators have also attempted to explore my hypothesis by eradicating HP in patients with DU disease. Thus, McColl et al (1989a and 1989b) have shown that following treatment with
colloidal bismuth subcitrate, metronidazole and amoxycillin (120 mg, 400 mg and 250 mg, respectively, each 3 times a day for one month), integrated meal-stimulated gastrin responses fall almost exactly as in my studies. In addition, as expected, HP eradication resulted in higher gastric juice pH during meals. However it should be noted that in situ pH monitoring may not necessarily reflect the acid secretion rate.

Sceptics (eg. Treiber, 1989) cite data which on initial inspection appear to contradict my findings. These apparently contradictory data largely relate to diseases other than DU (gastritis and non-ulcer dyspepsia). Furthermore, two studies (Karttunen et al, 1987; Brady et al, 1988) showing no consistent relationship between the presence of HP and either fasting gastrin or acid secretion fail to differentiate between acute and chronic HP infection in the study population, which may explain why clear-cut effects on gastrin and acid failed to emerge. It should be noted that in my study, although mean basal gastrin levels were higher in HP+ve compared with HP-ve patients with DU (n=51), statistical significance was achieved only when gastrin release was stimulated by a meal. I am therefore not altogether surprised by studies showing no consistent effects of HP on basal gastrin (Karttunen et al, 1987; Staub et al, 1989). Likewise, the measurement of gastric juice pH, rather than acid secretion rate, ignores the volume of secretion. In my study the HP+ve patients with DU disease produced significantly greater volumes of gastric juice, during pentagastrin infusion, than the HP-ve patients. This may explain why Wagner et al (1989) failed to detect a difference in pH in the 3 HP-ve patients he studied! The effects of HP colonisation on antral gastrin release in DU patients have not hitherto been addressed.

Our current work, and other studies of HP in DU disease, do not offer an explanation for presence of duodenal ulcers in HP-ve patients with normal gastric secretion. Presumably, ulcers in such patients are due to one of the other abnormalities, such as rapid gastric emptying of acid into the duodenum (Malagelada et al, 1977) and diminished duodenal prostaglandin synthesis (Ahlquist et al, 1983) that have been found in patients with DU disease (Soll and Isenberg, 1983).
It is also quite clear that the majority of patients with HP infection do not develop peptic ulcers or dyspepsia (Wyatt et al, 1988). First infection with HP is known to produce acute gastritis (Morris and Nicholson, 1987; Marshall et al, 1985) with temporarily diminished gastric acid secretion (Graham et al, 1988d), but it is not clear whether these events always occur on first exposure to HP. Furthermore, the proportion of individuals who become chronically colonised with HP following initial exposure is not known. Once the individual has become colonised he/she is very likely to have histological type B gastritis, but no symptoms. Type B gastritis affects the gastric antrum, and less commonly the gastric body and fundus. The prevalence of HP colonisation in type B gastritis is about 70%. At this stage, according to the work of Smith et al (1989), the individual will have normal gastric acid secretion but elevated plasma gastrin concentrations. The proportion of patients who progress from type B gastritis to DU disease is unknown. An obsessional autopsy study in Leeds showed active or healed DUs in about 13% of individuals (males 17%, females 8%) aged 40-49 years (Watson, 1969). About 33% of an American population were colonised with HP in the same age group (Graham et al, 1988c). The sequence of events between type B gastritis and DU disease also remains open to speculation: possibly, the HP-associated hypergastrinaemia increases acid secretion through its stimulatory (Maxwell et al, 1984) and trophic effects (Johnson, 1976), resulting in duodenal damage, duodenal gastric metaplasia (Wyatt et al, 1987), duodenal HP colonisation and ulceration (Goodwin, 1988). Whether this sequence of events occurs may partly depend on the presence or absence of type A gastritis, which preferentially affects the body and fundus of the stomach, and results in diminished acid secretory capacity (Strickland and Mackay, 1973). The prevalence of type A gastritis increases with age (Varis et al, 1979). Thus DU disease in an HP carrier may be initiated if HP colonisation occurs before type A gastritis develops and prevents hypersecretion. This view is consistent with the typical presence of HP and type B gastritis, and the absence of type A gastritis, in patients with DU disease (Kekki et al, 1984). Indeed, severe forms of type A gastritis lead to complete absence of gastric acid secretion; this state appears to protect the stomach from colonisation with HP, perhaps because HP prefers acid conditions to achieve neutralisation of the ammonia produced by its urease, or because other organisms which colonise
the achlorhydric stomach compete with HP (Warren and Marshall, 1983).

We find the intramucosal acid-neutralisation hypothesis outlined above is attractive because it offers a specific explanation for the defective inhibition of gastrin release by a low intragastric pH as originally described by Walsh et al in patients with DU disease (1975). In addition, prolonged alkalinisation of the antrum is already known to produce major increases in gastrin release, including greater postprandial release, as seen in patients with pernicious anaemia (Walsh, 1989). In addition to this intramucosal acid neutralisation hypothesis for HP-induced hypergastrinaemia, other possible mechanisms of HP-induced hypergastrinaemia deserve comment. Thus it may be the case that ammonia produced by HP may directly stimulate gastrin release from antral G cells. My results could also be explained by postulating that HP itself elaborates and releases into the blood a protein with immunological similarity to gastrin, but this seems unlikely as the organism fails to stain positively on gastrin immunostains. Recent findings of Wyatt et al (1989) have suggested a further possible mechanism: these investigators have obtained data which indicate that the hypergastrinaemia induced by HP may occur as a result of the inflammatory response stimulated by the organism in the antral mucosa. Acid secretion was not measured, so that it remains possible that the 4 HP-ve patients with antritis in her study had increased plasma gastrin levels because of diminished acid secretion. Nevertheless, their suggestion requires testing with larger numbers of HP-ve gastritic patients, and if confirmed will surely open new and exciting future avenues in this important area of gastrointestinal research.
CHAPTER 9

CONCLUSIONS AND IMPLICATIONS FOR FUTURE RESEARCH

The pathologic mechanisms behind two different risk factors for peptic ulceration have been considered:

1. Non-steroidal anti-inflammatory drugs (NSAIDs), which are associated with gastric as well as duodenal damage, and

2. Infection of the gastric antrum with Helicobacter pylori, which is strongly associated with duodenal ulceration.

STUDIES OF THE EFFECTS OF NSAIDs ON THE GASTROINTESTINAL MUCOSA

The aims of these studies were to assess gastrointestinal mucosal proliferation in man and in animals, in order to examine the changes in cell turnover associated with NSAID therapy in animals and in man.

Our studies naturally fell into three areas of enquiry:

1. What is the best method for assessment of proliferation in animals, where large blocks of well oriented tissue are readily available; and, what is the best approach in man? Efforts on the latter question led to the detailed appraisal of a simple and effective means of assessing proliferative status in small endoscopic biopsies from man.

2. What is the effect of NSAIDs, and of prostaglandins, on gastric and duodenal proliferation in animals, and in man?

3. Do NSAIDs inhibit the normal regenerative repair process at the edges of gastric and duodenal ulcers in the experimental animal, and in man?
Studies of gastrointestinal proliferation methodology

I studied and compared the merits and drawbacks of three methods for the rapid assessment of rat gastrointestinal epithelial cell turnover: the assessment of S-phase DNA synthesis by *in vitro* and *in vivo* 3H-thymidine (3H-TdR) incorporation, and the vincristine induced mitotic arrest (stathmokinetic) method. Using the latter method, new cells are generated in the gastric corpus and antral glands, and in duodenal, ileal and colonic crypts at a rate of 2.0 (0.5), 3.2 (0.9), 24.9 (3.1), 17.9 (3.1) and 11.8 (3.0) per hour (mean (SEM)). Our data cast doubt on the reliability and reproducibility of the *in vitro* 3H-TdR method for assessing DNA synthesis, particularly in the case of the ileum, where the variability was particularly marked. *In vivo* 3H-TdR studies, however, give a reproducible pattern of DNA synthetic rates, the profile obtained for the gastrointestinal tract being similar to that obtained with the stathmokinetic method. The stathmokinetic technique is the method of choice in the study of gut proliferation, but it is time consuming, requiring tissue microdissection, staining and metaphase counting. *In vivo* 3H-TdR labelling of DNA is a useful alternative, offering reliable and reproducible results where the experiment does not alter mucosal lymphocyte density; this makes it especially valuable to investigators who may not be experienced in gastric gland microdissection.

For the analysis of cell proliferation in single endoscopic biopsies from man, a simple and quick microdissection-based method was studied (Chapter 3). This involves staining of the biopsy by the Feulgen reaction, followed by microdissection of individual gastric glands or duodenal crypts and counting the total number of mitoses per gland or crypt. Data derived from human biopsies shows a good correlation between gland or crypt cross-sectional area and the number of mitoses per gland or crypt. The number of metaphases and the size of the proliferative compartments increased caudally in the human gastrointestinal tract, being maximal in the colon.

The advantages of the microdissection method for obtaining estimates of epithelial turnover are as follows:

1. Speed and ease with which it is possible to obtain 20 to 30 (or more) well orientated glands or crypts from a single biopsy,
2. Large number of readily identifiable mitotic figures seen in comparison with sectioned material,
3. Lack of those stereological problems associated with quantifying structures in section, and
4. The benefits of expressing results in terms of an all encompassing denominator (on a 'per crypt' or 'per gland' basis).

We believe this approach is a major advance in the assessment of mucosal proliferation in man, with substantial advantages over previous methods based on mitotic and labelling indices.

Studies of the effects of NSAIDs and misoprostol on gastric and duodenal proliferative status

We investigated the effects of indomethacin on epithelial cell proliferation in rat stomach and duodenum, using in vivo 3H-thymidine incorporation and stathmokinetic indices of cell renewal. A single high subcutaneous dose of indomethacin (30 mg/kg) universally induced an erosive gastritis within three to six hours, with concomitant reduction of DNA synthesis in the gastric corpus and antrum (by 60%, p<0.05, and 30%, p<0.05 respectively). There was no duodenal damage at this time, nor effect on duodenal proliferation. Single low doses of indomethacin (<5 mg/kg) produced no macroscopic gastric damage, and had no effect on proliferation. In contrast, chronic low dose indomethacin (2 mg/kg/d for eight weeks), markedly increased proliferative activity in the gastric corpus and antrum (by 56%, p<0.05, and 27%, p<0.05 respectively) as well as in the duodenum (26% increase in DNA synthesis, p<0.05, and 20% increase in crypt cell production rate); there was no macroscopic damage in the duodenum at this time point. It is concluded here that the blunted gastric regenerative response accounts in part for the acute gastric damage induced by high dose indomethacin, but that mucosal adaptation with stimulation of epithelial cell turnover occurs with chronic low dose administration (Chapters 4 and 5).

As expected, misoprostol in high doses (300 μg/kg/d) stimulated gastric corporal and antral proliferation in the rat. However, therapeutic doses (10 μg/kg/d) of misoprostol failed to influence
gastric or duodenal cell turnover either in the rat or in man (Chapters 5 and 6).

**Effect of NSAIDs on regenerative repair of peptic ulcers in animals and in man**

Indomethacin in low doses (2 mg/kg/d) exacerbated and reduced the rate of healing of experimental gastric ulcers in the rat by specifically inhibiting the regenerative response at the ulcer edge. This effect was substantial, and resulted in healing rates falling to below half that seen in control animals. Large doses (300 μg/kg/d) of misoprostol reversed the indomethacin-induced inhibition of regeneration at the ulcer edge in the rat. This suggests, but does not prove, that the inhibition of reparative regeneration results from inhibition of mucosal cyclo-oxygenase activity (Chapter 7). Local effects of prostaglandins and NSAIDs on mucosal blood flow could also explain our observations.

Similar studies were therefore conducted in patients with arthritis. Patients on NSAIDs with gastric and duodenal ulcers showed markedly lower rates of regeneration at the ulcer edge than patients with ulcers at the same locations not taking NSAIDs. These results suggest that the association of NSAIDs and peptic ulceration is not simply a consequence of the fact that these drugs cause ulcers, but also that they inhibit ulcer repair. This inhibition of healing may explain the greater prevalence of ulcers in patients taking NSAIDs (Chapter 7). It is not clear why NSAIDs should inhibit the normal regenerative function of the gastric and duodenal mucosae at sites of mucosal damage. The situation at sites of mucosal damage appears to be very different from other apparently 'normal' areas of mucosa, where adaptation to continuous low grade damage of superficial structures is accompanied by an effective increase in gland cell turnover (Chapters 4 and 5). It is possible that the mucosal adaptive process is may fail focally, at sites which are then rendered more vulnerable to erosive damage.

**Further research on the effects of NSAIDs on gastrointestinal epithelial cell turnover**

It would be of interest to determine the mechanisms by which NSAIDs, in high doses, interfere with the cellular DNA synthetic machinery. This inhibition is observed not only in the gastric
epithelium in vivo but also, in other studies, in the regenerating liver as well as a number of cell lines in culture (Bayer et al, 1979; Kirkpatrick et al, 1983; Hial et al, 1977). When given in low doses the gastrointestinal epithelium is clearly able to adapt to continuous low grade damage by increasing the rate of cell production, to compensate for increased cell shedding. However, when large increases in proliferation are required, as at the healing edge of a gastric or duodenal ulcer, even the 'low' doses used in the treatment of arthritis appear to suppress the normal regenerative response. The study of isolated human gastric glands in vitro using the methods developed by Tarnawski et al (1988) offers a system highly suited to the analysis of the effects of NSAIDs on cellular DNA synthetic pathways.

Likewise, the observed NSAID-induced stimulation of proliferation in apparently non-damaged gastric and duodenal mucosae lends itself to further investigation of the possible roles of extracellular growth factor induction, and of second messengers, in this process.

To minimize the effects of endoscopic biopsy error, it would be of value to confirm the observation that NSAIDs inhibit the regenerative response to gastric and duodenal ulcers by studying larger blocks of tissue obtained from patients undergoing surgery for perforated or bleeding peptic ulcers, when the procedure being undertaken involves resection of all or part of the ulcer.

It would also be most interesting to examine whether patients who present with major gastric or duodenal damage soon after commencing NSAID therapy adapt normally to these agents by increasing gastric and duodenal epithelial turnover. 'Poor adapters' may be more likely to develop acute, as well as chronic, NSAID side effects. As an offshoot of these studies, it would be useful to consider whether patients would benefit from commencing NSAID therapy at significantly lower (even sub-therapeutic) doses, and then gradually building up to the full dosage, in order to allow more time for adaptation to occur; this would be expected to minimize acute NSAID toxicity. Hawkey has recently highlighted the possibility that short term anti-ulcer prophylaxis - with either prostaglandin or H2 receptor antagonist co-therapy tiding a patient
over a period when an endogenous adaptive process takes place -
might have long term value (Hawkey, 1990).

Experiments presented in this Thesis also confirm that misoprostol,
a stable prostaglandin E1 analogue, increases the gastric gland cell
production rate in animals. This effect would reasonably be
expected to favour mucosal healing and resistance to erosive insult.
This occurred at relatively high doses of misoprostol (300
µg/kg/day). It is clear, however, in animals (Robert, 1979) as well
as man (Roth et al, 1989) that the protective and healing properties
of prostaglandins occur at much lower doses (10 µg/kg/day is
cytoprotective in humans). At this dose, no proliferative effect of
misoprostol was noted in patients with arthritis. Therefore, the
proliferative and trophic effects of prostaglandins may well be
purely pharmacological effects, noted in animals at high doses
during the course of routine pre-clinical toxicology studies.

The need to quickly and reliably assess proliferative rates in human
endoscopic gastric and duodenal biopsies led to the development of
a microdissection method to achieve this (vide supra). Microdissection of gastric glands and intestinal crypts may also be
used to study other gastrointestinal disease processes where
disordered cell proliferation occurs. Atrophic gastritis, ulcerative
colitis and familial polyposis coli are examples of pre-malignant
dysproliferative states where progression to malignancy is
accompanied by disordered cell proliferation. The ability to assess
epithelial turnover in small endoscopic biopsies, and the opportunity
afforded to gastroenterologists of obtaining sequential assessments
in the same cohort of patients, makes it possible to study carefully
the morphologic and proliferative parameters which may herald the
transition to frank malignancy.
ON THE RELATIONSHIP BETWEEN ANTRAL HELICOBACTER PYLORI COLONISATION AND DUODENAL ULCERATION.

In this Thesis I have also considered the relationship between antral Helicobacter pylori colonisation, antral gastrin release and gastric acid secretion in patients with duodenal ulcers (DUs) (Chapter 8).

Findings
I have demonstrated that pentagastrin stimulated gastric acid secretion and postprandial plasma gastrin concentrations are significantly higher in HP+ve compared with HP-ve patients with DU disease. Furthermore, suppression of antral HP with colloidal bismuth subcitrate and metronidazole leads to a significant drop in postprandial plasma gastrin responses. These findings may explain the strong association of duodenal ulcers with antral HP colonisation and the lower relapse rates of duodenal ulcer following treatments which suppress or eradicate this organism from the antrum.

Hypothesis
The hypothesis which led to this study was that HP, by virtue of its ability to produce large quantities of urease, enzymatically converts urea which is present in the gastric mucosa to produce ammonia. Ammonia would elevate the pH within the mucus layer which overlies the gastric antrum, resulting in impairment of the normal inhibition of gastrin release by intraluminal acid, as observed by Walsh et al (1975) in patients with duodenal ulcers. Increased gastrin release increases gastric acid secretion both by directly stimulating secretion and by its trophic effect on the gastric secretory cell mass (Crean et al, 1969; Johnson, 1976; Lam and Ong, 1980). Increased acid secretion then causes duodenal ulcers by producing a low intraduodenal pH (Malagelada et al, 1977). This intramucosal acid neutralisation hypothesis requires confirmation by direct pH estimation of gastric glands, gastric pits and gastric mucus, using microelectrodes and fresh endoscopic antral biopsies in patients with and without antral HP colonisation. HP urease can be inhibited by acetohydroxamic acid (Mobley et al, 1988); this offers a further opportunity to test this ammonia-dependent theory in animal models and in man.
An interesting alternative to this intramucosal acid neutralisation hypothesis has been suggested by Wyatt et al (1989): these investigators have obtained data which indicates that the hypergastrinaemia induced by HP may occur as a result of the inflammatory response stimulated by the organism in the antral mucosa. Acid secretion was not measured, so that it remains possible that the 4 HP-ve patients with antritis in their study had increased plasma gastrin levels because of diminished acid secretion. Nevertheless, this possibility requires testing with larger numbers of HP-ve gastritic patients, and if confirmed will surely open new and exciting future avenues in this important area of gastrointestinal research. There is already circumstantial evidence that this line of reasoning may be correct: exposure of immunised animals to immunogen causes gastrin release (Teichmann et al, 1984); the cytokines gamma interferon and interleukin 2 stimulate release of gastrin from the isolated canine gastric antrum (Reinhard et al, 1986); Cooper et al (1985) found that patients with hypergastrinaemic DU disease have G-cells that are abnormally sensitive to weak stimulants of gastrin release, at constant pH. These findings are consistent with synergistic stimulation of gastrin release by meals and some other intraepithelial factor. Furthermore, patients with pernicious anaemia, atrophic gastritis and gastric achlorhydria, who in addition have (non HP) gastric bacterial colonisation, have significantly higher gastrin levels than patients with normal stomachs receiving omeprazole. Therefore, patients with atrophic gastritis have higher gastrin levels than would be expected simply as a result of their achlorhydria, and one explanation is that the higher gastrin levels are at least partly attributable to bacterial infection and/or the immune response in the gastric mucosa.

**Future research on the relationship between Helicobacter pylori and gastrin**

These observations raise obvious questions which are readily answerable, at least in available animal models: (1) does the hypergastrinaemia in HP+ve subjects correlate with the degree of antral gastritis? (2) do lymphocytes, or the soluble factors they produce play a role in the latter? (3) is it possible to
'immunosuppress' an 'immunologically mediated' hypergastrinaemia? Extremely valuable information would also be obtained by directly determining the degree of omeprazole induced hypergastrinaemia in normal versus germ-free animals.

The reduction we have found in meal stimulated plasma gastrin responses following eradication of HP could explain the reduced likelihood of ulcer relapse in patients treated with agents that suppress or eradicate HP, as observed in several studies (Martin et al., 1981; Coghlan et al., 1988; Marshall et al., 1988; Smith et al., 1988). This fall in meal stimulated plasma gastrin levels would be expected to result in a diminished meal stimulated secretory drive on the gastric parietal cells. Another predictable long term result of the lower gastrin levels is a reduction of the parietal cell number, due to a reduction of the trophic effect of gastrin on these cells (Crean et al., 1969; Johnson, 1976; Lam and Ong, 1980). Peak acid output, which reflects the parietal cell mass, did not fall significantly in our patients after one month of anti-HP therapy, despite the lower gastrin levels. Presumably this reflects the need for a longer period of time to elapse before a significant reduction of gastric parietal cell numbers occurs. Longer term studies are urgently required in patients who remain free of the organism to reveal an effect on maximal acid secretory capacity. Follow-up every 6 months for 2 years would offer the opportunity of determining the acid / gastrin status and relating this to the HP status. A patient who remains HP negative with lower gastrin responses, and whose peak acid output falls into the normal range, may then be regarded as having been cured of his / her ulcer diathesis. Likewise it is equally important to study 'healthy' individuals without DUs but who are colonised with HP to determine whether the hypergastrinaemia seen in these individuals results, in the long term, in an increase in the parietal cell mass and acid secretion rate.

Many of these studies are already in progress at the Royal Postgraduate Medical School and at other medical centres.
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Additional references


Colour Plate 1. Two microdissected rat small intestinal crypts. The intestine was bulk stained by the Feulgen reaction. Note the darker stained, condensed chromatin of the arrested metaphases, and their migration towards the centre of the crypt. Magnification x 400.
Colour Plate 2. Microdissected human duodenal crypts 3 hours after incubation with vincristine sulphate. The crypts clearly contain anaphases and telophases, suggesting incomplete metaphase arrest. Magnification x 800.
**Colour Plate 3.** Small intestinal perforation in a rat 72 hours after three daily subcutaneous injections of indomethacin (10 mg/kg/day). Magnification x15.
Colour Plate 4. Cryo-probe used for the production of experimental gastric ulcers in the rat. Prior to photograph, the probe was cooled in liquid nitrogen, and has acquired a fine coat of frozen water vapour.
Colour Plates 5, 6 and 7. Edges of a rat gastric ulcer induced by cryo-probe 6 days earlier, showing distribution of BRdU uptake (brown nuclear reaction). The gastric ulcer is on the extreme right of the picture in each case. Plate 5 is from a control animal, showing intense regenerative activity at the ulcer edge. Plate 6 was from an animal receiving indomethacin (2 mg/kg/day), and shows very little regenerative activity at the ulcer edge. Plate 7 was from an animal treated with indomethacin (2 mg/kg/day) plus misoprostol (300 µg/kg/day), and shows restoration of regenerative activity at the ulcer edge, with a consequent increase in gland length. Magnification x 80 in each case.
FIGURE 2.1
THE STATHMOKINETIC METHOD FOR THE ASSESSMENT OF GASTROINTESTINAL PROLIFERATION

10 adult male Wistar rats

\[\text{vincristine sulphate}\]

\[1 \text{ mg/kg ip}\]

\[\text{kill at timed intervals and fix tissue of interest in Carnoy's solution}\]

\[\text{store in 70\% ethanol}\]

\[\text{Feulgen reaction}\]

\[\text{microdissection}\]

\[\text{metaphase counting}\]
FIGURE 2.2
QUANTITATIVE IN VIVO 3H-TdR METHOD FOR THE ASSESSMENT OF GASTROINTESTINAL PROLIFERATION

5 adult male Wistar rats

3H-TdR injection ip
125 μCi/kg

Sacrifice after 1 hr

Full thickness samples of gastric or intestinal wall

Store at -20 degrees C

DNA extraction

DNA assay

Scintillation counting

dpm/mcg DNA
FIGURE 2.3
QUANTITATIVE IN VITRO 3H-TdR METHOD FOR THE ASSESSMENT OF GASTROINTESTINAL PROLIFERATION

5 adult male Wistar rats

↓

full thickness samples of gastric or intestinal wall

↓

incubate in oxygenated MEM + 3H-TdR

37 degrees C for 1 hr

↓

DNA extraction

DNA assay

scintillation counting

dpm/mcg DNA
Figure 2.4. Rat gland and crypt cell production rates (CPR) at five gastrointestinal sites. Mean (SEM) derived from metaphase accumulation rates in 10 animals.

Figure 2.5. In vivo 3H-TdR labelling of DNA at five gastrointestinal sites in the rat. Mean (SEM) of five animals at each site.
Figure 2.6. 3H-TdR residual DNA radioactivity curves for gastric corpus, ileum and colon. Animals (n=5 per time point) were injected with 3H-TdR on day 0, and residual DNA radioactivity determined subsequently after 1 hour and on days 3 and 9. Results are mean (SEM).
Figure 2.7. In vitro 3H-TdR labelling of DNA at five gastrointestinal sites in the rat. Samples of tissue from each site were incubated in DMEM under conditions described in the text. Results are mean (SEM) of five animals per site.

Figure 2.8. Time course of in vitro 3H-TdR labelling of DNA in the rat gastric corpus. Mean (SEM) of 4 samples per time point.
Figure 3.1. Diagram to show the way in which gland and crypt outlines were traced prior to digitization.
Figure 3.2. Specific incorporation of 3H-TdR into DNA in human antral and duodenal cap biopsies in vitro in 8 patients with endoscopically normal mucosa. Incubation conditions are described in the text.
Figure 3.3. The Ki-67 index (please see text for definition) in the human gastric fundus, body, antrum (greater curve), antrum (lesser curve), duodenal cap (D1) and second part of the duodenum (D2). Mean (SEM) of 6 patients with endoscopically normal upper gastrointestinal mucosa.
3.4. Mitotic counts per gastric gland or intestinal crypt in human endoscopic biopsies, obtained by analysis of microdissected tissue stained by the Feulgen reaction. Results are expressed as mean (SEM) for tissue from endoscopically normal patients. Gastric fundus (n=5), gastric body (n=5), antrum lesser curve (n=19), D1 (n=22), D2 (n=5), jejunum (n=4), terminal ileum (n=5), caecum (n=5), transverse colon (n=4), rectum (n=4).
FIGURE 3.5. Areas of gastric glands and intestinal crypts in human endoscopic biopsies, obtained by tracing and then digitizing these structures following microdissection. Results are expressed as mean (SEM) for tissue from endoscopically normal patients. Gastric fundus (n=5), gastric body (n=5), antrum lesser curve (n=19), D1 (n=22), D2 (n=5), jejunum (n=4), terminal ileum (n=5), caecum (n=5), transverse colon (n=4), rectum (n=4).
Figure 3.6. Plot of the number of mitoses per gastric gland or intestinal crypt, versus their cross-sectional areas. $r = 0.893$, $P = 0.001$. 
Figure 3.7. Diagram of an intestinal crypt, showing an imaginary longitudinal plane of cross-section.

<table>
<thead>
<tr>
<th>*</th>
<th>mitosis in the plane of section</th>
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<td>*</td>
<td>mitosis outside plane of section</td>
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FIGURE 4.1. PLAN OF ACUTE INDOMETHACIN TOXICITY EXPERIMENT

20 ADULT MALE WISTAR RATS

↓

SINGLE INDOMETHACIN INJECTION (sc)

Control (n=5)
2 mg/kg (n=5)
5 mg/kg (n=5)
30 mg/kg (n=5)

↓ 6 hrs

3H-TdR 125 μCi/kg

↓ 1 hr

sacrifice and determine DNA specific radioactivity
Figures 4.2 A, B. Acute 6 hour effects of subcutaneous indomethacin, at the three test doses indicated, on in vivo 3H-TdR incorporation into gastrointestinal DNA in the rat. Results are expressed as mean (SEM) of data from 4 samples taken from each site in each of 5 animals. * indicates P < 0.05.
Figure 5.1. Effect of chronic (8 weeks) subcutaneous indomethacin (2 mg/kg/d) on in vivo $^3$H-TdR incorporation into gastro-intestinal DNA in the rat. Results are expressed as mean (SE) of data from 4 samples taken from each site in each of 5 animals. * indicates $P < 0.05$. 
Figure 5.2. Small intestinal and colonic crypt cell production rates: effect of indomethacin (2 mg/kg/d s.c. for 8 weeks). Mean (SEM) of data from 10 animals.
* P < 0.05.
Figure 5.3. Effect of indomethacin (5mg/kg/d s.c.) on rate of disappearance of 3H-TdR from the rat gastric corpus. Mean (SEM), 5 animals per time point. *P < 0.05. Indo, indomethacin.
Figure 5.4. Effect of misoprostol (300 μg/kg/d by gavage for 14 days) on corpus and antral gland length. Mean (SEM) * P < 0.05.

Figure 5.5. Effect of misoprostol (300 μg/kg/d for 14 days by gavage) on corpus and antral gland cell production rate. Mean (SEM); * P < 0.05.
Figure 6.1A,B,C. Effect of (A) NSAID alone, (B) NSAID plus misoprostol (200 mcg tid), and (C) misoprostol alone (200 mcg qid) on cell proliferation in endoscopic biopsies from endoscopically normal antral lesser curve. 'Adaptation index' (defined in text) was (A) 1.73 (0.06), (B) 1.84 (0.18), and (C) 1.08 (0.13) (mean (SEM)) in n=10, n=9, and n=4 patients, respectively.
Figure 6.2 A,B,C. Effect of (A) NSAID alone, (B) NSAID plus misoprostol (200 mcg tid), and (C) misoprostol (200 mcg qid) alone on cell proliferation in endoscopic biopsies from endoscopically normal duodenal cap. 'Adaptation index' (defined in text) was (A) 1.62 (0.23), (B) 1.50 (0.18), and (C) 0.93 (0.14) (mean (SEM)) in n=9, n=10, and n=4 patients respectively.
Figure 6.3. Effect of 2 weeks of NSAID therapy on number of fissures in gastric glands. '0' represents 'no fissures,' ie. 'single' gland. '1' represents 'one fissure,' ie. duplicating gland and so on. * P < 0.05. n=10 patients.
Figure 6.4. Effect of 2 weeks of NSAID therapy on number of fissures in duodenal crypts. '0' represents 'no fissures,' ie. 'single' crypt. '1' represents 'one fissure,' ie. duplicating crypt, and so on. * P < 0.05, n= 9 patients.
Figure 7.1. Gastric ulcer area following cryo-injury. Mean (SEM) of 6 ulcers in 3 animals, per time point.
Figure 7.2. Cryo-ulcer areas 3 and 6 days following ulcer induction. Effects of 10 day pre-treatment and continuing treatment with indomethacin and/or misoprostol. * signifies $P < 0.05$ for the test result with respect to the control result for the same day. Mean (SEM), n=4 animals per time point.
Figure 7.3. Effect of (A) control vehicles, (B) indomethacin, and (C) indomethacin + misoprostol on 3-day regeneration at cryo-ulcer edge. "Labelled cells" refers to BRdU MoAb staining. Mean (SEM) of data from 8 ulcers in 4 animals.
Figure 7.4. Effect of (A) indomethacin, (2mg/kg/d s.c.), (B) misoprostol (300mg/kg/d p.o.), and (C) indomethacin + misoprostol on 6-day regeneration at cryo-ulcer edge. "Labelled cells" refers to BRdU MoAb staining. Mean (SEM) of data from 8 ulcers in 4 animals.
Figure 7.5. Regenerative activity at the ulcer edge in patients with gastric ulcers, compared with proliferative activity in endoscopically normal mucosa nearby, in patients not taking NSAIDs. In these patients the 'regeneration index' at the ulcer edge (see text for definition) was 3.1 (0.61) (mean (SEM)) (n=9).
Number of mitoses per gland

NEARBY MUCOSA  ULCER EDGE

0  10  20

Figure 7.6. Regenerative activity at the ulcer edge in patients with gastric ulcers, compared with proliferative activity in endoscopically normal mucosa nearby, in patients regularly taking NSAIDs. In these patients the 'regeneration index' at the ulcer edge (see text for definition) was 1.49 (0.16) (mean (SEM)) (n=8).
Figure 7.7. Regenerative activity at the ulcer edge in patients with duodenal ulcers, compared with proliferative activity in endoscopically normal mucosa nearby, in patients not taking NSAIDs. In these patients the 'regeneration index' at the ulcer edge (see text for definition) was 3.64 (0.99) (mean (SEM)) (n=8).
Figure 7.8. Regenerative activity at the ulcer edge in patients with duodenal ulcers, compared with proliferative activity in endoscopically normal mucosa nearby, in patients regularly taking NSAIDs. In these patients the 'regeneration index' at the ulcer edge (see text for definition) was 1.20 (0.12) (mean (SEM)) (n=10).
Figure 8.1. Basal and peak acid output (BAO and PAO) in HP +ve and HP -ve patients with duodenal ulcers (n = 51). * indicates P<0.05.

Figure 8.2. Volume of gastric juice produced by 51 patients with duodenal ulcers during pentagastrin infusion (6 µg/kg/h, HP+ve, n=44; HP-ve, n=7). * indicates P<0.05.
Figure 8.3. Integrated meal stimulated plasma gastrin responses in patients with duodenal ulcers with (HP +ve) and without (HP -ve) antral H. Pylori (n = 44 and n = 7, respectively). * indicates P<0.05.
Figures 8.4 A,B. Changes in integrated postprandial gastrin responses (A) and peak acid output (B) in HP+ve patients before and after treatment with colloidal bismuth subcitrate (4 weeks) and metronidazole (2 weeks). * indicates a patient who remained weakly positive for antral urease after treatment.
Figure 8.5. Effect of HP eradication on integrated basal and meal-stimulated gastrin responses in 10 HP+ve patients with duodenal ulcers (mean (SEM)).
Figure 8.6. Hypothesis to explain the effect of H. pylori urease activity generating an alkaline microenvironment in the antral mucosa, and thereby interfering with the normal feedback inhibition of acid on gastrin release.