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Measurements of rat and mouse gastrointestinal pH, fluid and lymphoid tissue, and implications for in-vivo experiments

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Abstract

To use rodent models effectively in in-vivo investigations on oral drug and vaccine delivery, the conditions in the gastrointestinal tract must be understood. Some fundamental information is currently unavailable or incomplete. We have investigated the pH, water content and lymphoid tissue distribution along the gastrointestinal tract, as well as the stomach volume, as these were critical to our investigations on pH-responsive drug delivery and colonic vaccination. The observed values were compared with those in man as an indication of the validity of the rodent model. The mouse stomach pH was 3.0 (fed) and 4.0 (fasted), and the corresponding values in the rat were 3.2 (fed) and 3.9 (fasted). The mean intestinal pH was lower than that in man (< pH 5.2 in the mouse; < pH 6.6 in the rat). This brings into question the use of rodents in investigations on enteric-coated drug carriers targeted to the large intestine/distal gut. The water content in the gastrointestinal tract in the fed and fasted mouse was 0.98 ± 0.4 and 0.81 ± 1.3 mL, respectively, and in the fed and fasted rat was 7.8 ± 1.5 and 3.2 ± 1.8 mL. When normalized for body weight, there was more water per kg body weight in the gastrointestinal tracts of the mouse and rat, than in man. The stomach capacity was found to be approximately 0.4 and 3.4 mL for mice and rats, respectively. The low fluid volume and stomach capacity have implications for the testing of solid dosage forms in these animal models. Substantial amounts of lymphoid tissue analagous to small intestinal Peyer's patches were measured in the rat and mouse colon, showing the feasibility of colonic vaccination, a route which might prove to have different applications to the more commonly studied oral vaccines. The existence of lymphoid tissue in the mouse and rat caecum has also been reported.

Introduction

Animal models are used extensively in the pre-clinical testing of drugs and vaccines. Rodents (mainly rats and mice) are often used due to their small size and low cost. Rats, having a relatively larger size and greater capacity for blood samples, are more useful for bioavailability studies, whereas mice are often used for vaccination studies. Despite the extensive use of these animals, certain features are either unknown or inadequately characterized, although a number of aspects of the mouse and rat gastrointestinal (GI) physiology have been reviewed by Kararli (1995). During our investigations into pH-responsive drug release at different locations in the gastrointestinal tract, and into colonic vaccination, we identified several key elements of gastrointestinal physiology that needed clarification to enable the use of rat and mouse models in the in-vivo studies. These were the pH and fluid content along the gastrointestinal tract, the stomach volume and the presence of lymphoid tissue in the colon of the animal models.

The pH in the gastrointestinal tract is a crucial factor, affecting the stability and solubility of drugs and their absorption through the mucosa; unsuitable pH may cause the precipitation of acidic or basic drugs from solution, or the degradation of labile compounds. In addition, enteric-coated drug delivery systems for modified or targeted drug release are increasingly being investigated, for example using polymers such as polymethacrylate- and cellulose-based enteric coatings, which dissolve only when the pH of the environment exceeds a threshold level. In such a situation, knowledge of the

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gut pH of the experimental animal is critical. Previous reports on the pH of the rat gastrointestinal tract are conflicting (Smith 1965; Ward & Coaes 1987), while reports of pH in the mouse small and large intestinal tract were not found.

The fluid content of the gastrointestinal tract is another critical factor in the dissolution of drug from a dosage form, and the dispersion of solid-dosage forms. Lomas & Graves (1999) and Schiller et al (2005) suggested that water in the gut lumen of man was not homogeneously distributed; this implied that a dosage form would be in contact with varying amounts of fluid or indeed none at all during its passage through the gastrointestinal tract. To enable better animal study design and extrapolation to man, or to better explain dosage form/drug behaviour in the rodent model, knowledge of the water content in these animals is important.

In this study, we have investigated the stomach volume, fairly crudely, to give a rough indication of the volumes that may be administered orally to the animal models. To our knowledge, there are no reports of mouse and rat stomach volume, although maximum volumes to be administered by the oral route have been suggested (Wolfensohn & Lloyd 1994). Gelatin capsule shells and mini-tablets have been administered to rats (Hu et al 1999; Wong et al 2006). Knowledge of the animal stomach volume would enable calculation of dosage form:stomach volume ratio, which would give an indication of the likely fate of the dosage forms, with respect to disintegration and drug dissolution and absorption.

In our laboratories, we are also investigating colonic vaccination as it may have different applications to the more commonly studied oral vaccines, which are expected to be processed mainly by the small intestinal immunological system. Like the small intestine, the colon contains gut-associated lymphoid tissue. In man, there are approximately 339 Peyer's patches (Cornes 1965) in the small intestine, and approximately 12 000–18 000 follicles in the large intestine (Langman & Rowland 1986, 1992; Gebbers et al 1992). Presence of such a large number of follicles in man's colon implies the feasibility of vaccine uptake and processing in the colon. Very little is known, however, about colonic vaccine uptake, although significant differences between the large and small intestinal immunological environments have been reported. For example, a predominance of IgA2 cells over IgA1 cells is seen in the colon (as in the rectum and in the female genital tract) in contrast to a predominance of IgA1 cells in the small intestine (McGhee et al 1999). Other benefits of colonic targeting include the decreased proteolytic activity which may be beneficial for sensitive antigens and the higher transit time, which could lead to prolonged antigen contact with the lymphoid tissue and thereby increased uptake. Before mice and rats can be used in studies on colonic vaccination, the presence and density of lymphoid tissue in the colon must be established. Although the lymphoid tissue in the small intestine of mice and rats has been well quantified (Hillery et al 1994; Florence et al 1995; Abe & Ito 1977), the lymphoid tissue in the large intestine has not, and has been reported here.

Materials and Methods

Animals

All procedures were approved by the School's Ethical Review Committee and were conducted in accordance with the Home Office standards under the Animals (Scientific Procedures) Act 1986.

Adult female Balb/c mice (18–22 g) and adult female Wistar rats (160–190 g) were purchased from Harlan Olac Ltd. The animals were fed on Teklad Global 18% Protein Rodent Diet, from Harlan Olac Ltd.

Preparation and dissection procedure

Groups of animals (n = 5–8) were fasted overnight with free access to water, while other groups were allowed access to food and water at all times. The mice were killed by a Schedule One method (CO_2 asphyxiation), after which the intestinal tract was immediately removed and divided into sections: the stomach, the small intestine (into three sections approximating to the duodenum, jejunum and ileum), the caecum and the colon (into two sections approximating to the proximal and distal colon). Subsequently, the pH, water content and lymphoid tissue density of the different sections was measured as follows.

Determination of pH of gastrointestinal contents

The contents of each gastrointestinal section were removed, mixed and the pH was determined using a pre-calibrated pH 211 Microprocessor pH Meter (Hanna Instruments). pH measurements were taken a total of three times with the gastrointestinal tract contents being re-mixed, the pH meter being washed with distilled water and the calibration checked between measurements. An HI 1333 probe was used, with a spherical tip (diameter 7.5 mm); it was ensured that the sample covered the probe tip, and a stable reading acquired. The order in which the pH of the different gastrointestinal tract sections was read was varied within each group to minimize any influence of post-mortem time on pH.

Determination of pH of standard rat/mouse chow

To determine the influence of the animal feed on the pH of the gastrointestinal contents, the pH of standard rat/mouse chow was measured. Three pieces of standard mouse/rat chow (9.17 g) were mixed with 10 mL of tap water until the food pellet had disintegrated, and the pH of the resulting mixture was measured using the same pH meter.

Determination of water and solid contents of the gastrointestinal tract

To determine the gastrointestinal water and solid contents, the wet mass of the section contents was recorded, followed by lyophilization (Virtis-Advantage Freeze Drying Apparatus, Virtis, UK), measurement of the dry mass and calculation of water content.

Determination of stomach capacity

Approximate values for the volume of the mouse and rat stomach were determined by filling the stomach with distilled

water, and observing the results. The aim was to produce a rough estimate, as the method could only give a crude assessment of the volume, and was subject to investigator bias. The stomach, hand-held shut at the pyloric opening, was filled, using a syringe, via the oesophagus until it was considered to be comfortably full, with no obvious stress on the tissue (1), stretched (2), or to the point of bursting (or could no longer be filled) (3).

Determination of lymphoid tissue patches along the gastrointestinal tract

The method of Langman & Rowland (1986) was used. The emptied gastrointestinal sections were placed into glass vials containing 20 mL 10% v/v aqueous acetic acid and incubated overnight in the refrigerator. Acetic acid was used as it enhanced the visualization of the lymphoid tissue. The following day, the gastrointestinal tract sections were removed, opened lengthways, blotted dry and photographed, and the numbers of individual lymphoid follicles and patches (collections of follicles) were counted. The mean number of patches or follicles per cm was calculated from the data for the individual animals.

Statistical analysis

The data gathered from mice was analysed using parametric tests. The influence of fed (n = 8) and fasted (n = 7) states on mouse gastrointestinal pH, and water and solid contents were analysed using Student's Independent *t*-test. Differences between gastrointestinal tract sections for pH and water content were analysed using one-way analysis of variance, with post-hoc analysis using Tukey's test.

The data obtained from rats was analysed using non-parametric tests, as the data did not fulfil the assumptions required for parametric tests. The influence of fed (n = 5) and fasted (n = 5) state on rat gastrointestinal pH, and water and solid contents were analysed using the Mann–Whitney *U*-test. The differences between gastrointestinal sections for pH and water content were analysed using Kruskal–Wallis, with Nemenyi's post-hoc analysis.

All tests, apart from Nemenyi's test were carried out using SPSS Version 14.0 statistical software package. Nemenyi's test was conducted as described in Jones (2002). Results were considered statistically significant when $P < 0.05$.

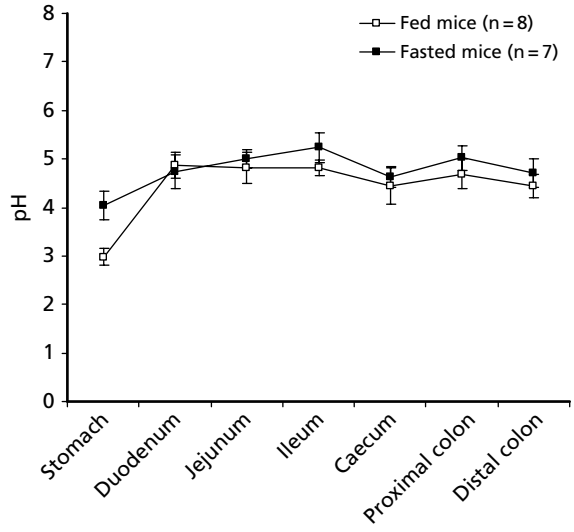

Figure 1 pH values along the mouse gastrointestinal tract. Mean and error bars are shown.

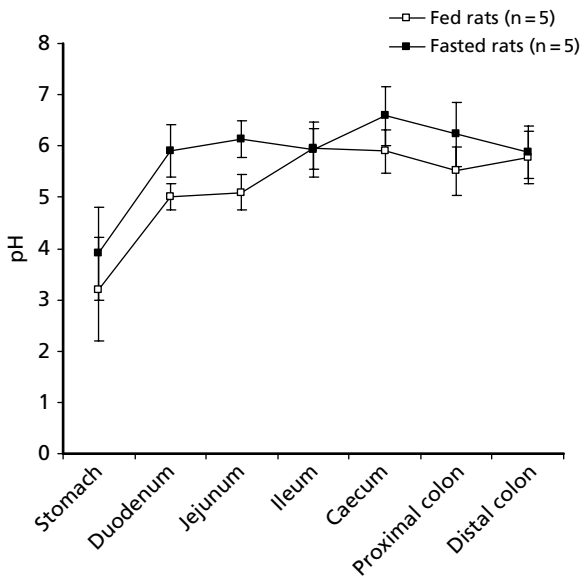

Figure 2 pH values along the rat gastrointestinal tract. Mean and error bars are shown.

Results and Discussion

The pH along the gastrointestinal tract of mice and rats

The pH of the contents of the different gastrointestinal sections of fed and fasted mice and rats are shown in Figures 1 and 2, and in Table 1. The standard deviations showed variability between individuals. Such variability has been observed in man (Evans et al 1988; Fallingborg et al 1989). The lowest pH was seen in the stomach, in both rats and mice. In both animals, the stomach pH appeared higher in the fasted state (3.9 compared with 3.2 in rats and 4.0 compared with 3.0 in mice), although the difference was only statistically significant in the mouse. Higher pH in the fasted state was surprising given that, in man, the fasted gastric pH is lower than the fed gastric pH (fasted pH 1.7 increasing to 5.0 after meal ingestion in healthy subjects (Dressman et al 1990; Russell et al 1993)) due to the buffering effects of food (Malagelada et al 1976). However, this was dependent on the meal type, with high protein meals having increased buffering effect over an isocalorific carbohydrate meal (Richardson et al 1976). In this study, the mice and rats were fed on a standard low protein (18%), low fat (5%) diet. The low

Table 1 The pH values of the mouse and rat gastrointestinal tract

Gastrointestinal section	pH mean (s.d.)			
	Mice		Rats	
	Fed	Fasted	Fed	Fasted
Stomach	2.98 (0.3)	4.04 (0.2)	3.20 (1.0)	3.90 (1.0)
Duodenum	4.87 (0.3)	4.74 (0.3)	5.00 (0.3)	5.89 (0.3)
Jejunum	4.82 (0.2)	5.01 (0.3)	5.10 (0.3)	6.13 (0.3)
Ileum	4.81 (0.3)	5.24 (0.2)	5.94 (0.4)	5.93 (0.4)
Caecum	4.44 (0.2)	4.63 (0.4)	5.90 (0.4)	6.58 (0.4)
Proximal colon	4.69 (0.3)	5.02 (0.3)	5.51 (0.5)	6.23 (0.4)
Distal colon	4.44 (0.3)	4.72 (0.2)	5.77 (0.5)	5.88 (0.5)

protein content of the animals' diet could be responsible for the absence of a food buffering effect. The pH of rat chow in water was 5.86 ± 0.06 and was therefore not responsible for the lower pH observed in the fed state. In addition, while the reasons for the difference between man and rodents are not clear, it is obvious that during experiments in man, fed and fasted states can be controlled more closely. In contrast, although the fed-state mice have free access to food, it is not known at what time they last ingested food, and in what quantity, and the immediate buffering effects of food may not have been observed.

The pH of the small intestinal contents also appeared to be higher in the fasted state than in the fed state, but this was not statistically significant in rats or mice. This suggested that the fed state of the animal had no effect on intestinal pH, which is similar to the situation in man, where the small intestinal and colonic pH are variable, but differences are not largely associated with the fed or fasted states (Kalantzi et al 2006). As expected, the small intestinal pH was higher than the gastric pH, due to the secretion of pancreatic juice and buffering with bicarbonate ions. In mice, there was a small drop in pH in the caecum. This may be associated with the increased presence of short chain fatty acids produced by bacterial polysaccharidases, bacteria being present in greater numbers in the caecum. Such a pH drop in the caecum also occurs in man (Evans et al 1988). Overall, the mean intestinal pH of both mice and rats does not reach the pH values reported in man i.e. 7.5, 6.4 and 7 in the distal small intestine, caecum and colon, respectively (Evans et al 1988).

The stomach pH values for the rat and mice were similar to that reported by Smith (1965). In contrast, the mean intestinal pH values of both animals were lower than expected and did not reach the values of pH 6–8 that have been reported in the literature for rats (Smith 1965; Ward & Coates 1987), though some individual rat pH values were found to be above pH 7. Differences in the methodology may help explain the different values obtained. Smith (1965) mixed distilled water with rat gut contents, while Ward & Coates (1987) inserted a pH probe into sections of excised rat gastrointestinal tract. In our investigation, mixing of undiluted contents was carried out, which may be more representative of the pH that a drug or delivery system is exposed to, due to the continually moving intestinal contents. To our knowledge, this is the first report of the pH of mouse intestinal tract contents.

The low intestinal pH in mouse and rat has implications for the in-vivo testing of oral pharmaceuticals in these animals. For example, drugs which require a basic pH to dissolve may precipitate at the lower pH values seen in the mouse or rat. This may prevent drug absorption and pharmacokinetic extrapolation to man would be inaccurate. The lower pH seen in mice and rat gastrointestinal tract also has implications when pH-responsive drug carriers are being investigated. For example, the pH responsive polymethacrylate polymers such as Eudragit S and FS, which dissolve at pH 7.0, but are water-insoluble at lower pH, are being investigated to target drug release to the distal intestinal tract e.g. for the treatment of diseases such as ulcerative colitis (Basit 2005; Ibekwe et al 2006). The low pH values for the mouse and rat gastrointestinal tract shown in this paper ($pH < 7.0$) suggest that rats and mice may not be the most appropriate models for the study of pH sensitive dosage forms targeted to the human lower intestine and colon, where pH is often greater than 7.0.

The water and solid contents of the mouse and rat gastrointestinal tract

The contents of the gastrointestinal tract are generally semi-solid. Water, either ingested or secreted, exists as fluid in the gastrointestinal tract. In this study, we measured the water content by freeze drying; the solid and water contents of the gastrointestinal tract of mice and rats are shown in Figures 3 and 4, respectively. As expected, total contents of the gastrointestinal tract were greater in rats than in the smaller mice. There were more solid contents in the fed rat gastrointestinal tract than in the fasted rat. Water content was also higher in the fed state, possibly due to increased secretions and water bound with the ingested food. The total amount of water present in the rat gastrointestinal contents was similar to that reported by Cizek et al (1954), who measured water by evaporating gastrointestinal contents to dryness and reported that gut water represented 1.8% (fasted) and 4.5% (fed) of total body weight (198–232 g) of female rats. In the mouse, differences between the total solid and water contents were less

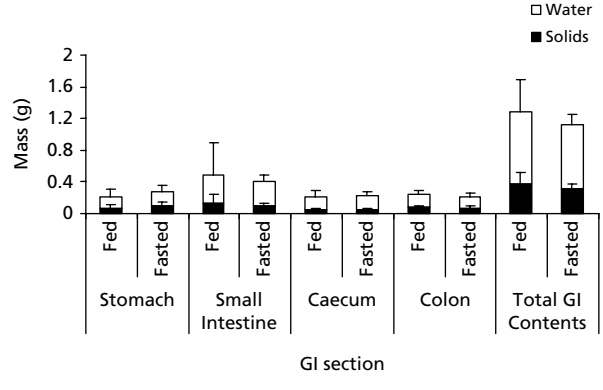

Figure 3 Water and solid compositions of the mouse gastrointestinal tract contents. Mean and error bars are shown.

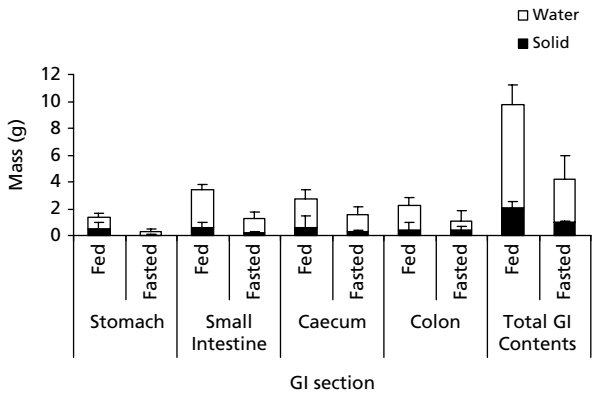

Figure 4 Water and solid content of the rat gastrointestinal tract contents. Mean and errors bars are shown.

obvious between the fed and fasted states, the small quantities making it more difficult to ascertain differences. As expected, the water concentration decreased along the length of the gastrointestinal tract from 82% w/w of the small intestinal contents to 71% w/w in the colon in rats, and from 74 to 67% w/w along the same segments in mice. The decreasing water content was observed visually as an increase in the viscosity of the gastrointestinal contents.

In Figures 3 and 4, the most striking observation was the very low levels of fluid present along the gastrointestinal tract, the total mass of water in the mouse gut being less than 1 mL (0.98 ± 0.4 mL fed, 0.81 ± 1.3 mL fasted). In experiments where mice are orally dosed with solid or semi-solid drug delivery systems, the latter may not come into contact with enough fluid to disperse and/or dissolve. The rat seems a more appropriate model for the dissolution of drug delivery systems, which require contact with sufficient water. The larger water content in the fed rat (7.8 ± 1.5 mL), compared with the fasted rat (3.2 ± 1.8 mL) suggests that if a dosage form is being investigated in the rat model, it may be beneficial to deliver it in the fed state, although interactions of food with drug or with dosage form may mean that this is not appropriate in all circumstances.

To compare with human data, the mass of rodent intestinal contents with respect to total body mass has been calculated. In man, the total large intestinal (colonic and caecal) water content post-mortem was found to average 187 g, or 2.6 g kg^{-1} body mass assuming a 70-kg body weight. For an average rat (175 g), the average (fed and fasted) colonic water content was 7.14 g kg^{-1} or 16.9 g kg^{-1} when the caecum was included. For an average (fed and fasted) mouse, the values were 7.8 g water kg^{-1} body weight and 16.3 g kg^{-1} when the caecal contents were counted. In man, the small intestine has been reported to contain a total of 206 g water or 3.8 g kg^{-1} (Gotch et al 1957). This compared with 11.1 g water kg^{-1} body weight in the rat small intestine, and 16.5 g water kg^{-1} body weight in the mouse small intestine. The same authors found 118 g water in the stomach or 2.2 g water kg^{-1} body weight. In our study, the corresponding values were 3.2 g kg^{-1} in rats and 8.5 g water kg^{-1} body weight in mice. Thus, when the values were normalized to take into account total body mass, more water per kg body weight was found in the gastrointestinal tracts of the mouse and the rat than in man.

Interestingly, although the total water content reported in the small and large intestine in man was high (206 g (Gotch 1957) and 187 g, respectively (Cummings et al 1990)), Schiller et al (2005), using magnetic reasonance imaging, measured a median free fluid volume of 105 ± 72 mL (fasted) and 54 ± 41 mL (fed) in the small intestine, and 13 ± 12 mL (fasted) and 11 ± 26 mL (fed) in the colon. These values indicated that most of the gut water was in the bound state. This suggested that only a proportion of the water content was available for drug or dosage form dissolution, and the same is likely to be true of the water content in the animal models discussed.

The volume of the mouse and rat stomach

Drug or vaccine formulations are often given to experimental animals by oral gavage. Consequently, the volume of the stomach is considered an important parameter for oral dosing, and the results are shown in Table 2. The mouse stomach was approximately one-tenth the volume of the rat stomach. Wolfensohn & Lloyd (1994) have suggested the upper limit for oral dosing in mice to be 20 mL kg^{-1}. Thus, for a mouse of 20 g, the maximum oral dosage volume would be 0.4 mL. For rats, the recommended maximum is 10 mL kg^{-1}; for a 200 g rat this would give a dosing volume of 2 mL. These values correlate to some degree with the 'comfortably full' volumes shown in Table 2, despite the fact that post-mortem results would be likely to differ from an in-vivo situation, since elasticity and responsiveness of gastric tissue to pressure may be altered.

Quantification of lymphoid tissue along the gastrointestinal tract of the mouse and rat

The lymphoid tissue along the gastrointestinal tract can be categorized broadly into, firstly, individual lymphoid follicles, which are seen as raised white areas, and secondly into patches, which are collections of individual follicles. In the small intestine these are referred to as Peyer's patches. No lymphoid tissue was observed in the stomach. However, significant amounts of lymphoid tissue were observed in the mouse and rat caecum (Table 3 ; Figure 5A, B). Thus, we confirmed previous reports on the presence of lymphoid tissue in mouse caecum (Owen et al 1991) and have reported, for the first time to our knowledge, the presence of lymphoid tissue in rat caecum.

Table 2 Fill volumes of mouse and rat stomach

	Volume (mL (s.d.))	
	Mice (n = 10)	Rats (n = 8)
1. Comfortably full	0.37 (0.09)	3.38 (0.52)
2. Stretched	0.55 (0.09)	4.63 (0.44)
3. On the point of bursting/could not be expanded further	0.71 (0.11)	6.63 (0.92)

Table 3 Quantification of lymphoid tissue in the intestinal tract of Balb/c mice and Wistar rats. The mean and (range) values are shown

	Mouse (n = 15)			**Rat (n = 10)**		
	Small intestine	Caecum	Colon	Small Intestine	Caecum	Colon
Mean length (range)	34.5 (29–39)	–	11.5 (9–14)	82.8 (70–97)	–	13.9 (12–18)
Mean number of patches (range)	10.1 (3–15)	1.4 (1–5)	11.6 (7–15)	9.4 (7–15)	1.2 (1–2)	3.8 (2–11)
Mean number patches cm^{-1}	0.3	–	0.8	0.33	–	0.3
Mean number of follicles (range)	57.5 (22–80)	18.1 (9–26)	39.4 (18–54)	207.5 (142–273)	13.5 (8–26)	38.6 (16–83)
Mean number follicles per patch	5.7	12.9	3.4	30.6	12	28.5
Mean number follicles cm^{-1}	1.6	–	3.4	2.1	–	3.4

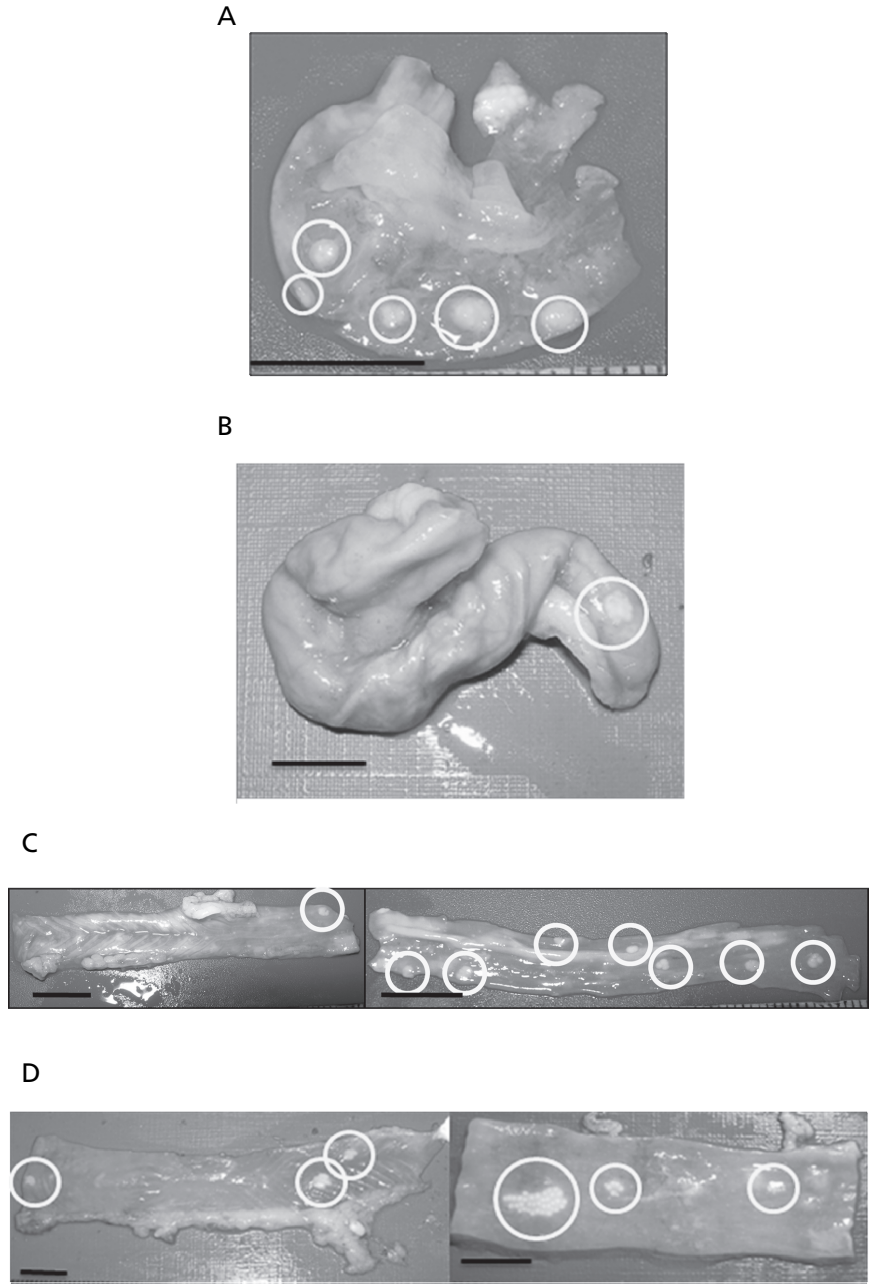

Figure 5 Lymphoid tissue patches in the (A) caecum of a Balb/c mouse (scale bar = 10 mm), (B) caecum of a Wistar rat (scale bar = 10 mm), (C) colon of the Balb/c mouse (left proximal; right distal) (scale bar = 10 mm), (D) colon of the Wistar rat (left proximal; right distal) (scale bar = 10 mm).

The numbers of Peyer's patches in the mouse and rat small intestine (Table 3) were similar to those reported in the literature: 6–12 Peyer's patches in mouse small intestine (Abe & Ito 1977) and 15 Peyer's patches in the rat small intestine (Hillery et al 1994; Florence et al 1995). The values for the number of lymphoid patches in the mouse colon, however, are slightly less than a previously reported value of 1.4 patches cm^{-1} (Owen et al 1991).

Examination of lymphoid tissue density along the gastrointestinal tract revealed that in rats and mice, Peyer's patches were distributed randomly along the sections of the small intestine, with no predilection for a particular area ($P > 0.05$). Examination of the three small intestinal sections (roughly duodenum, jejunum and ileum) within each animal showed that there were similar numbers of patches and follicles per cm, in all three sections (data not shown). Similarly, there was no difference between the number of patches per cm in the proximal and distal colon ($P > 0.05$), which correlated with the random distribution reported in man (Langman & Rowland 1986). Photographs illustrating the random distribution of patches in the mouse and rat colon are shown in Figure 5C, D.

There were, however, differences between the quantity of lymphoid tissue in the small intestine and colon. In mice, the number of patches in the small intestine and colon were similar, but the number of individual follicles was much greater in the small intestine. However, taking into account the lengths of the respective sections, there were actually more follicles and patches per cm in the colon ($P < 0.05$). In rats, there were significantly more patches and follicles in the small intestine, relative to the colon. Taking into account the large differences in intestinal tract length in the rat, similar numbers of patches per cm were seen between small intestine and colon ($P < 0.05$), and more follicles were found per cm in the colon ($P < 0.05$). Mouse colonic lymphoid patches tended to be smaller, containing fewer follicles than small intestinal ones. In contrast, rat lymphoid patches were of similar size in both the small and large intestine. The rat lymphoid patches were, in general, larger than mouse patches and contained a greater number of follicles. The presence of lymphoid tissue in the colon of mice and rats confirms that these animals could be used in colonic vaccination studies.

Conclusion

pH values of the small and large intestinal contents in mice and rats were lower than previously reported, and were lower than the pH levels in man. This has implications for the use of rats and mice in testing of drug formulations, such as pH-responsive drug carriers. The very low levels of fluid present in the mouse gastrointestinal tract cautions against the use of mice when drug dissolution from an oral dosage form is examined. The higher water levels in the rat, especially in the fed state, shows that the rat would be a more suitable animal model. Colonic lymphoid tissue was quantified and compared with small intestinal tissue, in both rats and mice. The significant quantity of lymphoid tissue in the colon in both animals highlights the colon as an immunologically important organ and shows that colonic vaccination may be studied in these animal models. Finally, the presence of lymphoid tissue in the mouse caecum was confirmed and its presence in rat caecum has been reported.

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