Short-term Toxicity Tests for Non-genotoxic Effects
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CHAPTER 8

The Gastrointestinal Tract and Short-term Toxicity Tests

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8.1 INTRODUCTION

The primary role of the gastrointestinal tract is the digestion and absorption of nutrients. This is critical for normal body homeostasis. The gastrointestinal tract also forms a barrier against unwanted materials, and is a major site of biotransformation and excretion. Its immunological function is defence against infection and the prevention of hypersensitivity reactions are dependent upon effective microenvironmental immunoregulation at the mucosal level. The implications of the symbiotic relationships of the gastrointestinal microflora are still being elucidated, while the complexities of the overall control of gut motility, secretion and growth are now recognized to depend on interactions between the autonomic nervous system and the neuroendocrine connections of the peptidergic nerves.

The importance of the gut with respect to toxicology arises from its central role in the absorption of ingested materials. It is the major portal of entry for a wide variety of compounds including those which have no nutritional or other functional value (xenobiotics). Most of our knowledge about absorption, transport and metabolism of xenobiotics relates to the normal adult gut, however, the expected effects following ingestion of a particular compound depend on the integrity of the intestinal mucosa. In infancy, many aspects of gut function are immature and, at any age, there are many situations in which mucosal function is compromised; infections, hypersensitivity reactions and malnutrition can all alter permeability of the gut due to epithelial cell damage, with varying effects on absorption of the compound in question.

8.2 THE NORMAL GASTROINTESTINAL TRACT

8.2.1 Structure

The inner surface area of the gastrointestinal tract is vastly increased by the

convolutions of the valvulae conniventes, villi and microvilli. The villi are covered by columnar epithelial cells (enterocytes) which are renewed every 24–48 hours. They originate in the crypts, proliferate and migrate up the villi, differentiating as they progress to the villus tip. These enterocytes are covered by microvilli whose surface membranes are phospholipid bilayers within which there are electronegative pores. These pores or discontinuities in the membrane are wider (7.5–8.0 Ångström units) in the upper small bowel than in the ileum or colon. Extending from the microvillus membrane is the glycocalyx which is characterized by hydrophilic molecules with lipophilic bases resting within the lipid membrane. An unstirred water layer external to the glycocalyx prevents immediate contact with the cell surface.

The enterocytes themselves are of similar origin to hepatocytes and contain comparable subcellular structures rich in enzyme activity relating to synthesis, metabolism and secretion. Between the epithelial cells lie goblet cells; they secrete mucins which contribute to local protection.

The muscularis mucosae is a thin layer of smooth muscle which separates the epithelium from the lamina propria. This extends into the core of each villus and contains capillaries, lacteals and numerous mononuclear cells including macrophages, polymorphs, mast cells and lymphocytes. The lymphocytes are also distributed throughout the epithelium itself and occur as aggregates in the Peyer's patches, appendix and tonxils. They, in effect, form a lymphoid organ. Thus gut associated lymphoid tissue (GALT) determines the recognition of antigen and controls a subtle balance of responses such as the generation of mucosal and/or systemic immunity, tolerance or hypersensitivity reactions of various types. The outer muscle layer of the gut contains both longitudinal and circular fibres which are richly innervated.

8.2.2. Uptake, transfer and metabolism

Digestion in the lumen is dependent upon secretions from the stomach, liver and pancreas. The enterocytes with their microvilli also produce numerous digestive enzymes. Access to the enterocytes will depend initially on the physicochemical characteristics of the substance concerned. Transport into cells may be by passive diffusion down a concentration gradient, by active carrier-mediated transport, by pinocytosis or, in the case of very lipophilic compounds, by micelle solubilization in bile acid mediated diffusion. Para-cellular entry may also be gained by diffusion through the tight junctions between cells.

Although our knowledge of normal digestive function is now extensive, the handling of foreign compounds which do not have nutritional value or functional importance is less well understood. Materials which are antigenic to the immune system have been particularly studied. They are preferentially taken up by specialized 'M' epithelial cells which overlie the Peyer's patches.

Discerning the actual fate of individual foreign compounds is further complicated by numerous other factors which may affect absorption and metabolism. These include binding to nutritional constituents, the generation of toxic metabolites, gastric emptying and small bowel transit times, the dilutional and pH effects of the lumenal contents, blood flow, interaction with the gut microflora, gastrointestinal disease states, and the nutritional and maturational status of the mucosa.

Once within the enterocytes, the fate of xenobiotics is dependent on the enzymatic processes of the subcellular membranes. Biotransformation into water-soluble products may result in transport into the portal circulation and hence to the liver. Lipophilic compounds may travel within chylomicrons into the lymphatics. Some compounds such as benzo(a)pyrene are metabolized in the cell and the products are secreted back into the lumen of the gut (Hietanen, 1980).

The enterocytes are rich in enzyme activity. Synthetic enzymes catalysing acetylation, *O*-methylation and glutathione transfer are present, as well as non-synthetic enzymes concerned with hydrolysis and reduction. The smooth endoplasmic reticulum contains cytochrome P-450 acting as an oxygen activator and NADPH cytochrome P-450 reductase supplying electrons for mono-oxygenation. The microsomes have the highest specific activity of metabolizing enzymes within the enterocytes. Several types of mono-oxygenases occur. They can act on lipophilic xenobiotics to produce polar hydroxyl groups which can be acted upon by the conjugation enzymes leading to hydrophilic products (Hoensch, 1982). Conjugation can lead to reduced bioavailability of compounds in the systemic circulation, for example, glucuronic acid conjugation of morphine and terbutaline, and sulphate conjugation of ethyl oestradiol (Back *et al.*, 1981). Conversely, deconjugation by intestinal bacteria may reduce water solubility and promote absorption (Hoensch and Hartmann, 1981).

8.2.3 Immunological considerations

Nutrients and xenobiotics may generate immunological reactions. It is currently suspected that immunologically mediated allergic mechanisms underlie many unexpected reactions to oral antigen. The responses of the GALT to antigen are complex. Humoral IgA immunity was initially studied, secretory IgA not only protects against infection by preventing bacterial adhesion and neutralizing viruses and toxins, but it can complex with specific antigens and prevent their absorption. Cellular immune mechanisms continue to be elucidated and it is clear that T cell control can result in immunity or tolerance that is a specific unresponsiveness to an antigen. This tolerance is particularly generated in certain circumstances by oral exposure to the antigen while the lack of tolerance may result in the production of clinically-apparent hypersensitivity reactions. This might occur when the mucosa is damaged, or possibly in the presence of adjuvants in the gut lumen.

In the first year of life, there is an increased incidence of hypersensitivity

reactions. The aetiology of these may relate to such factors as a transient deficiency of secretory IgA, an increase in receptor sites for antigen on immature enterocytes, increased macromolecular access, and decreased intralumenal degradation of antigenic material.

8.2.4 Maturational considerations

In infancy, many aspects of gut function are immature. Digestion of fats, proteins and carbohydrates are largely dependent on the development of exocrine pancreatic function. Even in full-term newborns, pancreatic function is relatively poorly developed. Lipase is low and amylase is absent, although proteases are developed fairly early and are present at birth. The neonatal pancreas is more often unresponsive to pancreozymin and shows a reduced sensitivity to secretin (Lebenthal *et al.*, 1981). Maturation of function is promoted by dietary exposure to nutrients. Brush border sucrase, maltase and alkaline phosphatase activities are high at birth; however, lactase activity is low (Moog, 1981). Different patterns of intestinal motility are also seen in infants and children, more rhythmic peristaltic waves being evident (Siegel and Lebenthal, 1981). One example of the effect of gut maturity on function may be seen in a study of lead uptake in rats in which there was a markedly reduced rate of uptake during the first 3–4 weeks of life (Barltrop, 1982).

Macromolecular transport across the intestine has been studied far more extensively in young animals than in man. In animals which receive partial passive immunity (e.g. rat) or more complete passive immunity (e.g. sheep, pig) postnatally, perinatal transport of immunoglobulin across the mucosa occurs (Morris and Morris, 1976). In animals receiving intrauterine passive immunity (e.g. rabbits, man), there is some evidence that intact proteins can penetrate the mucosal barrier of the gut of the immature animal (Udall and Walker, 1982).

8.2.5 Nutritional considerations

Malnutrition in children causes dramatic reductions in pancreatic enzyme production (Barbezat and Hansen, 1968). Animal studies have confirmed this, as well as the fact that recovery readily occurs following improvement in the diet (Schrader and Zeman, 1970). Malnutrition also comprises mucosal immunity. Thus, children suffering malnutrition encounter an increase in the number and duration of gastrointestinal infections; this further damages the mucosa and affects its capacity for absorption and metabolism.

Interaction between nutritional factors in the diet may be of importance in other respects. Lead absorption in rats has been increased by diets containing high fat, low mineral, low protein or high protein concentrations (Barltrop and Khoo, 1975; Bell and Spickett, 1983).

8.3 THE RECOGNITION OF TOXIC EFFECTS TO THE GUT

8.3.1 Introduction

Gastrointestinal symptoms are common and notoriously non-specific since their occurrence does not always imply a primary disturbance of gut function. Nausea, abdominal pain or diarrhoea frequently accompany stress in many adults, or urinary and respiratory infections in children. Similarly, substances encountered by inhalation or skin contact can be absorbed and cause secondary alimentary tract symptoms. In practice, toxic effects of many ingested compounds may not be completely evaluated since initial exposure clearly causes gastrointestinal disturbances, or macroscopic changes in the gut such as ulceration. Although the major emphasis in the literature has been placed on carcinogenesis and genotoxic mechanisms, xenobiotics have been studied in many other respects.

8.3.2 Histological studies

In man, mucosal biopsies from the oesophagus, stomach, jejunum, sigmoid colon and rectum have been studied over many years. Initially, general morphological changes were described. More exact quantification can now be applied by measuring villus height and crypt depth, by computing surface areas using light pens, and by recording cellular infiltration in terms of cell counts (related to enterocyte numbers, standard grids or calculated volumes). Individual cell types, such as mast cells, can be better identified using modern stains. Mitotic indices amongst crypt cells can be measured and monoclonal antibodies can be applied to identify lymphocyte subsets and their distribution through the mucosa. The advent of fibre-optic endoscopy has enabled macroscopic examination to complement histological appearances, and has allowed access to the rest of the colon and the terminal ileum.

Peptic ulceration is an important area of study; acrylonitrile is one substance which has a duodenal ulcerogenic action. This compound is also an interesting example since it points out the relevance of evaluating interactions with other factors. In this case, it was noted that polychlorinated phenols potentiate duodenal ulceration in rats given acrylonitrile (Szabo *et al.*, 1983). Polychlorinated phenols alone produce histological changes, gastric mucosal hyperplasia being observed in subhuman primates (Becker *et al.*, 1979).

Light microscopy may be supplemented with electron microscopy and polarizing light microscopy. The latter has been useful in demonstrating the penetration of the mucosa by particulate matter such as asbestos (Meek and Grasso, 1983). Electron microscopy of tissue residues and body fluids in man has shown that an oral intake of asbestos may results in its widespread distribution in the body (Carter and Taylor, 1980).

Two environmental toxins have been particularly implicated in causing histological damage to the mucosa and subsequent malabsorption: T-2 toxin and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The trichothecene T-2 toxin (derived from the

fungus *Fusarium sporotrichioides*), which may occur in stored grains, causes alimentary toxic aleukia. An enteropathy is succeeded by systemic and neurological disturbances and leukopenia (Lutsky *et al.*, 1978). The mechanisms of toxicity are not known, although T-2 toxin is recognized as an inhibitor of protein synthesis and mitochondrial respiration (Schiller and Yagen, 1981). 2,3,7,8-TCDD alters fat absorption so that the mucosal cells become filled with large lipid droplets (McConnell and Shoaf, 1981).

8.3.3 Organ weights

Determination of organ weights is another long-established practice in toxicological studies. Usually, no changes have been reported in the gut except for the caecum. Many compounds, including food flavourings such as 4-methyl-1-phenylpentan-2-ol, have been shown to increase the weight of the caecum in rats (Ford *et al.*, 1983). The cause of this is disputed but physiological mechanisms involving changes in the microflora and the osmotic activity of caecal contents have been postulated (Leegwater *et al.*, 1974).

8.3.4 Gut microflora

The normal gastrointestinal tract is colonized by bacteria. Many of these are anaerobic Gram-negative organisms; their metabolism is largely reductive and hydrolytic and they may interact with a wide variety of chemicals. Enzymes present in the anaerobic flora of the caecum include nitrite reductase, azoreductase, imidazole reductase and glucosidase. In some situations, toxic compounds may be broken down; in other situations, they may be generated as in the action of bacterial glucosidase in the conversion of glycosides to toxic aglycones (Rowland, 1981; Williams, 1972). Bacterial flora can change readily for instance, in response to oral antibiotics or the ingestion of a high fibre diet, the gut microflora in rats has been shown to increase bacterial enzyme activities (Rowland *et al.*, 1983).

8.4 INTESTINAL ABSORPTION STUDIES

8.4.1 Whole body techniques

A basic approach to the clinical assessment of ingested compounds is to study absorption kinetics. This was significantly advanced when isotope labelling techniques were introduced. In man, after an oral dose of the test substance, analysis of faeces, urine, blood, saliva and expired air can be readily carried out (Walson *et al.*, 1981). Collection may be necessary for several days to ensure that delayed excretion is recognized. However, whole body analysis fails to detect localized concentrations of compounds of their metabolites in specific organs of the body.

In animals, pharmacokinetic evaluation may be extended to include tissue

distribution and metabolism studies by measuring the radioactivity levels in various tissues and body fluids over time (Abou-Donia *et al.*, 1983)

8.4.2 *In vivo* techniques in animals

Transport and uptake studies may be undertaken in animals by using gut loops. A classic approach has been to construct isolated segments of small intestine. Thiry-Vella loops consist of a length of ileum, *in situ*, retaining its blood supply but externally cannulated at each end to allow perfusions to be carried out (Keren *et al.*, 1975). These preparations require a considerable amount of maintenance. For this reason, various other perfusion techniques have been developed. Triple lumen tubes may be inserted orally or, in animals, by means of a chronic jejunal fistula. After constant infusion to achieve a steady state, proximal and distal collections may be carried out to determine transport kinetics (Barbezat, 1980). Various animal models have been devised to allow *in vivo* perfusions of various lengths of gut within the abdominal cavity (Sandhu *et al.*, 1981) or in exteriorized loops for short periods; additional variants such as the effects of blood supply may also be studied (Granger *et al.*, 1976).

8.4.3 *In vitro* techniques

A basic approach has been to simply excise gut segments, tie their ends and place them in suitable media. Perfusion may be carried out by cannulating the ends of short lengths of excised gut, and perfusing them while immersed in an oxygenated medium. Various assays may be devised involving translocation of labelled compounds from the diffusing fluid to the serosal bath. Using this technique, Lyons *et al.* (1983) found that cadaverine, a substance present in spoiled fish, was able to inhibit the intestinal detoxification of histamine.

Ion transport may be studied by taking sheets of mucosa stripped of their serosal and muscle layers and mounting them in Ussing or Lucite chambers. The mucosa separates two reservoirs of oxygenated Ringer's solution, one bathing the serosal and the other the mucosal surfaces (Binder *et al.*, 1973). The potential difference across the mucosa may be monitored, and ion fluxes measured using, for instance, isotopes ^{22}Na and ^{36}Cl. Numerous compounds affect sodium, chloride and bicarbonate secretion and absorption. This can be demonstrated by adding them to the mucosal or serosal reservoirs and measuring changes in short-circuit current that reflect ion transport. Alpha-adrenergic stimulants have both anti-motility and anti-diarrhoeal activity; their effects in promoting sodium and chloride absorptions have been shown using this system (Durbin *et al.*, 1982).

Subcellular fractionation allows more detailed studies of intracellular enzyme function and dysfunction to be undertaken. The gut mucosa may be scraped off and homogenized, and subcellular fractions isolated by differential centrifugation. Studies using mitochondria, microsomes, nuclear and microvillus preparations have

become practicable. Microsomes are vesicular structures visible under the electron microscope which represent the smooth endoplasmic reticulum within the cell. They are of particular importance in the current context since they carry a very high specific activity of metabolizing enzymes. Biotransformation of xenobiotics may be evaluated by incubating different substrates with microsomes (Stohs *et al.*, 1976). Amongst the various types of monooxygenase activity which have been shown to occur in intestinal tissues are: aromatic ring-hydroxylation; O-dealkylation; N-demethylation; sulphoxidation; and N-amine hydroxylation (Hoensch, 1982). Microsomal action may be influenced by many factors, for example, they are located primarily in the epithelial cells of the upper villus and may be dependent on additional factors such as dietary iron (Hoensch *et al.*, 1976). The biological effects of drugs and xenobiotics may be significantly affected by changes in intestinal metabolism, for example, phenacetin has been extensively studied and it has been found that its oxidative metabolism is increased in rats exposed to cigarette smoke (Welch *et al.*, 1972) and brassica vegetables (Pantuck *et al.*, 1976).

Enterocytes may be dissociated from the mucosa by chemical or mechanical means, and maintained in various culture systems (Hartmann *et al.*, 1982). The toxic effects of various xenobiotics on cell or organ cultures may be evaluated directly by measuring protein synthesis, enzyme activities and morphological changes using electron microscopy (L'Hirondel *et al.*, 1976). Over the last decade, enterocytes obtained from coeliac patients have been used to identify toxic fragments of ∝-gliadin; however, the difficulties encountered in obtaining sufficient cells from biopsy specimens and in standardizing the culture techniques have rendered many of these studies difficult to interpret (Falchuk *et al.*, 1974; Howdle *et al.*, 1981; Wood *et al.*, 1983).

8.5 FUNCTIONAL STUDIES IN MAN

Indirect evidence of mucosal damage may be gained from the use of conventional absorption tests and other parameters of gut function.

8.5.1 Absorption

Fat absorption is still most consistently evaluated by accurate but time-consuming measurements of intake and excretion in the stool over 3–5 days. Over the years, numerous tests to detect an increased faecal fat content have been described. More recently, specific monitoring of absorption has been undertaken using ^{14}C or stable ^{13}C labelled lipids and measuring the excreted labelled carbon dioxide in breath tests.

Changes in carbohydrate absorption can be a useful way of demonstrating enterocyte brush border damage. Lactase is a particularly vulnerable enzyme; more extensive injury will reduce sucrase and isomaltase activity, and eventually the absorption of the monosaccharides glucose and fructose can be affected. Lactose intolerance

can be demonstrated on clinical challenge by the generation of watery stools of acid pH containing glucose, and confirmed by breath H_2 analysis. Disaccharidase activity may be measured directly in mucosal biopsy homogenates.

Protein absorption can be investigated laboriously using balance studies measuring N_2 excretion. A more elegant approach is to use a stable ^{15}N isotope and measuring urinary excretion after a labelled oral load. However, these methods would only indicate fairly gross evidence of mucosal damage.

8.5.2 Mucosal integrity

Various tests aim to evaluate mucosal integrity. The xylose absorption test is time honoured, but subject to various theoretical criticisms. Recently the differential absorption of two non-metabolized carbohydrate probe molecules of different molecular weight has been advocated. Lactulose, rhamnose, cellobiose or mannitol may be given orally and the urinary excretion ratio calculated. If the mucosa is damaged, more of the higher molecular weight carbohydrate is present in the urine. Polyethylene glycols of various molecular weights have also been used as probe materials. Another indirect indicator of enterocyte damage is protein loss in the stools; $\propto_1$ antitrypsin is a non-specific marker protein which can be measured.

8.5.3 Specific function

If more specific functional information is required, gastric acidity, pancreatic secretion and vitamin B_{12} absorption can all be investigated. Pancreatic function may be quantified by measuring duodenal juice lipase, trypsin, amylase and bicarbonate after intravenous injection of pancreozymin (CCK) and secretin, or after a test meal. Pancreatic function may also be measured indirectly by the *p*-aminobenzoic acid screening test. Similarly, bile acid secretions and pool size may be studied using direct analysis and isotope breath tests.

Gut motility is an area of increasing interest. Transit time measurements using carmine dye or polystyrene shapes of different sizes have been superseded by pressure manometry, electrical monitoring and the use of telemetry capsules. The normal pattern of electrical activity and motility of both the small and large bowel is now being better defined; however, disturbances associated with disease states have still not been well characterized.

8.6 CONCLUSION

It is evident from this review that a wide range of potential and practical approaches exist for the detection and evaluation of the toxicity of compounds to the gastrointestinal tract itself, and for the evaluation of factors which influence the absorption of ingested xenobiotics. At the cellular and subcellular levels, mucosal cells with intense metabolic activity and rapid turnover may be particularly appropriate for

developing assay techniques. Although the intra-cellular enzyme systems of enterocytes are shared by cells in many other tissues, application to short-term *in vivo* and *in vitro* toxicity studies has yet to be adequately exploited. The demonstrable preservation of mucosal function would, nevertheless, seem to be a logical prerequisite for the evaluation of the safety of substances which may enter the body by the oral route or otherwise affect the gastrointestinal tract.

The gut is particularly vulnerable to dysfunction as a result of perturbations in both its internal and external milieu, thus enhancing its potential value for the evaluation of toxicity. Many of the organ's components and functions have similar but less accessible equivalents elsewhere in the body so that a continuous and economical alternative may be available for some test systems.

The development of new approaches to toxicity testing and safety evaluation involving the gut should, however, take account of its highly dynamic nature and overall lack of structural and functional homogeneity. Moreover, human subpopulations with developmental immaturity, particular nutritional status and with coexisting inflammatory or other enteropathic changes in the mucosa may experience modified toxicity of ingested substances. Toxicity may be modified in the gut itself, or in other organs when absorption from the gut is altered. Many of the problems identified here are at the limits of current knowledge; their elucidation will have scientific, clinical and toxicological implications.

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