AN EXPERIMENTAL MODEL OF GASTROINTESTINAL CANDIDIASIS

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BACTERIAL overgrowth occurs in the upper small intestine of malnourished children (Mata *et al.*, 1972; Gracey *et al.*, 1973; Heyworth and Brown, 1975) and species of *Candida* are found more frequently in the gastric and small-intestinal contents of such children (Gracey *et al.*, 1974). The possible importance of *Candida* species as a cause of diarrhoea in malnourished children has been suggested by El-Gholmi, Aboul-Dahab and El-Essawi (1961); this is supported by our findings that there is decreased absorption of sugar (Burke, Houghton and Gracey, 1977) and water (Thelen, Burke and Gracey, 1978) in rat intestine perfused *in vivo* with cell-free broth cultures of *Candida* species isolated from the upper intestinal contents of malnourished children.

We have now studied the effects on intestinal transport and activity of brush-border enzymes in rats experimentally infected with *C. albicans* to clarify the relationship between diarrhoea and intestinal candidiasis.

MATERIALS AND METHODS

Infection of animals

Intestinal infection with *C. albicans* was established in 150-g inbred albino Wistar rats by the intragastric inoculation of 1 ml of broth culture containing 1×10^9 organisms/ml. The broth was prepared by inoculation of *C. albicans* into Sabouraud broth and incubation at 37°C for 48 h. Intragastric inoculation was performed by means of a sterile syringe attached to sterile polyethylene cannula of internal diameter 0.4 mm and external diameter 0.8 mm (Dural Plastics, Dural, NSW, Australia). Intragastric inoculation was repeated daily for 3 days. A second group of animals was maintained on a protein-depleted diet (Takano, 1964) for 3 weeks before inoculation with *C. albicans*. Eighty percent of the animals developed soft, pale stools the day after the first inoculation; only these animals were used in subsequent investigations. Control animals were given broth only, without added yeasts.

Two days after the third dose of *Candida*, absorption of sugar and water and activity of the intestinal disaccharidases and enterokinase were measured. Gastric and upper intestinal contents were cultured for yeasts and specimens of mucosa were taken for light and electron microscopy.

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Absorption experiments

Animals were fasted overnight and used for in-vitro or in-vivo studies of sugar and water absorption.

In-vitro experiments. Animals were killed with anaesthetic ether. The stomach and duodenum were removed, opened and placed in 2 ml of transport medium (glucose broth with 10% v/v glycerol). The small intestine was removed, rinsed through with isotonic saline and everted. Areas containing Peyer's patches were discarded and the remainder was cut transversely into segments approximately 1.5 cm long. These were randomised (Crane and Mandelstam, 1960) in a beaker of Krebs Henseleit (KH) buffer (Krebs and Henseleit, 1932) (0.9% NaCl 3 litres, 1.15% KCl 120 ml, 1.22% CaCl₂ 90 ml, 2.11% KH₂PO₄ 30 ml, 3.8% MgSO₄ 7 H₂O 30 ml, and 1.3% NaHCO₃ 630 ml) at 37° and pH 7.4 then, after gentle blotting, mounted in plexiglass chambers (Semenza, 1969). Specimens were incubated for 2 min in KH buffer, then transferred to beakers containing 30 ml of incubation medium with four specimens per beaker. Incubation medium consisted of KH buffer which was gassed with O₂:CO₂ (95:5 by vol.) for 60 min. before and during the experiments and contained 3 mM of arbutin (p-hydroxy-phenyl β -glucoside) and 2 mM of 2-deoxy-glucose. Specimens were incubated in a Dubnoff-type shaking water bath (Paton Industries P/L, Stepney, South Australia) at 37°, shaking at 90 oscillations/min. After 10, 20, 30, 40 or 50 min. the chambers were rinsed in cold KH buffer and the exposed tissue was punched out, blotted to remove excess fluid, then weighed. Specimens were homogenised and deproteinised by the method of Somogyi (1952). Arbutin was determined by the method of Folin and Ciocalteu (1927) and 2-deoxy-glucose as described by Waravdekar and Saslaw (1957). Results were expressed as μ mol of arbutin per mg of tissue after correction for 2-deoxy-glucose space.

In-vivo experiments. Solutions consisted of KH buffer pregassed with O₂ and CO₂ (95:5 by vol.) containing 5 mM of arbutin and 5 μ g per ml of phenol red as a non-absorbable marker. Recovery of phenol red was $98 \pm 5\%$.

At laparotomy under urethane anaesthesia, a polyethylene tube (internal diameter 2 mm, external diameter 3.5 mm) was tied into the proximal jejunum. An exit tube was introduced in the same way 20 cm distal to the first tube. The segment was washed with perfusion solution then connected to a constant-rate perfusion pump delivering the fluid at 10 ml/h. After 30 min. for equilibration, fluid was collected for 1 h. At the end of the experiment the gut segment was removed and, with a standard weight attached to one end, its length was measured against a vertical rule.

Arbutin was measured by the method of Folin and Ciocalteu (1927) and phenol red was determined spectrophotometrically at 560 nm after alkalinisation with 1N NaOH.

Arbutin absorption was expressed as μ mol absorbed per cm of intestine per hour, and net water flux as the change in volume per cm of intestine per hour.

Enzyme assays

Specimens of mucosa from the first 5 cm of jejunum were used for assay of β -galactosidase (EC.3.2.1.23), sucrase (EC.3.2.1.26) and enterokinase (EC.3.4.4.1) activity.

Disaccharidases were measured by the method of Dahlqvist (1964) with glucose determined by the glucose-oxidase method of Huggett and Nixon (1957). Enterokinase was assayed by the method of Barns and Elmslie (1977), and protein by the method of Lowry *et al.* (1951). Results were expressed as units of disaccharidase per g of protein where 1 unit hydrolyses 1 mol of substrate per minute.

Statistical methods

For in-vitro absorption experiments, results were transformed to logarithms because variance was proportional to the size of the observations. In the other comparisons, untransformed data were used with Student's *t* test and results were considered significant at the 95% level.

Microbiological methods

The stomach and duodenum were separately opened and placed in transport medium (10%

v/v glycerol in glucose broth). After homogenisation, serial dilutions were cultured in Sabouraud's dextrose agar and incubated at 25° for 48 h.

Mucosal adherence of C. albicans

Adherence of *C. albicans* to intestinal mucosa was compared in normal and protein-deprived rats by the technique described by Freter (1974). Sections of rat jejunum longitudinally slit and about 1 cm long were incubated in KH buffer with broth cultures of *C. albicans* to give a final concentration of *Candida* 1×10^6 /ml. After incubation for 1 h. in a shaking water bath at 37° in an atmosphere of O₂ and CO₂ (95:5 by vol.) tissues were washed in sterile saline, homogenised with 1 ml of sterile isotonic saline in a geared electrical stirrer (Betts & Co. P/L, Milperra, NSW, Australia), then cultured in Sabouraud's medium. The numbers of viable organisms found in normal tissues were compared with the numbers found in mucosa from protein-deprived rats.

Histology and electron microscopy

Transverse sections approximately 1 cm long were taken at the level of upper, mid and lower jejunum. Those for light microscopy were fixed in formol saline and carefully orientated before mounting and staining with haematoxylin and eosin.

For electron microscopy, tissues were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) for 16 h. They were then post-fixed in 1% osmium tetroxide in cacodylate buffer for 1 h. and dehydrated in graded solutions of ethanol. Specimens were embedded in Araldite and sections were cut on an LKB III ultramicrotome (LKB, Stockholm, Sweden) and examined on a Philips 201 electron microscope (Philips, Eindhoven, Holland) at an accelerating voltage of 60 kV.

RESULTS

Significant growth of *C. albicans* was found in the stomach and duodenum from all the animals considered to have had abnormal stools. The mean population of yeasts was 10^5 /ml of gastric and intestinal contents (range 8×10^3 /ml– 4×10^6 /ml)

In-vitro uptake of arbutin

There was a decrease of arbutin uptake throughout the period of incuba-

TABLE I
Intestinal uptake of arbutin after incubation for 30 min. in isolated intestine from candida-fed rats and controls

Animals: diet (number in group)	Arbutin content of intestinal tissue μmol/g (SEM)
Normal (16)	11.01 (0.92)
Normal, candida-fed (15)	5.72* (0.65)
Protein deprived (18)	12.24 (0.70)†
Protein deprived, candida-fed (11)	7.20* (0.70)†

SEM = Standard error of mean.

* Results significantly different from control values; $p < 0.05$.

† Results significantly different for group 1 versus group 3; group 2 versus group 4.

TABLE II

Intestinal absorption of arbutin and net water flux in vivo in candida-fed rats and controls

Animals: diet (number in group)	Arbutin absorption $\mu\text{mol/cm}$ of intestine per h (SEM)	Net water flux $\mu\text{litre/cm}$ of intestine per h $\ddagger$ (SEM)
Normal (12)	2.01 (0.03)	+28.7 (1.3)
Normal, candida-fed (12)	1.52* (0.03)	+2.4 (0.8)
Protein-deprived (12)	2.53 (0.05) $\ddagger$	+32.2 (2.1) $\ddagger$
Protein-deprived, candida-fed (12)	1.30* (0.06) $\ddagger$	-14.9 (2.8) $\ddagger$

SEM = Standard error of mean.

* Results significantly different from control values; $p < 0.05$. $\ddagger$ Results significantly different for group 1 *versus* group 3; group 2 *versus* group 4. $\ddagger$ Positive results indicate net absorption of water and negative results indicate net secretion.

tion in normal and protein-deprived animals fed *Candida*. Results after 30 min of incubation are shown in table I. Comparison of normal and protein-deprived animals with and without candida feeding showed arbutin uptake to be significantly greater in the protein-deprived groups.

In-vivo studies of absorption

Uptake of arbutin was decreased in normal and in protein-deprived animals fed *Candida*.

Similarly, net water transport was decreased in candida-fed animals, with net secretion of water in the candida-fed protein-deprived group. Results are shown in table II. Arbutin absorption was greater than controls in protein-deprived animals without candida feeding, but less than controls in protein-deprived animals fed *Candida*. Similar differences were found by comparison of water flux in normal and protein-deprived animals. Differences were statistically significant.

Activity of intestinal enzymes

There was no difference in activity of lactase, sucrase or enterokinase in candida-infected rats and no differences between normal and protein-deprived groups (table III).

TABLE III

Intestinal enzyme activity in candida-fed rats and controls

Animals: diet (number in group)	Intestinal activity as units/g of protein (SEM) of		
	lactase	sucrase	enterokinase
Normal (12)	6.5 (1.54)	22.7 (2.31)	32.7 (3.8)
Normal, candida-fed (13)	7.1 (1.45)	31.4 (2.41)	33.4 (4.2)
Protein deprived (12)	6.8 (1.20)	32.9 (4.77)	37.3 (4.1)
Protein deprived, candida-fed (12)	7.2 (1.28)	31.5 (4.82)	34.1 (5.0)

Histology

Light microscopy showed no evidence of tissue invasion by *C. albicans* and no inflammatory changes. Trophozoites of *Giardia lamblia* were found in two of the protein-deprived animals.

Electron microscopy showed that enterocytes from the protein-deprived animals contained fewer mitochondria and smaller strands of ergastoplasm than normal controls, with slight reduction in the number of microvilli and a less developed terminal web. Enterocytes from candida-fed rats that were not protein-deprived showed some dilatation of ergastoplasm when compared with normal controls, with slight reduction in microvillar concentration and some disorganisation of the terminal web when compared with normal rats.

Adherence to intestinal mucosa

Candida 10^4 – 10^5 /ml were grown from washed mucosa after incubation with the organisms in normal and in protein-deprived animals. In the mucosal adherence of *Candida*, there was no difference between the two groups.

DISCUSSION

Absorption of sugar and net water flux from the intestinal lumen are decreased in rats that have been fed *C. albicans*. Differences were more obvious in protein-deprived animals; in the protein-deprived group there was, in fact, net secretion of water into the intestinal lumen. These abnormalities occurred in the absence of histological evidence of invasion of the tissues by yeasts. This is consistent with the finding that perfusion of rat intestine *in vivo* with cell-free broth cultures of *Candida* species decreases absorption of sugar, water and electrolytes (Burke *et al.*, 1977; Thelen *et al.*, 1978). Toxin production by *Candida* species could explain these observations.

Histological changes in the small-bowel mucosa are known to occur in malnourished children and may follow gastroenteritis even in well nourished children (Burke, Kerry and Anderson, 1965). Disaccharidase deficiency, an important cause of diarrhoea, particularly in malnourished children (Bowie, Brinkman and Hansen, 1965) is common in such patients. Bishop and Barnes (1974) suggested that *C. albicans* may depress intestinal lactase activity even when the mucosa appears normal on light microscopy and there is no invasion of the tissue by the organisms.

Enterokinase, the peptidase necessary for activation of pancreatic trypsinogen to trypsin, is found in the brush border of the intestinal mucosa (Nordström and Dahlqvist, 1971) in the same way as disaccharidases (Lebenthal, Antonowicz and Shwachman, 1976). Decreased intraluminal enterokinase activity has been reported in patients with bacterial overgrowth in the small bowel (Lutgeerts and Eggermont, 1976). Impaired pancreatic function is known to be associated with malnutrition (Barbezat and Hansen, 1968) and it may be that enterokinase deficiency contributes to defective proteolytic

activity in malnourished children. In our studies lactase, sucrase and enterokinase activities were not depressed in candida-fed rats.

Bishop and Barnes (1974) found that inoculation of yeast cells 10^8 /ml into loops of small intestine in infant rabbits produced a slight decrease in lactase activity 20 h. later. This technique was very different from our model and it seems that the numbers of yeasts in that system were much higher than in the candida-fed rats in which stasis was not produced in the intestinal lumen. In analysing their results Bishop and Barnes (1974) included only those loops in which more than 10^6 organisms were grown after the experiment. In the candida-fed rats, the mean population of yeasts was 10^5 /ml of gastrointestinal contents which corresponds with our experience in isolating *Candida* species from upper intestinal contents of malnourished children (Gracey *et al.*, 1974) and significantly less than the concentrations used in the experimental system used by Bishop and Barnes (1974). It seems that the present model may have more clinical relevance than that earlier study.

Although histological changes were not found on light microscopy in the intestinal mucosa of candida-fed rats, some ultrastructural changes were apparent with dilated ergastoplasm and mild reduction in the number of microvilli. Furthermore, no differences in adherence of *C. albicans* to the mucosa could be found in normal and protein-deprived rats. There was no evidence of invasion of the mucosa by yeasts.

This study has shown that experimental infection with *C. albicans* affects intestinal transport of sugar and water without invasion of mucosa by the organisms. There were no differences in mucosal adherence of *Candida* in protein-deprived rats although effects on transport were more obvious in these animals. Candida-fed animals showed no significant decrease in activity of intestinal disaccharidases or enterokinase. However electron microscopy showed some reduction in microvillar concentration and it may be that experiments of longer duration would demonstrate an effect on brush-border enzymes.

Our findings are consistent with the production of enterotoxin by *C. albicans*. We have already shown that cell-free cultures of *Candida* species can affect intestinal transport (Thelen *et al.*, 1978) *in vivo* and our preliminary investigations suggest that this is the effect of a heat-stable toxin (unpublished observations).

It seems that the overgrowth of *Candida* species often found in the upper intestine of malnourished children has the potential to decrease intestinal absorption of sugar and water, even without tissue invasion, and contributes to diarrhoea which is so common in these children. In our experiments, results of candida feeding were more obvious in the protein-deprived animals.

El-Gholmi *et al.* (1961), emphasise that the *Candida* found in the intestinal tract of malnourished infants "should not be regarded merely as a transient passer-by in the gut" even in the absence of hyphae or mycelium. Our results suggest that *Candida* contributes to the production of diarrhoea without invading the intestinal mucosa. While other organisms found in the intestinal contents of malnourished children may affect intestinal absorption (Thelen *et*

al., 1978), antifungal treatment of such children should be considered, as El-Gholmi *et al.* (1961) have suggested. Our findings provide a possible basis for the success of such treatment.

SUMMARY

Intestinal absorption of arbutin (p-hydroxyphenyl- β -glucoside), a non-metabolised analogue of *D*-glucose, and net flux of water out of the small intestinal lumen were found to be decreased *in vitro* in rats fed with *Candida albicans*. In rats on a protein-deficient diet and fed *C. albicans* there was net secretion of water. Sugar uptake and net water transport were also impaired in infected animals *in vivo*. These abnormalities were present although there was no histological evidence of invasion of the small-intestinal mucosa by *C. albicans*. The findings suggest that *C. albicans* has significant enteric pathogenicity and that this is particularly important in malnutrition.

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