Rectal organ culture as a model for the investigation of bacterial adhesion and invasion

RJ DICKINSON, WJ BRANCH, RE WARREN, G NEALE

From the Department of Medical Gastroenterology and the Clinical Microbiology Laboratory, Addenbrooke's Hospital, Cambridge, and the MRC Dunn Clinical Nutrition Centre, Cambridge

SUMMARY A system was developed for the *in vitro* culture of human rectal mucosa. Its viability was proved by histological appearances and by metabolic studies. Biopsy samples were cultured in the presence of appropriate bacteria isolated from the faeces of patients with ulcerative colitis or with dysenteric illnesses. Attempts to show adhesion of bacteria to the mucosa or invasion of the cultured tissue failed. Problems with the use of this model are discussed.

The ability of bacteria to adhere to or to invade host epithelia are of major importance in the pathogenesis of enteric infection.^{1,2} These properties have been assessed *in vitro* using HeLa and Hep 2 cell monolayers and suspensions of fetal colonocytes.³⁻⁵ The invasion of isolated cells by *Escherichia coli* and other pathogens correlates with *in vivo* properties, but the importance of bacterial adhesion to isolated cells remains unclear because bacterial strains showing *in vitro* adhesion do not necessarily possess other properties associated with pathogenicity.^{1,4-10} In view of these doubts and the probable complexity of host-parasite interactions *in vivo* we have investigated the ability of bacteria with known properties in the HeLa monolayers to adhere to or to invade biopsy samples of rectal mucosa maintained in organ culture.

Material and methods

Two rectal biopsy samples were obtained with standard forceps from outpatients undergoing sigmoidoscopy for the investigation of irritable bowel syndrome or for the management of ulcerative colitis. Each patient gave informed consent to the study, which had been approved by the appropriate ethical committee. One sample from each patient was processed routinely for histological examination and the other was placed immediately in culture medium for transport to the laboratory at ambient temperature. The samples were subsequently washed with fresh medium and divided into 1-2 mm

squares with a scalpel. Two to three explants were obtained from the average sample.

ORGAN CULTURE SYSTEM

Each explant was orientated mucosa-side up in a peripheral area of a 6 cm plastic Petri dish, which had been abraded to encourage adherence. A maximum of two explants were mounted in each dish and 4 ml of culture medium was added. The culture medium was that used by Defries and Franks¹² as modified by Autrup *et al.*¹³ Waymouth's MB 752/1 medium was supplemented with 10% fetal calf serum, hydrocortisone sodium succinate (1 µg/ml), ascorbic acid (300 µg/ml), ferrous sulphate (0.45 µg/ml), and 20 mM tricine buffer (pH 7.4). The pH of the medium was adjusted to 7.4 with 1 M NaOH. In control cultures (no bacteria to be added) the medium was supplemented with benzyl penicillin (100 U/ml), streptomycin (100 µg/ml), gentamicin (50 µg/ml), and amphotericin B (0.25 µg/ml). Organ cultures were maintained in an atmosphere of 95% O₂, 5% CO₂, at 37°C in a rocking chamber. Mucosal viability was assessed against cultures maintained in an atmosphere of 100% CO₂ or in air by histological examination and by the incorporation of ¹⁴C-leucine.

ASSESSMENT OF VIABILITY

Histological studies

Randomly selected explants were removed from culture at intervals of 3, 6, 12, and 18 h, fixed in buffered formal (pH 7.4), routinely processed, and stained with haematoxylin and eosin for examination under the light microscope.

Incorporation studies

At the start of culture 200 μ L (U- 14 C) - leucine (Radiochemical Centre, Amersham) was added to each dish. Explants were removed at intervals of 2½, 5, 8, 12, and 18 h and washed in Waymouth's MB 752/1 medium containing 10 mM N-acetyl cysteine (Airbron, Duncan Flockhart) to remove surface mucus. Each explant was homogenised manually in a Potter homogeniser in 1 ml 1 M NaCl. One millilitre of 10% trichloroacetic acid (TCA) was added to each homogenate and allowed to stand for 10 min or overnight at 4°C. The homogenates were centrifuged at 3000 rpm for 10 min and the supernatant was removed. The pellets were resuspended in 5% TCA and recentrifuged before solubilisation of protein in 1 ml 0.1 M NaOH. Aliquots (0.2 ml) of each preparation were added to 10 ml scintillation fluid (Instagel-Packard Instruments) and counted for 10 min in a Phillips PW 450 liquid scintillation counter. Radioactive decay, as disintegrations per minute (dpm), was calculated by using 10 μ L of 14 C-toluene of known specific activity as an internal standard. The remaining 0.8 ml of solubilised protein was analysed for protein content by Lowry's

method using bovine serum albumin as a standard.¹⁴ The results for each explant were recorded as dpm/ μ g protein.

EXPERIMENTS WITH ORGAN CULTURE AND BACTERIA

Bacterial strains

E coli strains with known properties in HeLa cell monolayers were used. All had been shown to be non-enterotoxigenic in Y₁ adrenal cell and suckling mouse assays.^{11 15 16} The strains and their properties on HeLa cell monolayers were as follows: *E coli* 021:H47 (invasive), 01:H7 (adhesive), 064:H- (adhesive), 01:H? (adhesive), 08:H- (non-adhesive/non-invasive), 01:H? (non-adhesive/non-invasive). In addition, *E coli* strains SC13 (01:H7 non-invasive CFAI-/CFA II-) and E12860/0 (*E coli* 024:H-invasive) and one each of strains of *Campylobacter jejuni* and *Shigella sonnei* isolated from patients with diarrhoea were also investigated. The two reference *E coli* strains were kindly provided by Dr B Rowe (Central Public Health Laboratories, Colindale). All the strains were stored on Dorset egg medium in the dark until use.

Fig. 1 Organ culture of rectal mucosa from a patient with the irritable bowel syndrome after 6 h incubation in test conditions. Haematoxylin and eosin. Original magnification $\times 100$.

Fig. 2 Organ culture from a patient with ulcerative colitis after 18 h incubation in test conditions. Haematoxylin and eosin. Original magnification $\times 100$. There is epithelial attenuation and degeneration of the crypt bases but the overall architecture is preserved.

Infection experiments

Bacteria for each experiment were prepared from overnight peptone water cultures. They were centrifuged, washed, and resuspended in antibiotic free culture medium immediately before use. The concentration of the bacteria was estimated on the assumption that an overnight culture comprises 10^8 bacteria/ml and were diluted accordingly to yield suspensions of about 10^6 , 10^7 , and 10^8 /ml. Each test and reference strain were tested at least twice in separate experiments.

The rectal biopsy samples for the infection experiments were kept in medium containing antibiotics for at least 1 h. They were then washed three times with 4 ml aliquots of phosphate buffered saline (PBS) and once with 4 ml of fresh medium containing no antibiotics. They were then mounted in plastic Petri dishes directly on to abraded areas and orientated mucosa side up with forceps or a fine needle. Antibiotic free culture medium (4 ml) was then added containing either 10^6 , 10^7 , or 10^8 bacteria/ml and the cultures were incubated at 37°C as previously described. After varying intervals of culture (1, 3, or 6 h) they were washed three times with 4 ml aliquots of fresh medium at room temperature to remove proliferating non-adherent bacteria and reincubated at 37°C . After 3, 6, and 18 h of incubation the organ cultures were washed three times with 4 ml aliquots of PBS and fixed in phosphate buffered formalin.

Some variations to the technique were applied. Some organ cultures were mounted on to fibrin clots

or blobs of clear agar in an attempt to prevent bacteria entering subepithelial tissues. This was technically more difficult and did not appear to encourage epithelial adhesion or invasion. In a few instances the reference invasive *E coli* strain was added in saline solution and the biopsy samples were fixed after one hour, and in other instances the culture medium was further supplemented with 20 mM sodium salicylate and 10 mM N-acetyl-cysteine in an attempt to reduce and degrade surface mucus.^{17 18} These variations were applied in case the medium contained molecules which were blocking receptor sites for bacteria and in case the large amounts of mucus produced by the organ cultures mitigated against the success of the system. As a final modification the organ cultures were centrifuged with the infected culture medium for 30 min at 2000 rpm before incubation. This was attempted in order to encourage contact between the test bacteria and the epithelial surface of the organ cultures.

Results

ASSESSMENT OF VIABILITY OF ORGAN CULTURES Histological studies

Biopsy samples were taken both from intact mucosa of five patients with ulcerative colitis with inactive or moderately active disease activity and from six patients with normal rectal mucosa in whom a clinical diagnosis of the irritable bowel syndrome was made.

¹⁴C incorporation in each biopsy sample (dpm/μg protein)

Atmosphere	Origin of biopsy sample of rectal mucosa	Subject no	Time (h)				
			0	2½	5	8	18
95% O ₂ /5% CO ₂	Patients with irritable bowel syndrome	1	ND	63	499	274	993
		2	ND	131	186	215	589
		3	ND	50	198	149	715
		4	ND	ND	279	349	616
		5	4	337	627	ND	ND
		6	5	ND	ND	193	488
		n	2	4	5	5	5
		Mean	45	145	358	236	680
		SEM	0.5	66	88	35	81
	Patients with ulcerative colitis	7	7	174	343	ND	819
		8	5	106	213	ND	1108
		9	4	69	232	ND	444
		10	ND	168	ND	360	1723
		11	ND	102	ND	429	862
		n	3	5	3	2	5
		Mean	5.3	124	263	395	991
		SEM	0.8	20.3	41	35	212
	Combined results	n	5	9	8	7	10
		Mean	5	113	322	281	836
		SEM	0.6	29	57	39	119
100% CO ₂	Patients with irritable bowel syndrome	12	ND	19	61	67	411
		13	ND	12	9	9	12
		n	0	2	2	2	2
		Mean	—	15.5	35	38	212

ND = not done.

Organ cultures from either source were intact after incubation for 6 h in 95% O₂ 5% CO₂ (Fig. 1). At 18 h there was usually some attenuation of the surface epithelium and degeneration of the crypt bases (Fig. 2). In contrast, organ cultures incubated in air or 100% CO₂ showed cytolysis and epithelial destruction after culture for 6 h.

Metabolic studies

The data are given in the Table. Biopsy samples of rectal mucosa derived both from control subjects with irritable bowel syndrome and from patients with ulcerative colitis continued to incorporate leucine into protein throughout the 18 h of incubation. Samples maintained in 100% CO₂ or in air did much less well. These results confirm that rectal biopsy samples from patients with ulcerative colitis are as viable in culture as those from "controls".¹⁹ The combined results show a linear incorporation of leucine into protein throughout the 18 h of culture ($y = 44.3x + 17$, $r = 0.98$, $n = 6$).

EXPERIMENTS WITH ORGAN CULTURE AND BACTERIA

Fifty eight attempts were made to show a close relation between mucosal biopsy samples and proliferating bacteria in the culture system described above.

An intimate relation was seen in only one instance where *E. coli* (strain SC13) appeared to be invading the epithelium of rectal mucosa obtained from a patient with the irritable bowel syndrome. In all other cases epithelial adhesion or invasion was not evident, although bacteria were often seen in the subepithelial tissue, where the mucosa had been denuded of epithelium or in surface mucus. Variations in the incubation time and means of mounting of the samples and prior centrifugation appeared to make no difference, and when sodium salicylate and N-acetyl-cysteine were added to the medium to try to reduce and degrade mucus this led to premature morphological demise of the organ cultures without evidence of bacterial adhesion or invasion of the epithelium. Similarly, when the bacteria were added in saline solution and the organ cultures were fixed after 1 h of incubation there was no evidence of bacterial adhesion or invasion.

Discussion

These results confirm previous findings that rectal mucosa from normal patients and from patients with ulcerative colitis can be maintained in organ culture for at least 18 h.^{13 19}

None of the bacterial strains tested, whether they were proven or putative pathogens was seen consistently to adhere to or invade the epithelium. Bacteria were seen in the deeper tissues but this is considered to be of doubtful pathological importance. Thus this technique cannot be used to show pathogenicity of suspected or proven large intestinal pathogens. At first sight this result is surprising because Candy *et al*²⁰ have successfully used jejunal biopsy samples in short term culture to show the adhesion of pathogenic *E. coli*. There are several possible explanations.

Organ cultures of the rectum, unlike the jejunum, produce large amounts of mucus which may have a protective role,^{6,21} but attempts to reduce the mucus barrier by the addition of sodium salicylate and N-acetyl-cysteine to the medium were unsuccessful. Next, it is probable that bacterial adhesion to the colonic mucosa depends both on chemical attractants normally present in the intestine and on the presence of other indigenous bacteria to which the pathogenic strains might initially adhere.⁶ It is possible that the pretreatment of the organ cultures with antimicrobials mitigated against the use of this system. It is also possible that appropriate receptors for adhesion were not expressed on the organ cultures because of some constituents of the culture medium. Receptors on the cell surfaces which allow bacterial adhesion may, for example, be blocked by carbohydrates competing for the binding sites.^{6,22} But bacterial adhesion could not be shown in saline solutions either, and this is an unlikely single explanation for the failure of the model.

Thus we have failed to establish a role for organ cultures of rectal mucosa in the investigation of the action of pathogenic bacteria in the large intestine. We have established a culture system which is reliable and easy to maintain, but no pathogen, whether proven or putative, was found to damage consistently the mucosa of rectal biopsy samples. This failure is probably related to one or more factors which operate in vivo but not in vitro.

We thank Dr DGD Wight and B Disbrey for help with the histology and Professor EM Cooke, under whose direction and in whose laboratory the *E. coli* strains were investigated by one of us (RJD). We also thank the technical staff of the Clinical Microbiology Laboratory, Addenbrooke's Hospital, for their assistance. This study was supported in part by a grant to one of us (RJD) from the East Anglian Regional Health Authority.

References

- ¹ Evans N. Pathogenic mechanisms in bacterial diarrhoea. *Clin Gastroenterol* 1979;**8**:599-623.
- ² Anonymous. Microbial adhesion, colonisation, and virulence

- (Editorial). *Lancet* 1981;ii:508-10.
- ³ Hartley CL, Robbins CM, Richmond MH. Quantitative assessment of bacterial adhesion to eukaryotic cells of human origin. *J Appl Bacteriol* 1978;**45**:91-7.
- ⁴ Dickinson RJ, Varian SA, Axon ATR, Cooke EM. Increased incidence of faecal coliforms with *in vitro* adhesive and invasive properties in patients with ulcerative colitis. *Gut* 1980;**21**:787-92.
- ⁵ McNeish AS, Fleming J, Turner P, Evans N. Mucosal adherence of human enteropathogenic *Escherichia coli*. *Lancet* 1975;ii:946-8.
- ⁶ Freter R. Mechanisms of association of bacteria with mucosal surfaces. In: *Adhesion and microorganism pathogenicity*. Ciba Foundation Symposium No 80. Tunbridge Wells: Pitman Medical, 1981:36-45.
- ⁷ Bergman MJ, Updike WS, Wood SJ, Brown SE, Guerrant RL. Attachment factors among enterotoxigenic *Escherichia coli* from patients with acute diarrhoea from diverse geographic areas. *Infect Immun* 1981;**32**:881-8.
- ⁸ Sugarman B, Donta ST. Specificity of attachment of certain Enterobacteriaceae to mammalian cells. *J Gen Microbiol* 1979;**115**:509-12.
- ⁹ Gianella RA, Washington O, Gemski P, Formal SB. Invasion of Hela cells by *Salmonella typhimurium*: a model for the study of invasiveness of salmonella. *J Infect Dis* 1973;**128**:69-75.
- ¹⁰ Mäki M, Grönroos P, Vesikivi T. In vitro invasiveness of *Shigella enterocolitica* isolated from children with diarrhoea. *J Infect Dis* 1978;**138**:677-80.
- ¹¹ Dickinson RJ. Enteric infection and ulcerative colitis. London: University of London, 1983. MD Thesis.
- ¹² Defries EA, Franks LM. An organ culture method for adult colon from germ free and conventional mice: effects of donor age and carcinogen treatment on epithelial mitotic activity. *J Natl Cancer Inst* 1977;**58**:1323-8.
- ¹³ Autrup H, Barrett LA, Jackson FE, *et al*. Explant culture of human colon. *Gastroenterology* 1978;**74**:1248-57.
- ¹⁴ Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951;**193**:265-75.
- ¹⁵ Donta ST, Moon HW, Whipp SC. Detection of heat labile *Escherichia coli* enterotoxin with the use of adrenal cells in tissue culture. *Science* 1974;**183**:334-5.
- ¹⁶ Dean AG, Ching Y-C, Williams RG, Harden LB. Test for enterotoxin production using infant mice: application in a study of diarrhoea in children in Honolulu. *J Infect Dis* 1972;**125**:407-11.
- ¹⁷ Lukie BE, Forstner GG. Synthesis of intestinal glycoproteins. Inhibition of I-¹⁴C Glycosamine incorporation by sodium salicylate *in vitro*. *Biochem Biophys Acta* 1972;**273**:380-8.
- ¹⁸ Gilman AG, Goodman LS, Gilman A. *Goodman and Gilman's the pharmacological basis of therapeutics*. 6th ed. New York: Macmillan, 1980:960.
- ¹⁹ MacDermott RP, Donaldson RM, Trier JS. Glycoprotein synthesis and secretion by mucosal biopsies of rabbit colon and human rectum. *J Clin Invest* 1974;**54**:545-54.
- ²⁰ Candy DCA, Leung TSM, Phillips AD, Harries JT, Marshall WC. Models for studying the adhesion of enterobacteria to the mucosa of human gastro-intestinal tract. In: *Adhesion and microorganism pathogenicity*. Ciba Foundation Symposium No 80. Tunbridge Wells: Pitman Medical, 1981:36-55.
- ²¹ Florey HW. Observations on the functions of mucus and the early stages of bacterial invasion of the intestinal mucosa. *Journal of Pathology and Bacteriology* 1933;**37**:283-9.
- ²² Ofek I, Mirelman D, Sharon N. Adherence of *Escherichia coli* to human mucosal cells mediated by mannose receptors. *Nature* 1977;**265**:623-5.

Requests for reprints to: Dr RJ Dickinson, Department of Medicine, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QQ.