

Cell recovery during segmental intestinal perfusion in healthy subjects and patients with Crohn's disease

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Abstract

The recovery of cells arising from small intestinal mucosa alone was studied during continuous perfusion of a closed segment of jejunum. The perfusion technique minimised the contamination of the perfused segment with, for example, proteolytic enzymes from pancreas, allowing recovery of viable cells. The use of hyaluronidase in the perfusion fluid increased the recovery of cells fivefold, the median recovery being 8×10^6 cells. The cells were analysed with monoclonal antibodies and flow cytometry. Nearly all cells (98–99%) recovered during perfusion of healthy control subjects and patients with Crohn's disease were epithelial cells. The jejunal cells expressed HLA-DR in similar proportions – around 30% – in patients and control subjects. The ratio between CD4⁺ and CD8⁺ lymphocytes was similar (0.2) in control subjects and patients with inactive Crohn's disease but decreased (0.03) in patients with active Crohn's disease in the ileum.

Characterisation and functional studies of cells in the intestinal mucosa have been based on biopsy specimens. Immunohistochemical staining of biopsy sections have given valuable information on the cellular events in health and disease. Some of the surface markers, however, may disappear with the use of the immunoperoxidase technique on frozen sections of the intestine.¹ Isolated cell populations from gut specimens have been used for various functional studies. In inflammatory bowel disease, mitogen-stimulated proliferation of isolated lymphocytes has been found to be both increased^{2,3} and decreased,^{4,5} possibly owing to the isolation procedure.⁶ Thus the methods used today are limited by problems of trauma during collection of tissue specimens and time consuming cell isolation procedures.⁷

One aim of this study was to elucidate cell recovery during jejunal perfusion of healthy control subjects and patients with Crohn's disease. A further aim was to characterise jejunal cells recovered during perfusion: the HLA-DR expression of epithelial cells and lymphocyte reactivity to various monoclonal antibodies. For this purpose we used a flow cytometry technique.

Methods

PATIENTS AND CONTROLS

Fourteen patients with Crohn's disease were examined (10 women, four men; mean age 40 years, range 21 to 62 years). Four patients had had colectomies or ileostomies and had no sign of

recurrence; the Crohn's disease activity index was <150.⁸ Ten patients had had ileocaecal resections. Five of these had no radiological signs of recurrence and a Crohn's disease activity index of <150; five had radiological signs of Crohn's disease in the terminal ileum and a mean index of 230 (range 170 to 280). None of the patients, apart from one taking metronidazole, had received any medical treatment for Crohn's disease as steroids, antibiotics, or non-steroidal anti-inflammatory drugs within the previous three months. Nine healthy volunteers (two women, seven men; mean age 30 years) were given a jejunal perfusion. An ileal perfusion was performed on four patients with Crohn's disease who had had a colectomy or an ileostomy (two women, two men; mean age 40 years, range 25 to 54 years). Three men (mean age 42 years, range 25 to 53 years) with ulcerative colitis, who had had a colectomy or an ileostomy served as controls for the ileal perfusion. Patients and control subjects gave informed consent to the study, which was approved by the Ethic Committee of the Medical Faculty, Uppsala University.

SEGMENTAL INTESTINAL PERFUSION

Segmental intestinal perfusion was achieved by a small diameter tube (LOC-I-GUT, Pharmacia, Uppsala, Sweden) containing six channels and two balloons for isolation of a 10 cm segment.⁹ The balloons were inflated when the tube was in place in the proximal jejunum or by retrograde intubation of the ileum, on average 40 cm, proximal to the stoma. The segment was rinsed with 120 ml 154 mM NaCl solution, at 37°C over 30 minutes. After that recovery of cells started during continuous perfusion of the segment at 3 ml per minute with a solution (37°C) containing 10 mM glucose, 5.4 mM KCl, 120 mM NaCl, 2 mM Na₂HPO₄, 35 mM mannitol, osmolality 290 mOsm/l; 1 g/l of ¹⁴C-labelled polyethylene glycol (¹⁴C-PEG, MW 4000 daltons, 2.5 µCi/l, Amersham, Buckinghamshire, UK) was added to the perfusion solution as a volume marker.¹⁰ Hyaluronidase, 200 IU/l (Hyalas, Ferrosan, Sweden) was added to increase the release of cells. Ten ml of aprotinin, 10 000 KIU/l (Bayer AG, Leverkusen, FRG) was added to every litre of perfusion solution to inhibit proteolytic activity that might have contaminated the perfusate. Another 0.6 ml of aprotinin was added to every 60 ml of effluent. The duration of the perfusion was usually 180 minutes. To prevent leakage at the proximal balloon, phenol red solution (50 mg/l in 154 mM NaCl) was infused into the stomach through the sump line of the Salem-sump tube (jejunum), or in a proximal channel (ileum), at a rate of

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Monoclonal antibodies used for characterisation of cells

Antibody	Antigen (cluster designation)	Predominant reactivity
Anti-Leuco	CD45	Leucocytes
Anti-Leu-M1	CD15	Monocytes/granulocytes
Anti-Leu-M3	CD14	Monocytes/macrophages
Anti-Leu-2	CD8	T cytotoxic/suppressor cells
Anti-Leu-3	CD4	T helper/inducer cells
Anti-Leu-4	CD3	T cells (mitogenic)
Anti-Leu-7	-	T cells and NK cell subsets
Anti-Leu-11	CD16	Fc IgG receptor on NK cells and neutrophils
Anti-Leu-12	CD19	B cells
Anti-Leu-15	CD11	T suppressor cells, NK cells, monocytes, and granulocytes
Anti-Leu-18	CD45R	T suppressor/inducer subset, NK cells, B cells
Anti-HLA-DR	-	B cells, monocytes, macrophages, activated T cells, and epithelial cells

NK = natural killer.

1 ml/min and analysed in the effluent from the intestinal segment. The effluent was continuously drained by gravity suction and collected on ice every 20 minutes. Each collected fraction was immediately centrifuged. The cell pellets were resuspended and pooled in a culture medium (RPM 1640, Flow industries, UK) with the addition of HPG (Hepes-penicillin/streptomycin-glutamine) and 10% fetal calf serum. The cell suspension was centrifuged and resuspended in phosphate buffered saline before cell counting and analysis, which was performed within four hours of perfusion.

ANALYTICAL MEASUREMENTS

¹⁴C-PEG was determined by liquid scintillation counting of 1 ml aliquots (LKB Rackbeta II, liquid scintillation counter, Wallac Oy, Turku, Finland) in duplicate for 15 minutes.¹¹ Phenol red was measured spectrophotometrically at 520 nm (Hitachi Ltd, Tokyo, Japan) after alkalinisation (pH 11) with Na₃PO₄ buffer.¹² Measurements were performed on cells obtained over the whole perfusion time.

ANTIBODIES

The monoclonal antibodies (Becton Dickinson, Mt View, California, USA) are listed in the Table. They were directly conjugated to fluorescein isothiocyanate or phycoerythrin. Some were also used as unconjugated antibodies and combined with a Fab₂ rabbit anti-mouse (RAM) antibody conjugated with phycoerythrin (Serotec Ltd, Oxford, UK).

STAINING OF CELLS

For direct immunofluorescence staining, 0.5 × 10⁶ cells were incubated with 25 μl fluorescein isothiocyanate or phycoerythrin conjugated antibodies, or both, for 10 minutes at room temperature. The cells were then washed in phosphate buffered saline (PBS), suspended in 300 μl PBS with 0.1% sodium azide and 2% fetal calf serum, and fixed in 300 μl PBS with 1% paraformaldehyde and 1% fetal calf serum. For indirect immunofluorescence staining, cells were incubated at room temperature with 25 μl of unconjugated primary monoclonal antibody for

10 minutes. The cells were washed as above followed by incubation with 25 μl of RAM-phycoerythrin antibody. After washing, free binding sites on the RAM antibodies were blocked by a 5 minute incubation with normal mouse serum (diluted 1:100). After washing, the fluorescein isothiocyanate conjugated monoclonal antibody was added for 10 minutes, with a final wash before fixation as above.

FLOW CYTOMETRY

Flow cytometric analyses were performed with a FACStar (Becton Dickinson), equipped with a 5W Argon ion laser run at 488 nm for an output of 200 mW. Green fluorescence (fluorescein isothiocyanate) was collected through a 530 per 30 nm bandpass filter, and red (phycoerythrin) through a 585 per 42 nm bandpass filter. A 560 nm dichromic mirror was used to split red and green signals. All these features are part of the standard configuration on the FACStar. The forward scatter, corresponding to granularity, and the two fluorescence signals were collected in logarithmic mode and evaluated with Consort 30 software on a Hewlett-Packard 9000 series model 310 computer: 5 × 10⁵ cells were analysed.

Results

RECOVERY OF CELLS

The method of perfusing a closed jejunal segment was successful, with a contamination of less than 5% according to phenol red, and the ¹⁴C-PEG recovery was more than 80% over the whole study period. The perfusion of distal ileum was technically more complicated owing to the viscosity of the intestinal secretion, which clogged the drainage channel. Only the results from the best periods of ileal perfusion, usually at the start of the perfusion, are presented. The contamination of phenol red was 23% and the mean recovery of ¹⁴C-PEG 75% during these periods.

Cell recovery during jejunal perfusion increased on average five times with the addition of hyaluronidase to the perfusion fluid. Cell recovery was therefore studied with enzyme present. The median recovery of cells was 8 × 10⁶

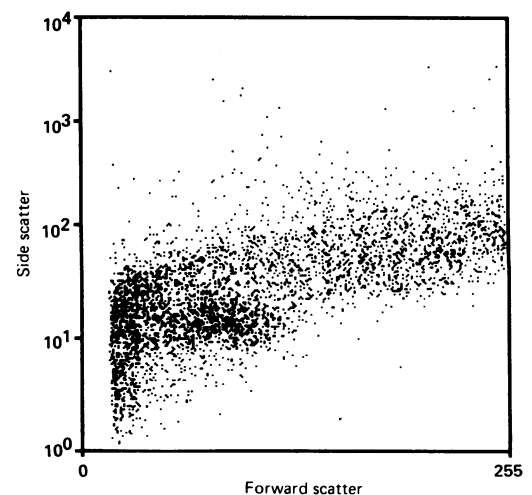


Figure 1: Scatter pattern of cells obtained with intestinal perfusion.

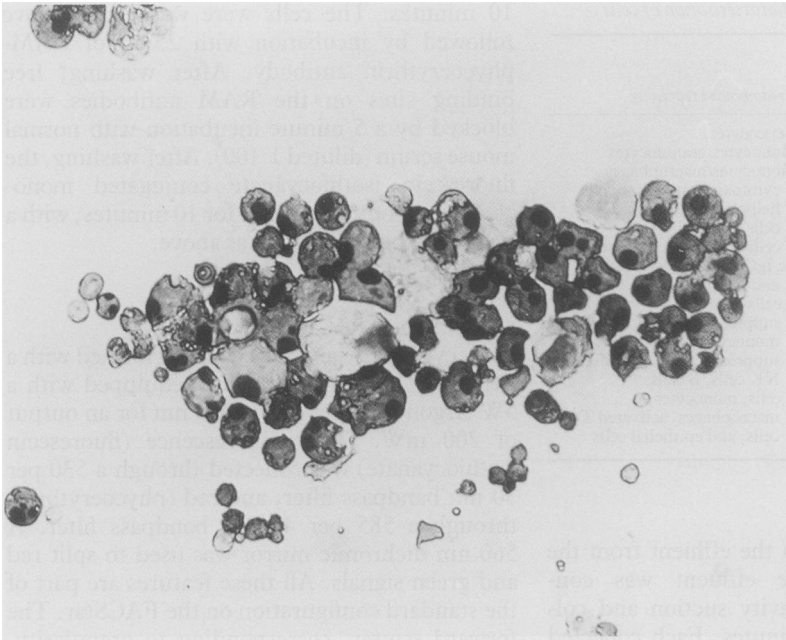


Figure 2: A cytocentrifuge preparation of cells recovered during intestinal perfusion of a healthy volunteer. The cell preparation is dominated by epithelial cells. (May-Grünwald-Giemsa staining).

cells. Cell recovery was similar in perfused jejunum and ileum and no difference in the number of recovered cells was found between control subjects and patients with Crohn's disease.

EPITHELIAL CELLS

Epithelial cells were identified as separate cell clusters with the flow cytometry technique (Fig 1). The epithelial cells predominated (Fig 2), representing on average of 99% of the total cells recovered in control subjects and patients with inactive Crohn's disease (in jejunum as well as in ileum). In perfused jejunum from patients with active Crohn's disease 98% of the cells recovered were epithelial cells.

LEUCOCYTES

Jejunal recovery of CD45⁺ leucocytes in healthy control subjects was 0.8 (0.3)% (mean (SEM)) and in patients with Crohn's disease 1.1 (0.3)%. The recovery of these cells was related to disease activity; patients with active disease had a twofold increase of CD45⁺ cells (1.4 (0.4)%), while patients with inactive disease had a similar recovery to the control subjects (0.9 (0.3)%).

The lymphocytes were grouped according to their reactivity with CD4 and CD8. The proportion of CD4⁺ cells was decreased in active Crohn's disease, giving a low CD4⁺/CD8⁺ ratio (0.03) compared with the ratio in healthy controls (0.2). The ratio of lymphocytes recovered during ileal lavage of patients with stoma and inactive Crohn's disease or ulcerative colitis was 0.5 and 0.3, respectively. A few cells positive for CD3, Leu-7, CD14, CD15, and CD19 were found in patients and control subjects (data not shown). No recovered cells expressed the antigens CD11, CD16, and CD45R.

CELLS EXPRESSING HLA-DR

Cells recovered during jejunal lavage expressed HLA-DR in similar proportions in control subjects (mean (SEM) 29.6 (7.7)%), patients with inactive disease (25.9 (19.4)%), and patients with active Crohn's disease (30.6 (5.4)%).

Discussion

This study has shown that large numbers of viable epithelial cells are recovered from healthy control subjects and patients with Crohn's disease during perfusion of a jejunal segment provided that hyaluronidase is present in the perfusion fluid. This enzyme is specific for the glycosaminoglycan hyaluronan (hyaluronic acid or hyaluronate by older nomenclature). Large amounts of hyaluronan are found in jejunal lumen,^{9,13,14} indicating considerable local synthesis of this connective tissue component, for which a role in intercellular adhesion has been suggested.¹⁵ Recently we have visualised hyaluronan in the loose connective tissue of the villi.¹⁶ Furthermore, hyaluronan accumulates in the subepithelial structure. This histochemical localisation of hyaluronan together with our findings of increased cell recovery in the presence of hyaluronidase suggest that hyaluronan is involved in the epithelial cell attachment to lamina propria. The site from which exfoliated epithelial cells are derived during continuous jejunal perfusion in humans has not been identified. The recovered cells, however, are likely to be villous tip cells, since intestinal epithelial cells divide in the fundus of the villi and move continuously towards the tip of the villi where they are shed. In experimental studies the chelating agent ethylenediaminetetra-acetate has been used in perfusion fluid to increase the recovery of epithelial cells in view of the role of calcium ions in cell adhesion.¹⁷ But the epithelial cells recovered were trapped in mucus and therefore not suitable for analysis by flow cytometry. The cells recovered during jejunal perfusion in the presence of hyaluronidase evidently escaped from the mucus, while the perfusion of distal ileum in patients with stoma often was complicated by the high viscosity of the intestinal secretion.

Most of the cells recovered during jejunal lavage were epithelial cells, as in previous experimental studies.¹⁷⁻¹⁹ About a third of the epithelial cells from healthy control subjects expressed HLA-DR. Previous immunohistochemical studies of jejunal biopsy specimens have also claimed the presence of activated epithelial cells in healthy specimens.²⁰ A similar proportion of jejunal epithelial cells staining with anti-HLA-DR was seen in our patients with Crohn's disease affecting the distal ileum.

Attempts to collect cells from small intestinal washings have been made in experimental animals. The methods used have included simple aspiration of intestinal fluid and the conventional small intestinal perfusion technique.^{17,21} Specimens obtained by these methods, however, contained cells that were completely disrupted and converted to an amorphous mass, probably owing to the high enzyme content of the intestinal fluid in the lumen. This problem

was partly overcome in experimental studies by occlusion of the small intestine and rapid washing of the small intestine.¹⁷ A few washings of small intestine in humans have been performed by using a triple lumen tube,¹⁹ but the recovery of small intestinal epithelial cells was unsatisfactory because they were damaged and impossible to identify by light microscopy. The success of the present six lumen catheter technique with two occluding balloons is based on the principle that material from the stomach, biliary tract, and pancreas is prevented from entering the perfused segment. Furthermore, the addition of protease inhibitors to the perfusion fluid prevents a cell damaging effect owing to a possible contamination of the perfusate with minute amounts of proteolytic enzymes.

The number of lymphocytes recovered during jejunal perfusion of healthy control subjects was about 8×10^4 cells – that is, about 1% of the total cell recovery. The jejunal recovery of lymphocytes was similar in patients with inactive Crohn's disease but was on average 2% in patients with active disease in the distal ileum. The paucity of recovered lymphocytes was surprising but in accordance with previous perfusion studies.¹⁸ It seems that the recovery of lymphocytes is not representative of the physiological populations covering the villi but may present cells derived from lymphoid aggregates and Peyer's patches. Therefore, the phenotypic characterisation of recovered cells must be interpreted with caution. The ratio between CD4⁺ and CD8⁺ cells (helper/inducer cells and cytotoxic/suppressor cells, respectively) was similar in control subjects and patients with inactive Crohn's disease but apparently lower in patients with active Crohn's disease owing to a relative decrease of CD4⁺ cells. Interestingly, this relation between CD4⁺ and CD8⁺ cells has also been reported in immunohistochemical studies of biopsy specimens from Crohn's lesions.²² The altered cellular response in an area of the intestine not apparently affected by the disease may reflect a more generalised nature of the disease as previously suggested by an increased secretion of complement components and histamine in non-affected areas of the intestine in patients with Crohn's disease.^{23,24} The few lymphocytes recovered did not allow conclusive flow cytometry analyses with other monoclonal antibodies with reactivity for less frequent appearing antigen structures on lymphocytes. Nor did flow cytometry analyses using monoclonal antibodies directed against monocytes/macrophages give reliable data owing to the small number of such cells in the perfusion fluid. The CD4⁺/CD8⁺ ratio for lymphocytes recovered during ileal perfusion in patients with ulcerative colitis and active Crohn's disease with a stoma was similar to the corresponding jejunal ratio for healthy control subjects.

This study has shown a new way to obtain viable epithelial cells from the small intestine in

vivo. The cell recovery obtained by jejunal lavage minimises the time from harvest to analysis of cells. The observation that epithelial cells appear in more than 98% of all cells recovered is of potential value for characterisation of epithelial cell function in a primary highly purified epithelial cell preparation. The use of the technique for the characterisation of lymphocytes seems more uncertain because of the limited recovery of such cells.

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