

# Phagocytes in cell suspensions of human colon mucosa

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**SUMMARY** Because little is known of the phagocytes of the human colon we enumerated these cells in mucosal suspensions and studied their phagocytic activity. Phagocyte rich suspensions were made by EDTA collagenase dissociation followed by elutriation centrifugation. Phagocytosis was evaluated by measuring cellular radioactivity after incubation of phagocytes with  $^3\text{H}$ -adenine labelled *E coli* ON2 and checked microscopically. Dissociation of normal mucosa from colorectal neoplasms yielded means of  $1.9 \times 10^6$  eosinophils,  $1.4 \times 10^6$  macrophages and  $2 \times 10^5$  neutrophils per gram of mucosa. Visually normal mucosa of inflammatory states yielded  $2.2 \times 10^6$  eosinophils,  $2.3 \times 10^6$  macrophages and  $7 \times 10^5$  neutrophils per gram of mucosa. Phagocyte rich suspensions of normal mucosa from tumour patients phagocytosed 21.8% of a pool of opsonised tritiated *E coli* ON2 and by microscopy 100% of mucosal neutrophils ingested bacteria, 83% of eosinophils were phagocytic, and 53% of macrophages contained bacteria. These results suggest that in the human colonic mucosa, the eosinophil is more abundant than the macrophage and the per cent of those cells exhibiting phagocytosis is intermediate between that of the macrophage and the neutrophil. Thus these three types of cells are actively phagocytic and share the potential for a major role in host defence against invasive enteric bacteria.

Phagocyte populations of human peripheral blood have been well characterised, but information about these cells in the human colon is sparse. Macrophages and the 'occasional' eosinophil and neutrophil<sup>1,2</sup> are reportedly found in normal mucosal specimens. Macrophages reside in the subepithelial region,<sup>1</sup> and are thought to be 12 times as numerous as eosinophils and neutrophils.<sup>3</sup> Recent studies of mucosal cell suspensions derived by enzymatic or mechanical dissociation techniques report that macrophages comprise from 1 to 30% of the population, neutrophils from 0 to 11% and eosinophils from 2 to 8% of all cells.<sup>4-8</sup> Nylon wool filtration,<sup>3</sup> and gradient centrifugation,<sup>6,7</sup> methods frequently used in these studies, tend to deplete neutrophils and eosinophils from mononuclear cell populations, and consequently these phagocytes have not been intensively studied in mucosal preparations. We previously reported the preparation of macrophage suspensions from colonic mucosa by collagenase-DNAase dissociation, Ficoll-

Hypaque gradient centrifugation and countercurrent centrifugation (elutriation).<sup>9</sup> We have modified this technique to recover not only macrophages, but also neutrophils and eosinophils from human colonic mucosa, and we have compared phagocytic activity of these cells with phagocytes of peripheral blood.

## Methods

### SUBJECTS

Colon mucosa and peripheral blood were obtained from patients with large bowel neoplasms and colonic inflammatory diseases. Comparative studies were conducted on blood of healthy volunteers. In the neoplasm group there were 10 women and 14 men between the ages of 44 and 82 years (mean 63). Nineteen had large bowel cancer, four had resections for polyps and one had carcinoid tumour. There were four women and five men between 20 and 79 years of age (mean 53) with colonic inflammatory diseases, three of whom had diverticular disease, three had Crohn's disease, two had ulcerative colitis, and one had radiation enteritis. Normal blood donors were laboratory personnel, house staff, and faculty. All

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subjects gave informed consent and the investigation was approved by the University of Vermont Committee on Human Experimentation.

#### PERIPHERAL BLOOD SPECIMENS

Fifty to 100 millilitres blood was obtained by venapuncture and heparinised. Monocyte rich mononuclear cells were obtained by Ficoll-Hypaque centrifugation.<sup>10</sup> The monocyte rich mononuclear leucocytes, were removed and washed three times in phosphate buffered saline (PBS) before assay. The neutrophil eosinophil rich pellets were cleared of erythrocytes by hypotonic lysis in 0.85% ammonium chloride and used for phagocytic assays.

#### PREPARATION OF CELL SUSPENSIONS FROM COLON MUCOSA

Surgical specimens were collected from the operating room immediately after removal and segments containing 5–20 g of normal mucosa were removed distant from neoplastic tissue and visible inflammation and placed in chilled calcium-magnesium-free Hanks' balanced salt solution (Whittaker MA Bioproducts, Walkersville, MD) containing 100 U/ml penicillin, 50 µg/ml gentamicin and 1 µg/ml amphotericin B (CMF-HBSS-AB) and processed by a modification of methods previously described.<sup>6,9</sup> In brief, debris was dislodged from the specimen by gentle agitation for one minute in each of four sequential washings. The mucosa was separated from the muscularis by sharp dissection and cut into 1–2 mm<sup>2</sup> pieces, weighed, washed again, and stirred in 1 mM EDTA in CMF-HBSS-AB (10 ml/g tissue) for six 60 minute periods, maintaining pH above 7 by dropwise addition of 1 M NaOH. Lamina propria denuded of epithelial cells was then incubated for eight to 12 hours in RPMI 1640 medium with 25 mM Hepes (Whittaker MA Bioproducts) with 10% heat inactivated fetal calf serum, 20 U/ml collagenase (Type IV, Sigma Chemical Co, St Louis, MO or CLSPA-Worthington, Cooper Biomedical, Malvern, PA) and 2 U/ml of DNAase (Sigma), using 10 ml/g tissue with constant agitation at 37°C. At the end of the incubations cell suspensions were filtered through nylon mesh to remove clumps and washed three times in RPMI 1640 medium. Cells were resuspended and counted in a haemocytometer, and cytocentrifuge preparations were made before elutriation centrifugation.

#### ELUTRIATION CENTRIFUGATION

Lamina propria phagocytes were further separated by elutriation centrifugation in a Beckman JE6 elutriator rotor with a standard 4.2 ml elutriation chamber using the Beckman J2-21 centrifuge equip-

ped with a sensitive speed control potentiometer as previously described.<sup>9</sup> In brief, 50–300×10<sup>6</sup> cells in 10–20 ml of chilled medium were loaded into the centrifuge at 700 g with a flow rate of 18 ml/minute. Lymphocytes were collected in five 90 ml fractions before the speed was reduced to 100 g for collection of phagocytes in two 90 ml fractions, and the centrifuge was then stopped and any cells remaining in the chamber were collected by flushing for the eighth fraction. Fractions were centrifuged for 10 minutes at 400 g and the sedimented cells resuspended and aliquoted for further study.

#### CELL ENUMERATION AND IDENTIFICATION

Cells were counted in a haemocytometer and cytocentrifuge preparations were air dried, fixed and stained by Diff Quik (Dade Diagnostics, Aguada, PR). Percentages of macrophages, eosinophils, neutrophils, lymphoid cells and other elements were determined by counting at least 200 cells by light microscopy.

#### PHAGOCYtic ASSAY

The percentage of radiolabelled bacteria ingested by phagocytic populations was measured by the technique of Peterson and coworkers.<sup>11</sup> *E coli* ON2 (kindly provided by P K Peterson, University of Minnesota), were incubated overnight in 20 ml Mueller-Hinton broth containing 20–40 µC <sup>3</sup>H-adenine, washed three times with PBS and diluted to 5×10<sup>8</sup> CFU/ml using a spectrophotometric method previously calibrated by pour plate counting. Tritium labelled bacteria were opsonised for 15 minutes at 37°C in 10% pooled normal human serum frozen at –70°C immediately after separation to preserve complement components, washed and resuspended to 5×10<sup>7</sup>/ml HBSS-BSA. Washed phagocytes were resuspended at a concentration of 5×10<sup>6</sup> cells/ml in HBSS. Assays were conducted in duplicate with 5×10<sup>6</sup> opsonised and non-opsonised bacteria (0.1 ml) and 5×10<sup>6</sup> phagocytes (0.1 ml), giving a 10:1 bacteria:phagocyte ratio, for 30 minutes at 37°C with constant shaking. Incubations were terminated by the addition of chilled PBS, cells were sedimented at 160 g×10 min, washed three times and phagocytosis determined by measuring radioactivity in cell pellets solubilised in Hydrofluor scintillation fluid (National Diagnostics, Somerville, NJ). The percentage of the pool of labelled bacteria ingested was calculated as follows:

$$\% \text{ phagocytosis} = \frac{\text{CPM in leucocyte pellet}}{\text{CPM of total bacteria}} \times 100$$

Cyocentrifuge preparations of phagocyte populations were also examined microscopically to determine directly the percentage of cells ingesting

bacteria and the efficiency of removal of uningested bacteria from the cells by washing.

DETERMINATION OF THE EFFECT OF PREPARATIVE TECHNIQUES OF PHAGOCYTOSIS

Phagocytosis by peripheral blood monocyte and neutrophil-eosinophil enriched suspensions after standing overnight at 4°C was compared with phagocytosis by each cell type held stationary, shaken or stirred at 37°C with or without collagenase. Phagocytic assays were also conducted before and after elutriation centrifugation with monocyte populations.

STATISTICAL ANALYSIS

Data were stored and analysed on the University of Vermont General Clinical Research Center CLINFO system. Results are expressed as means and standard errors of the means unless stated otherwise.

Results

PHAGOCYTES IN MUCOSAL SUSPENSIONS

Enzymatic dissociation of colon lamina propria from the neoplasm group yielded a mean of  $20.2 \times 10^6$  cells per g of colon mucosa. These populations consisted of  $8.5\% \pm 1.5$  eosinophils,  $7.5\% \pm 1.4$  macrophages, and  $1.3\% \pm 0.4$  neutrophils; mast cells comprised less than 1% and the remaining cells were almost all lymphocytes and plasma cells (lymphoid cells). As expected, slightly more cells were found in the inflammation group probably because of patchy leucocyte infiltration (Table 1). After washing, these cell suspensions were separated into lymphoid and phagocytic populations by elutriation. Eosinophils were the predominant phagocyte in resections from both the neoplasm group and from patients with inflammation.

PHAGOCYTE ENRICHMENT BY COUNTERCURRENT CENTRIFUGATION

Elutriation centrifugation resulted in a considerable enrichment of the various phagocyte populations at 100 g. The major populations in the neoplasm group were distributed according to the elutriation pattern shown in the Figure, and the pattern for the inflammation group was practically identical. The various phagocyte populations after enrichment in both groups are given in Table 2. After elutriation total recovery was 89.3% (5.1) in the neoplasm and 64.6% (10.6) in the inflammatory group.

MEASUREMENTS OF PHAGOCYTOTIC FUNCTION

Active phagocytosis of opsonised *E coli* ON2 was exhibited by each type of phagocyte studied. Few

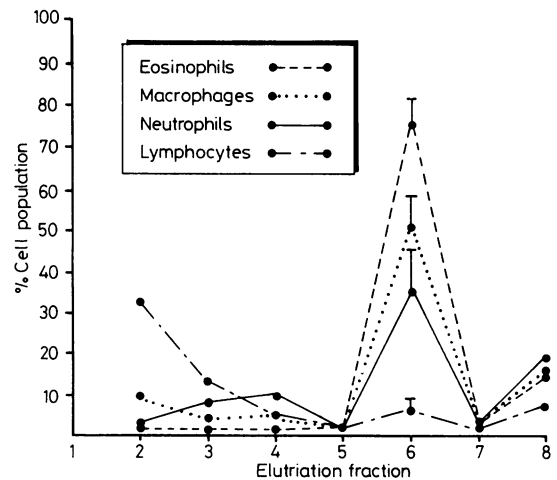


Figure Elutriation pattern of major cell types in suspensions of histologically normal human colonic mucosa (n=13).

Table 1 Cell populations in EDTA-collagenase dissociated mucosal cell suspensions

	All cells	Macrophages		Neutrophils		Eosinophils		Lymphoid cells		Others		
		N*	I†	N	I	N	I	N	I	N	I	
Total cells: mean %	100	7.5	8.4	1.3	4.4	8.5	12.9	72.0	67	9.7	6.7	
SEM		1.4	2.1	0.4	2.5	1.5	4.3	2.1	6.5	1.8	2.1	
n		24	9	24	9	24	9	24	9	24	9	
		N	I									
Mean cells $\times 10^6$ per gram mucosa	20.2	23.1	1.4	2.3	0.2	0.7	1.9	2.2	14.8	15.7	1.8	1.8
SEM	1.5	4.9	0.2	0.9	0.0	0.5	0.4	0.7	1	3.8	0.4	0.8
n	19	7	19	7	19	7	19	7	19	7	19	7
Mean cells $\times 10^6$ per resection	268	324	18.6	33.4	2.7	10.3	25.3	32.6	196.8	231.1	23.9	26.3
n	19	7	19	7	19	7	19	7	19	7	19	7

\*N=Mucosa from resections for neoplasms; †I=Mucosa from resections for inflammatory disease.

*Phagocytes in cell suspensions of human colon mucosa*Table 2 Cell types in phagocyte enriched elutriator fraction 6 ( $10^6$  cells per  $100 \times 10^6$  cells elutriated)

	Macrophages		Neutrophils		Eosinophils		Lymphoid cells	
	N	I	N	I	N	I	N	I
Mean	2.31	1.24	0.31	1.06	5.0	8.74	3.28	1.74
SEM	0.54	0.29	0.12	0.38	0.89	5.12	0.61	0.59
n	16	7	16	7	16	7	16	7

N=Neoplasm group; I=Inflammation group.

Table 3 Per cent  $^3\text{H-E}$  coli ON2 ingested by elutriator fraction 6

	Neoplasm		Inflammation	
	Op*	Non-op†	Op	Non-op
Mean	21.8	3.9	8.4	1.8
SEM	3.5	1.0	2.6	0.6
n	16	12	5	5

\*Opsonised; †Non-opsonised.

Table 4 Per cent of  $^3\text{H-E}$  coli ON2 ingested by autologous blood and mucosal phagocytes from neoplasm group

Subject	Blood				Mucosa	
	Monocytes		Neutrophils		Mixed phagocytes*	
	Op†	Non-op‡	Op	Non-op	Op	Non-op
A	24.7	2	35.2	1.4	6.7§	1.5§
B	15.1	1.7	50.6	1.3	35.6	2.9
C	65.6	9.2	56.7	5.4	43.0	11.9
D	30.0	5.9	36.9	3.4	27.1	5.6
E	36.5	7.4	45.5	4.6	37.1	7.3
Mean	34.4	5.2	45.0	3.2	29.9	5.8
SEM	8.6	1.5	4.1	0.8	6.3	1.8

\*Mean phagocyte composition=21% macrophages, 5% neutrophils and 74% eosinophils, present in elutriator fraction 6; †Opsonised; ‡Non-opsonised; §Unelutriated.

non-opsonised organisms were ingested. Peripheral blood neutrophils from normal subjects phagocytosed 32.1% (3.8, n=16) of  $^3\text{H-E}$  coli ON2 and monocytes ingested 28.4% (3.4, n=16). The percentages of  $^3\text{H-E}$  coli ingested by mucosal phagocytes (elutriator fraction 6) are shown in Table 3. Phagocytosis by cells from neoplasms seemed higher than by cells from the inflammatory group, but the latter group was small and the differences were not statistically significant. Table 4 compares phagocytosis by blood monocytes and neutrophils with autologous mucosal populations containing an average of 74% eosinophils, 21% macrophages, and 5% neutrophils.

Microscopic examination indicated that by 30 minutes mucosal neutrophils invariably contained ingested bacteria and 95% of eosinophils were phagocytic but only 66% of the macrophages contained bacteria. Phagocytic activity at 15 minutes was nearly identical to that at 30 minutes and by 60 minutes the number of visible bacteria began to decline.

## EFFECT OF ISOLATION METHODS ON PHAGOCYTOSIS

The preparative measures did not diminish phagocytosis. Although phagocytosis by monocytes decreased 38% after stationary incubation in collagenase at 37°C compared with standing at 4°C without the enzyme, shaking and stirring in collagenase resulted in slightly greater phagocytic activity. Elutriation did not affect the magnitude of phagocytosis, but consistently resulted in maximum activity at 15 minutes rather than at 30 minutes as seen in unelutriated phagocytes.

## Discussion

These results cannot necessarily be extrapolated to the normal state for the obvious reason that all donors of colon tissue had an abnormality requiring resection. Moreover, grossly normal tissue from inflammatory states may have contained areas of inflammation not seen because of limited sampling. In addition the populations in these suspensions may not precisely reflect the proportions *in vivo*, because some cells, especially fragile macrophages, may have been disrupted during dissociation. Nonetheless, the populations in these mucosal suspensions likely reflect the proportions in the colon *in vivo* in general. Macrophages were a major cell group and neutrophils comprised a much smaller proportion. The large numbers of eosinophils was not anticipated, however, probably because their presence in clinical biopsy specimens is often overlooked. Eosinophils were much more numerous than neutrophils and slightly more numerous than macrophages.

Phagocytosis by mucosal macrophages varied from subject to subject and may have been influenced by ingestion of debris from cells lysed during dissociation<sup>9</sup> resulting in membrane receptor internalisation and a postphagocytic refractory period.<sup>12,13</sup> We previously found that approximately 70% of mucosal macrophages in suspension internalise opsonised erythrocytes.<sup>14</sup>

Neutrophils were quantifiable and were readily identified by microscopy in cytocentrifuge preparations, but too few were obtained from mucosa to permit complete assessment of phagocytosis. As in

other tissues, however, they were the most avidly phagocytic of the three populations when examined by microscopy. Because they are present in relatively small numbers, their role in the initial phases of bacterial invasion would be expected to be limited. Because large numbers quickly migrate to the mucosa in response to chemotactic and other stimulating factors in disease, however, their subsequent role in mucosal defence would be major.

Phagocytosis by blood eosinophils *in vivo* is debated.<sup>15</sup> Recent studies showed complement dependent phagocytosis by eosinophils *in vitro*, although these cells were less active than neutrophils.<sup>16</sup> We almost never encountered peripheral blood eosinophils with internalised bacteria during microscopic checks of our assays. Phagocytosis by 95% of intestinal eosinophils, not reported previously, was presumably related to factors such as cell maturity and stimulation in the mucosal environment.<sup>17</sup> Thus, in addition to their recognised role in hypersensitivity reactions and helminthic infections, eosinophils also seem potentially capable of a role in the defence against bacterial invasion of the human colon similar to that of the traditional phagocytes, the macrophages and neutrophils. Our studies show active phagocytosis by all three of these cell populations.

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